CITRULLINE METABOLISM IN CULTURED FIBROBLASTS: CITRULLINEMIA ANALYSIS AND NITRIC OXIDE PRODUCTION.

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A dissertation submitted in fulfilment of the requirements for the degree of Master of Science (Med.) in the department of Chemical Pathology in the Faculty of Medicine, University of Cape Town, South Africa.

December 1993.

To: The Higher Degrees Committee 14 April 1994

The following corrections were made to my Masters thesis in accordance with the specifications of the 3 examiners and the changes were approved by my supervisor Prof. E.H.Harley:

Several general corrections were made to the entire thesis:

- uM, ug symbols were changed to µM and µg.
- °C replaced all 'C symbols.
- Argininosuccinate synthetase, canavanine-resistance, tetrahydrobiopterin, nitric oxide, nitric oxide synthase were referred to by their abbreviated forms after their inital definition.
- The possessive form of "it" was corrected from "it's" to "its".
- The purpose of the error bars was defined in figures 22, 23, 38, 40, 42, 44, 46.
- The use of the non-standard abbreviations for CO_2, NO_2^-, Ca^{2+}, Mg^{2+} etc. was corrected.
- The Y-axis scale on figures 9, 11, 22, 31 and 40 were redefined as suggested.
- Product names such as Falcon, Southern, Luria, Sephadex were capitalised.
- Bacterial names and restriction enzymes were italisized.
- The incorrect spelling of cycloheximide was altered throughout the thesis.
- DNA marker names and sizes were given where appropriate.

The following specific corrections were also made:

Spelling errors:
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pg 43: aperture.

pg 50/61: electrophoresed.

pg 51: thiocyanate, isoamyl alcohol.

pg 112: iron-protoporphyrin, carbon monoxide.

pg 115: relaxation.

pg 123: hydroxyl.

pg 127: in turn.

pg 128: peroxynitrite.

pg 129: threatening.

pg 134: transcarbamylase.

pg 138: aqueous, whereas.

pg 139: solvent.

pg 142: isocratic.

pg 158: fumarate.

pg 165: ice cold.

pg 177: ketoglutarate.

pg 195: immunology.

- The section entitled " Development of amino acid separation tecniques" was more appropriately entitled "evaluation of amino acid separation techniques".

pg 8: References to the G->A mutation were more correctly referred to as G->A transitions.

pg 10/18: The figure from reference [1] was found to be incorrect and was altered accordingly.

pg 17: The appropriate sentence was restructured.

pg 21: Phosphate was changed to pyrophosphate.

pg 24: This figure was reproduced to improve the quality, the bacterial names were put into italics and the full names of the bacteria were given.

pg 25: The number of remaining ASS exons was corrected.

pg 27: It was noted on the figure that the sequence given in this figure is +3 base pairs when compared to the Genbank sequence used throughout the rest of the study. The figure was also reprinted to improve the quality.

pg 28: References were given to the various forms of evidence.

pg 36: The description of atypical citrullinemia was redefined.

pg 37: The reference to Table 2 was corrected and the description of the range of mutations altered. A sentence was included to explain why the exact frequencies of the various mutations have not been given.

pg 40: The process in which the diagnosis of citrullinemia was achieved was more accurately detailed.

pg 42: F535 was characterised in more detail.

pg 43: The concentrations of the various antibiotics were given as mg/ml.

pg 45: The method of maintaining the specific activity in this experiment was clarified.

pg 46: The origin of the pAS1 plasmid was referenced.

pg 50: Liquid scintillation spectrometer.

pg 52: "Solubility" was changed to "dissolution".

pg 55: The correct PCR program was inserted.

pg 57/58: Reference 45 was relocated to indicate it as the reference for SmaI cloning.

pg 58: The reasoning behind the incubations in the transfection of competent cells was simplified.

pg 59: The reference to the alpha fragment of the beta-galactosidase gene was corrected and the description of the "larger" plasmids" improved upon. The description of the PEG precipitation was rewritten to clarify the process.

pg 60: The volume of the DNA sample and the molarity of the Tris.HCl solution were given.

pg 61: "Respective" mixes.

pg 69: "attack" was replaced with "approach".

pg 75: "results not shown" was added.

pg 77: It was stated that the original primers were not designed with "oligo" and that a Hot-start was only employed with the new set of primers.

pg 79: A more accurate quantitation of the number of ASS target molecules was given, and the quantity of DNA used in the magnesium titration was also indicated..

pg 84: "Possibility" was substituted for " hypothesis".

pg 87: The reasoning behind the use of SmaI in the cloning reaction was clarified with regards to the prevention of plasmid religation.

pg 89: The DNA was digested with *PstI* and *ECoRI*, and an uncut pUC18 band was indicated in figure A.

pg 96: 1-1.5 fold and "up to a 0.5 fold decrease".

pg 99: The term Km/Vmax mutant was found to be incorrect and consequently the term "altered" was used.

pg 103: The logic behind the role of glycine in the folding process was rewritten, and the evidence that the mutation was not a sequencing artifact was given.

pg 104: The figure references were corrected and "whole cell" studies were performed. The possibilty that the mutation may cause the protein to become unstable and become more rapidly degraded was included in this discussion.

pg 115: The abbreviation for acetylcholine was corrected and the abbreviation for soluable guanylate cyclase was given.

pg 123: The remnant sentence was removed.

pg 134: The unknown function of BH_4, referred to cytokine-induced BH_4 specifically.

pg 146: Radioactive emission.

pg 147: The purpose of the dashed argininosuccinate lines was explained.

pg 150: The program was descibed in the methods section and was referred to in this figure.

pg 156: The numbers of the various figures were corrected.

pg 157: The last sentence was corrected as suggested.

pg 162: The description of the "dilution effect" was clarified.

pg 172: It was clarified in the text as to the reasoning behind the shortened incubation time for F500 and the length of the incubation was given in the graph legend.

pg 173/176: References to peak Z were corrected to peak Y. The X-axis was also labelled.

pg 178: The number of the diagram showing urea formation was corrected.

pg 185: The correct incubation times were given for the nitric oxide assay and the assay given in the methods section was referred to as the finalised assay.

The following points were not corrected:

pg 19: Table 1 was not a composite table and was taken entirely from reference 11.

pg 20: Glutamate is correct in this context.

pg 32: The word used was pre-translational not pre-transcriptional as suggested.

pg 34: A figure showing the areas of homology between the various mammalian species was not available.

pg 60: KGB buffer was defined on page 48.

pg 71: A diagram of the various primers indicated what fragments they produced.

pg 82: Although the figure could not be altered, the legend was changed to indicate that the two gels are the same indicating that the concentrations given in figure 17B also represent 17A.

pg 93/98: As with all the results sections a brief summary of the experimental procedure was given to explain certain parts to the experiment. The brief introduction to ASS activation was shortened as recommended but was included to explain the rationale of the experiment.

pg 116: The sentence was corrected but the suggested correction changed the meaning of the sentence.

pg 118: The sentence refers to the neurons in the pituritary gland rather than neurons and the pituritary gland.

pg 130-132: It is important to realise that NO itself may not be the biological agent but that other redox forms may play a significant role, that is why the section was included. A description of the p53 gene seemed unnecessary in this context.

pg 159: The section referred to was only a brief introduction and an explaination of the mode of action of cycloheximide and valine was inappropriate. These discussions were given in detail when the experiments were discussed.

pg 163: At this stage cellular supernatant fractions were being collected, the cells were precipitated at a later stage with TCA.

Pg 167: The logic behind the statment was that the method of collecting the counts was inadequate and therefore the results unreliable.

pg 195: 300 dpm does equate to 2.4nM, as the labelled metabolite was mCi/mmol rather than Ci/mmol (given in the methods).

I have checked this point and I am perfectly satisfied that the corrections have been dealt with comprehensively and adequately

E. H. Harley

(Supervisor)

PROFESSOR E. H. HARLEY
DEPT. OF CHEMICAL PATHOLOGY

This thesis is dedicated to Sean and my loving family, for all their patience, love and support. Special thanks to Tony and Ingrid for their encouragment and guidance.

I, **Karen Lesley Shires**, hereby declare that the work on which this thesis is based is original (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or in any other University.

I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate Signature Removed

10 December 1993

CONTENTS:

ABBREVIATIONS:

ADP : Adenosine 5′-diphosphate

amp : Ampicillin

AMP : Adenosine 5′-monophosphate

ASL : Argininosuccinate lyase

ASS : Arginiosuccinate synthetase

ATP : Adenosine 5′-triphosphate

BH_4 : Tetrahydrobiopterin

BME : Basal medium Eagle

bp : Base pairs

BSA : Bovine serum albumin

CanR : Canavanine resistant

cDNA : Complementary deoxyribnucleic acid

cGMP : Cyclic guanosine 3′,5′-monophosphate

cNOS : Constitutive nitric oxide synthase

CO_2 : Carbon dioxide

cpm : Counts per minute

CPS : Carbamyl phosphate synthetase

dCTP : Deoxycytidine 5′-triphosphate

DEPC : Diethylpyrocarbonate

DMEM : Dulbecco's modified Eagles medium

dNTP : Deoxyadenosine, thymidine, cytidine and guanosine 5′-triphosphate mix

dpm : Disintegrations per minute

eNOS : Endothelial nitric oxide synthase

FAD : Flavin adenine dinucleotide

FCS : Foetal calf serum

FMN : Flavin mononucleotide

GTP : Guanosine 5′-triphosphate

HPLC : High performance liquid chromotography

IDO : Indoleamine 2,3 dioxygenase

IFN-γ : Gamma-interferon

IL-2 : Interleukin-2

iNOS : Inducible nitric oxide synthase

IPTG : Isopropyl-B-D-thiogalactopyranosid

LPS : Lipopolysaccharide

LTP : Long-term potentiation

mRNA : Messenger ribonucleic acid

NA-PP : Sodium pyrophosphate

ng : Nanogram

NMDA : N-methyl-D-aspartate

NO˙ : Nitric oxide

NOS : Nitric oxide synthase

OKT : Ornithine keto-amino transferase

OTC : Ornithine transcarbamylase

P-450 : NADPH-cytochrome P-450 oxireductase

pAS1 : Plasmid pBR322 containing human ASS cDNA

PCR : Polymerase chain reaction

PEG: Polyethylene glycol

pg : Picogram

pI: Isoelectric point

PPi : Pyrophosphate

PSN : Penicillin, streptomycin, neomycin

rRNA : Ribosomal ribonucleic acid

SDS : Sodium dodecyl sulphate

TCA : Tri-chloroacetic acid

TLC : Thin-layer chromatography

TNF : Tumour necrosis factor alpha

µg : Microgram

µM : Micromolar

X-gal : 5-Brom-4-chlor-3-indolyl-B-D-galactopyranosid

LIST OF TABLES:

LIST OF FIGURES:

ABSTRACT:

A citrullinemic fibroblast cell line was used in this study to investigate two biochemical pathways involving citrulline. In the first section, the genetic mutation responsible for the argininosuccinate synthetase (ASS) deficiency (1-5% activity) in this cell line was investigated. PCR analysis of the ASS cDNA revealed that the mRNA coding region (1236bp) was intact, showing no signs of major rearrangements. The ASS cDNA (1307bp) was cloned and sequenced and showed the presence of a single base mutation at position 1045bp, which represented a G->A transition. This mutation resulted in a glycine -> serine amino acid substitution at position 324 in the ASS subunit protein sequence. Although this glycine residue was not found to occur in any potential substrate binding sites, it was shown to be highly conserved among species, indicating a possible role of this amino acid in ASS catalytic activity. In the second section, the presence of the nitric oxide pathway in fibroblasts was investigated. Inducible nitric oxide synthase activity was assayed by measuring the production of ^{14}C-citrulline from ^{14}C-arginine after cytokine stimulation. By using the citrullinemic cell line (ASS deficient) any citrulline that may be produced by this pathway would accumulate, allowing detection. Under the assay conditions that were tested, no detectable ^{14}C-citrulline was formed. Evidence suggests that human fibroblasts have the potential to synthesise nitric oxide, although a more sensitive assay system may need to be employed (longer cytokine activation, nitrite/nitrate detection).

INTRODUCTION: CITRULLINE METABOLISM, THE INS AND OUTS.

Citrulline is a basic amino acid that is not directly utilised for the production of protein, but rather plays a role in the synthesis of endogenous arginine and ureagenesis, the process by which waste nitrogen is converted to urea and excreted from the body [1]. The enzymes that are responsible for the metabolism of citrulline include argininosuccinate synthetase and argininosuccinate lyase, two enzymes of the urea cycle shown in figure 1.

UREAGENESIS AND ENDOGENOUS ARGININE SYNTHESIS.

The major source of circulating citrulline is supplied by the intestine which converts glutamine into the urea precursors - ammonia and citrulline [1,2,3], both of which are secreted into the portal circulation (figure 2). Although the liver and a few other tissues possess the enzymes that are able to synthesize citrulline [1,4], extra-intestinal citrulline is not normally found in the circulation [3]. Under abnormally low citrulline concentrations in the blood however, it has been found that citrulline storage pools in the cerebrospinal fluids and skeletal muscle can be stimulated to release citrulline [3].

The major site of ureagenesis is in the liver, where ornithine is used directly to produce the citrulline necessary for the urea-cycle to proceed [4,5]. The circulating intestinal citrulline passes straight through the liver, with little uptake being evident [3].

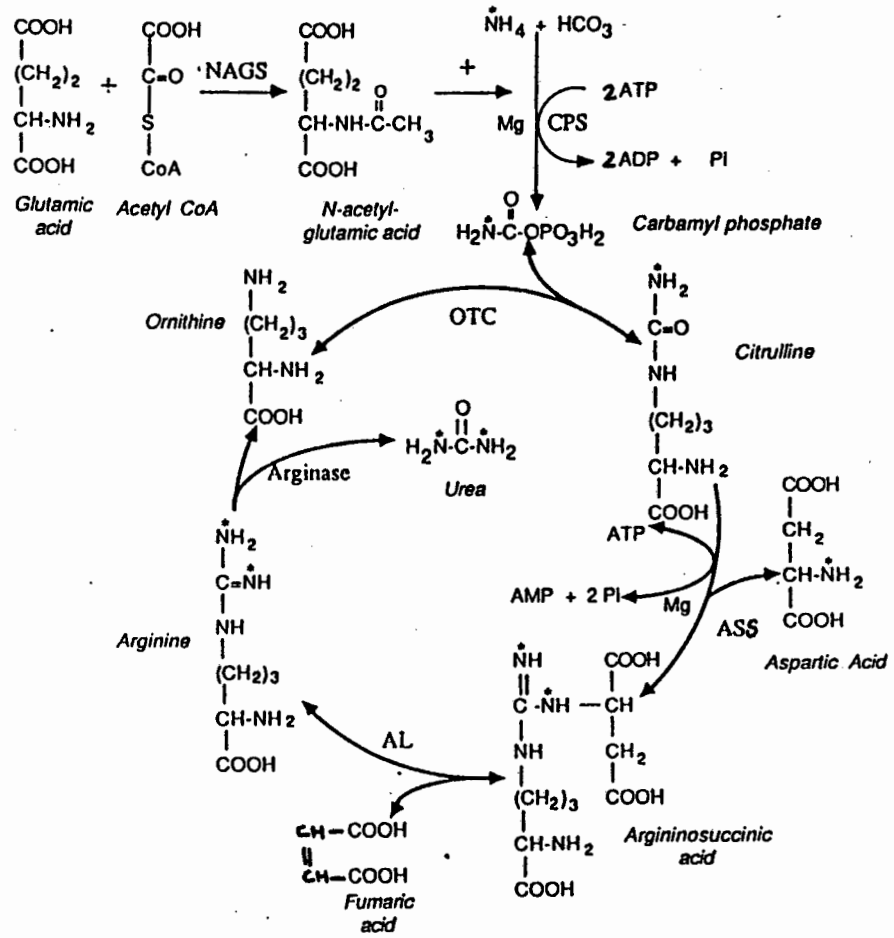

FIGURE 1: UREA CYCLE.

The substrates, products and co-factors required for ureagenesis. ASS = argininosuccinate synthetase, AL = argininosuccinate lyase, CPS = carbamyl phosphate synthetase, NAGS = N-acetylglutamate synthetase, OTC = ornithine transcarbamylase. This figure was a modification from the figure given in reference [1].

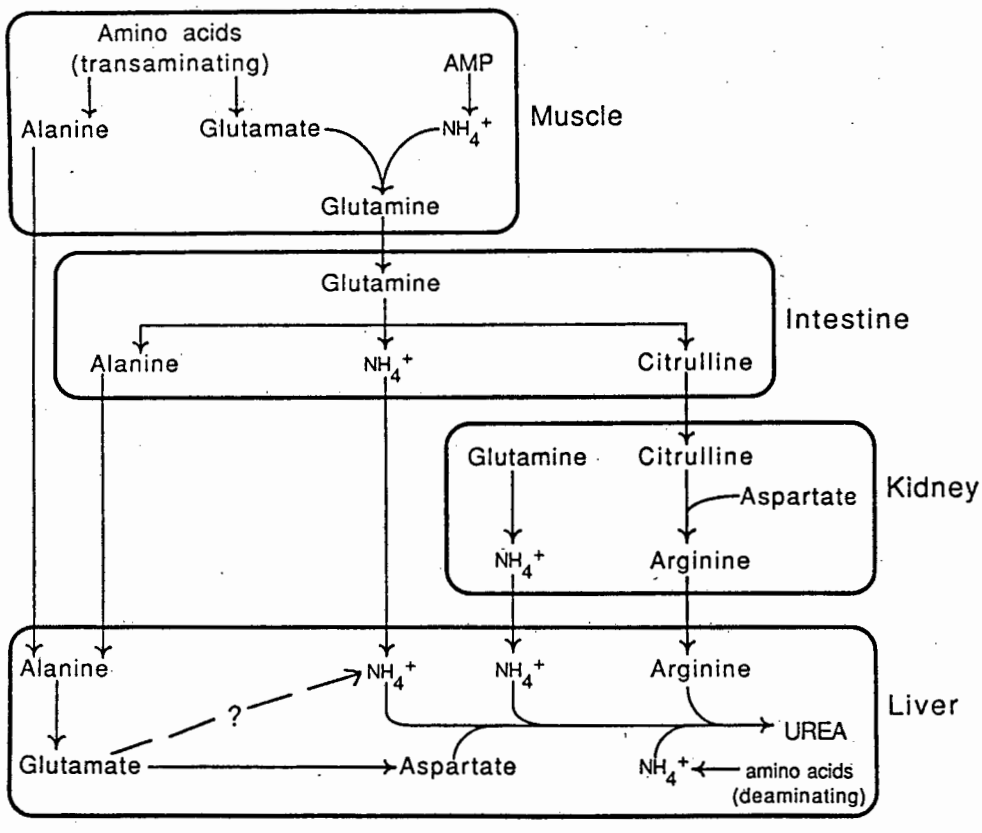

FIGURE 2: The pathway of waste nitrogen synthesis from amino acids.

Diagrammatic representation of the participation of the intestine , kidney and the liver in the utilisation of citrulline [1].

The fate of circulating citrulline under normal conditions resides in its utilisation by the kidney, in the production of endogenous arginine for protein synthesis. It has been reported that up to 83% of the citrulline released from the intestinal tract is taken up by the kidney, most of which is converted to arginine [3]. The kidney is thus an important organ in the metabolism of citrulline.

However, the kidney is probably not the only organ with the ability to extract and use large amounts of citrulline. In a study performed on rats whose kidneys had been removed [3], the amount of circulating citrulline rose by only 17% thirty minutes after kidney removal, instead of the expected 80%. This result suggested that under these conditions, other systems are brought into operation to compensate for the arginine loss and the excessive citrulline build-up. Several pathways were proposed in this study [3] such as the utilisation of citrulline by the liver or the use of enzymes in other tissues.

The brain is able to participate in citrulline metabolism as it possesses both argininosuccinate synthetase and argininosuccinate lyase enzymes, allowing for the synthesis of arginine from citrulline [1,3]. Other tissues and individual cell types (fibroblasts [1,6] and lymphoblasts [1]) that possess these two urea cycle enzymes are also capable of producing endogenous arginine for protein synthesis and other metabolic pathways.

NITRIC OXIDE PATHWAY.

In the past, citrulline was only known to be associated with ureagenesis and arginine synthesis. However, since the discovery of the nitric oxide pathway, an important new source of citrulline has been discovered. The nitric oxide pathway involves the metabolism of arginine by nitric oxide synthase to produce equal quantities of $NO^{\cdot}$ and citrulline. The $NO^{\cdot}$ produced by this pathway is the active metabolite, participating in signal transduction and the immune defense system. The citrulline is probably released into the circulation where it is utilised by other tissues for arginine synthesis. Citrulline may in fact play a direct role in the perpetuation of the $NO^{\cdot}$ response, since extracellular arginine (0.2-0.4mM) [7] is essential for nitric oxide synthase activity. Thus in tissues that possess the necessary urea cycle enzymes (ASL and ASS), a constant level of arginine for $NO^{\cdot}$ production can be maintained.

CITRULLINEMIA.

Citrullinemia is one of the diseases associated with defective citrulline metabolism and is caused by argininosuccinate synthetase deficiency. The disease is an autosomal inherited metabolic disorder characterised by elevated ammonia and citrulline levels in the urine, plasma and cerebral spinal fluids and often leads to retardation and in severe cases, death [1].

The cause of the argininosuccinate synthetase deficiency has been studied in several cases of citrullinemia [1,8,9,10],

revealing that the mutations leading to enzyme inactivity are heterogenous. The mutations range from gene-rearrangments to single base mutations that are scattered along the length of the argininosuccinate synthetase coding region. These mutations also have a variety of effects from complete gene inactivation to reduced levels of mRNA production and protein inactivation.

THESIS AIM.

The purpose of this thesis was to study several aspects of citrulline metabolism, using normal and mutant fibroblast cell lines in order to gain insight into elements of the urea cycle in these cells and to assess their ability to synthesize nitric oxide. Each section contains a review and a detailed introduction explaining the aims and rationale behind each set of experiments. A brief overview is given below.

The first section of the study involves the investigation of the mutation responsible for the argininosuccinate synthetase deficiency in the citrullinemic fibroblast cell line F25. The purpose being to gain insight into the catalytic mechanisms of argininosuccinate synthetase, which are ill-defined at present. Molecular biology techniques were employed to investigate this mutation at the gene level.

The second section investigates whether activation of the nitric oxide pathway can be demonstrated in fibroblasts. The argininosuccinate synthetase deficient cell line F25 was used in the study, allowing for the correlation of citrulline

accumulation in these cells, to $NO^{\cdot}$ production under specific conditions.

In an attempt to optimize the conditions necessary for studying the nitric oxide pathway in fibroblasts, other aspects of urea cycle metabolism in fibroblasts were investigated. Ornithine transcarbamylase activity as well as arginase activity and protein synthesis were assessed in this respect.

SECTION 1

CITRULLINEMIA: THE PIN-POINTING OF A POINT MUTATION.

1.1 REVIEW: ARGININOSUCCINATE SYNTHETASE - CITRULLINE METABOLISM.

1.1.A: ARGININOSUCCINATE SYNTHETASE - THE PROTEIN.

A.i: Expression of Argininosuccinate synthetase.

Argininosuccinate synthetase (ASS) is one of five enzymes of the urea cycle (shown in figure 3) and is responsible for the catalysis of the following reversible reaction:

ATP + citrulline + aspartate -> argininosuccinate + AMP
<- + PPi

This reaction occurs in the cellular cytoplasm, as do the reactions catalysed by argininosuccinate lyase (ASL) and arginase. The reactions cataylsed by the other urea cycle enzymes: carbamyl phosphate synthetase (CPS) and ornithine transcarbamylase (OTC), occur in the mitochondrial matrix [11].

In the liver, ASS is produced in large quantities as a component of the complete urea cycle, that serves to dispose of ammonia. However, much lower levels of ASS are found in peripheral tissues that do not possess the full complement of urea cycle enzymes. Table 1 [11] shows the levels of ASS activity found in various rat and human tissues. In these peripheral tissues, ASS in combination with ASL serves to synthesize arginine from citrulline [12]. This arginine is then used for protein synthesis and other metabolic pathways.

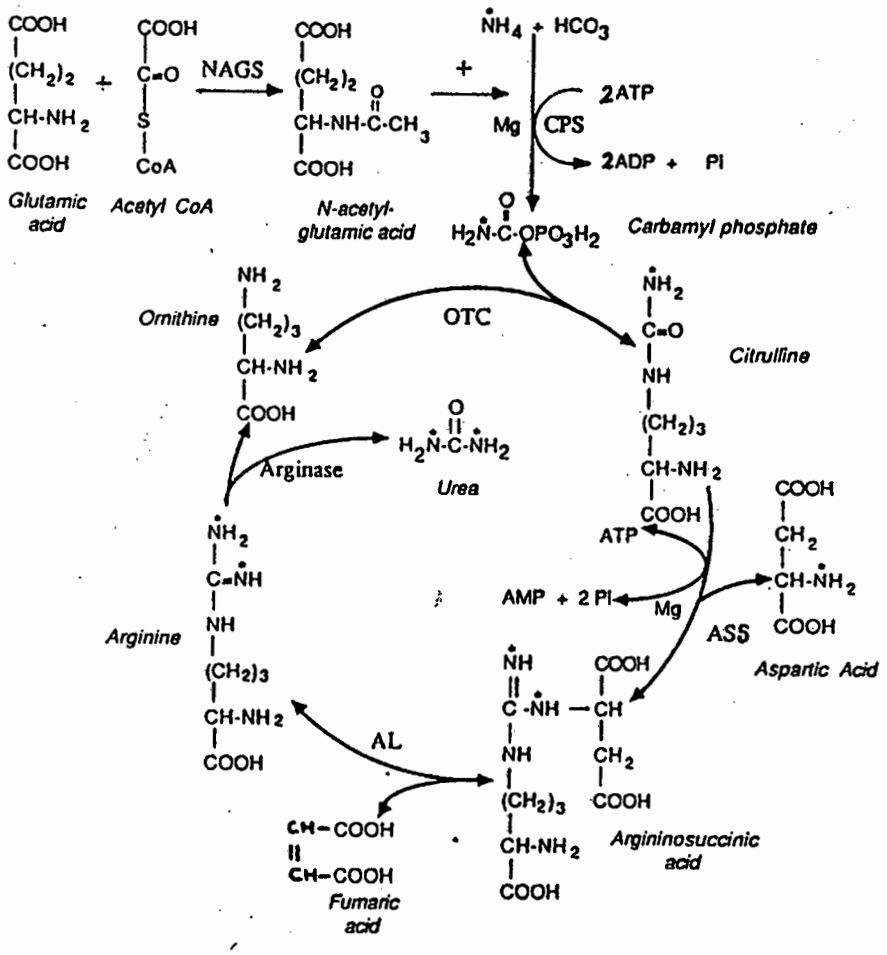

FIGURE 3: UREA CYCLE.

The substrates, products and co-factors required for ureagenesis. ASS = argininosuccinate synthetase, AL = argininosuccinate lyase, CPS = carbamyl phosphate synthetase, NAGS = N-acetylglutamate synthetase, OTC = ornithine transcarbamylase. This figure was a modification from the figure given in reference [1].

TABLE 1: ASS ACTIVITY IN MAMMALIAN TISSUES.

SOURCE:	SPECIFIC ACTIVITY: (nmol/hr/mg) at 38'C
RAT:	
LIVER	1386.0 +/- 95
KIDNEY	667.0 +/- 55
SMALL INTESTINE	68.3 +/- 2.5
LARGE INTESTINE	35.3 +/- 3.4
BRAIN	50.6 +/- 4.6
LUNG	21.7 +/- 2.1
SPLEEN	10.5 +/- 0.6
HUMAN:	
LIVER	1080.0 +/- 174
FIBROBLAST(MGF1139)	81.2 +/- 5.3
FIBROBLAST(MGF591)	192.5 +/- 19

The active human ASS enzyme of 183000Da in size, exists as a tetramer of four identical subunits (42,800Da each) of 412 amino acids each [13], with the two pairs of subunits linked via s-s bonds [14,15]. It has an unusually high content of basic amino acids resulting in a pI of 9 [13] and appears to undergo no post-translational modification [16].

A.ii: ASS enzymology.

The exact nature of the catalytic mechanisms and the amino acid residues involved in the active site of this enzyme have not yet been clearly defined. However, the following information is available: The enzymatic reaction proceeds in a two-step manner as follows [17]:

A1) citrulline + MgATP -> citrulline-AMP + MgPPi + 2H+

B2) citrulline-adenylate + aspartate -> argininosuccinate + AMP.

This two-step reaction occurs in an ordered manner, shown below in figure 4 with the sequential addition of the substrates: MgATP, citrulline, aspartate, followed by an ordered release of the products: argininosuccinate, MgPPi, AMP [18,19]. No products are released until all the substrates have bound to the enzyme [19].

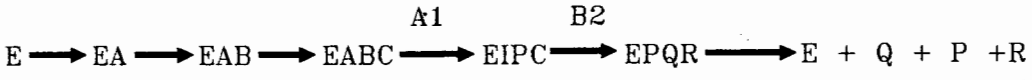

FIGURE 4: Proposed kinetic scheme of the argininosuccinate synthetase reaction [19].

E = ASS, A = MgATP, B = citrulline, C = aspartate, I = citrulline-adenylate, P = PPi, Q = argininosuccinate, R = AMP.

The first reaction **(A1)** represents the activation of citrulline by ATP to form a tightly bound enzyme-citrulline-adenylate complex [17]. Although the citrulline-adenylate complex can be formed in the absence of the enzyme-bound aspartate, aspartate increases the rate of production of this complex 600-fold. Glutamine synthetase also exhibits similar enhancement, with the rate of formation of g-glutamyl phosphate from glutamine and ATP being increased 35-fold in the presence of NH_3 [17].

The PPi formed in this reaction **(A1)**, is not released at this stage, but remains tightly bound to the enzyme. This is unusual as amino acid activation normally proceeds with an amino acid dependant ATP-PPi exchange [14]. It is only after

condensation of aspartate with the iso-ureido group of the citrulline-adenylate complex that argininosuccinate is formed as PPi and AMP are released in the second step of the reaction **(B2)** [19]. Kumar, Lennane and Ratner [15] suggested that upon condensation with citrulline, aspartate causes a conformational change, which results in the weakening of the binding affinity between ASS and PPi. This then allows the release of the pyrophosphate group.

The active ASS protein exists as a tetramer, with one active site per subunit. Contained within each subunit there exists a cysteine residue that has been shown to be essential for catalytic activity (all four cysteines are required) [18]. Each subunit of the tetramer also contains an arginine residue that is essential for ATP and PPi binding and that for optimum catalysis all four active sites must be occupied by either ATP or PPi [15]. Arginine residues 153 and /or 157 have been implicated in ATP binding through modification studies, and these residues occur in a region that has homology with other ATP-binding domains for other enzymes [9]. Enzymes such as aspartic and ornithine carbamyl transferases, as well as glutamine and carbamyl phosphate synthetase, possess arginine residues that are important in binding anionic co-factors and other substrates [15].

ASS is activated by the presence of divalent cations. One role of these cations is the formation of complexes with ATP. However, it has also been shown that the synthetase requires the binding of an additional uncomplexed Mg^{2+} ion to achieve maximum activity. The

function of this additional cation is not yet known. It could either be involved in the catalytic mechanism or fulfill a structural role [20]. Glutamate synthetase, carbamyl phosphate synthetase, pyruvate kinase as well as several other enzymes also require an additional uncomplexed metal ion, but at present its function in these enzymes is also unknown [20].

A.iii: ASS regulation at the protein level.

ASS displays negative co-operativity for its three substrates. This is when the binding of one substrate molecule to an oligomeric protein with two or more catalytic sites, causes additional substrate molecules to bind with diminished affinity at the other catalytic sites [14]. This mechanism enables an effective kinetic response over a wide range of substrate concentrations. Due to the amplitude of the kinetic response exhibited by this enzyme, it suggests that negative homotrophic behaviour may play a significant role in regulation of ASS activity [14].

In kinetic studies on the human liver ASS enzyme, it has been demonstrated that ASS, like many other synthetases is inhibited by ADP and AMP. These nucleotides act as competitive inhibitors of ATP-binding when aspartate and citrulline are at saturating concentrations. However, this effect is diminished at lower non-saturating concentrations of citrulline and aspartate and affinity data suggest that this is due to the affinity of the enzyme for the inhibitors decreasing at the lower substrate concentrations. This

suggests that a conformational change causes the loss of affinity for the ADP and AMP nucleotides. It is interesting to note that the affinity of the enzyme for ATP remained unchanged over the concentration range of citrulline and aspartate [14].

Comparisons of amino acid sequence and cDNA sequence data of enzymes from various organisms, makes it possible to indicate regions that may be important for enzyme function by their high degree of homology among the various organisms. Comparisons have been done between various organisms for the ASS enzyme and although they show large differences at the overall amino acid primary sequence level (*E.coli*/man = 26% homology, rat/man = 97%, yeast/human = 49%), several regions of high homology have been located - figure 5. Using the information about the presence of a cysteine and an arginine residue at the active site, these conserved areas were evaluated as possible active sites. Although no cysteine residues were seen in these regions, three possible reactive arginines were found to occur in the subunit. Using copmputer modelling programs to locate possible substrate binding domains, two of the highly conserved regions showed substrate-binding capabilities [18].

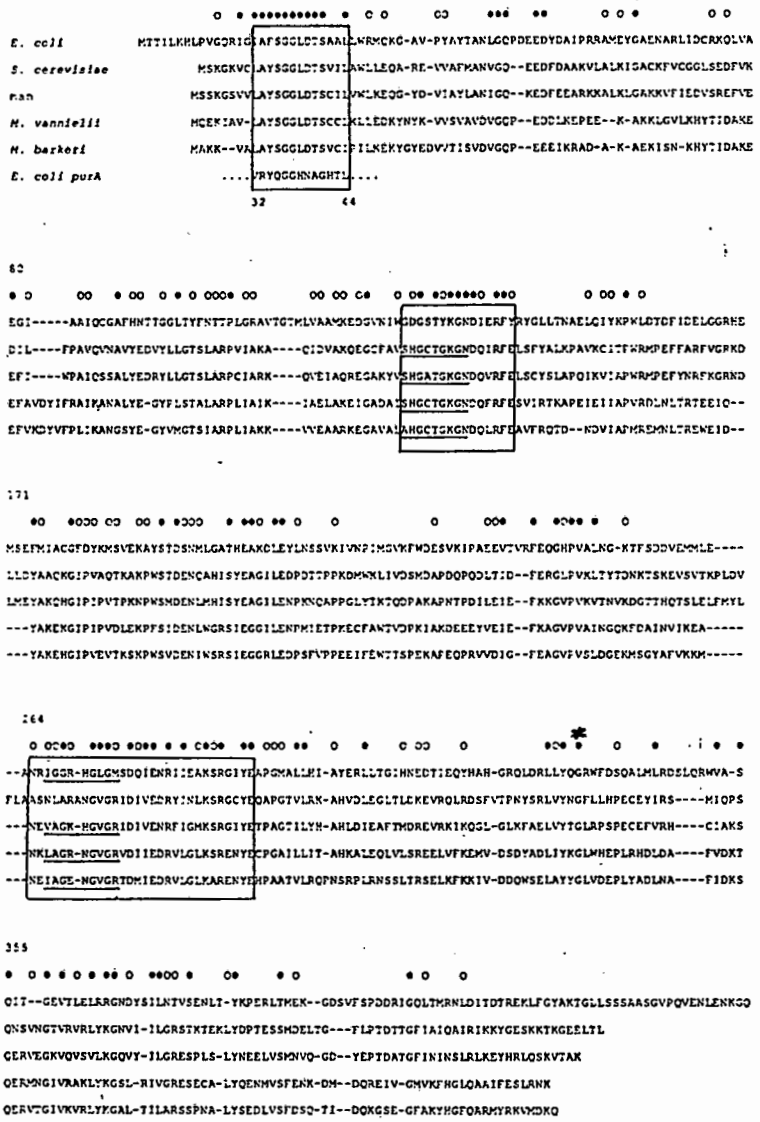

FIGURE 5: Comparison of the ASS-subunit amino acid primary sequences [18].

The ASS amino acid sequences from the following organisms were aligned: Human, *Escherichia coli*, *Saccharomyces cerevisiae*, *Methanosarcina barkeri* and *Methanococcus vannielii* (archeobacteria), revealing several areas of homology. The boxed areas indicate areas of high homology. The under-lined regions are possible substrate binding sites within the ASS subunit and the symbols indicate possible reactive arginine residues (one arginine per subunit is involved in ATP/ADP binding). The mutated glycine residue in F25 is indicated by a * symbol.

1.1.B: ARGININOSUCCINATE SYNTHETASE - THE GENE.

B.i: Location and gene product.

The actively transcribed human ASS gene has been located to the q34 region of chromosome 9 [1] and is 63 kB in size. Some controversy seems to have developed over the years as to the number of exons in the active gene, with researchers reporting between 11 and 16 [1,9,16,21,23]. However, the consensus at the moment seemas to be that there are 16 exons. The first two exons code for untranslated regions and are alternatively spliced [21]. In most human cell lines exon 1 and 3 are spliced together (type B mRNA), deleting the second exon. This alternative splicing however, seems to be species specific with baboon liver expressing 99% type A mRNA (exon 1-2-3) while human liver 99% type B mRNA (exon 1-3), but the levels vary a little between human cell types [21]. The biological significance of this alternative splicing has not yet been elucidated [16]. The other 13 exons encode a mRNA of 1600 bases, that has an open reading frame of 1236 nucleotides [1]. Exon 3 possesses the translational start site for the production of a 412 amino acid protein subunit.

B.ii: Pseudogenes.

There are 14 different pseudogenes of the ASS gene, and they are located at multiple loci in the genome. These include two loci on chromosome 9, where the active gene is located, two on the X chromosome and one on the Y chromosome [22]. Seven of these pseudogenes have been investigated and they are all

less than 2Kb in length (as opposed to the expressed gene of 63Kb), belong to the second class of pseudogenes (intronless) and represent processed pseudogenes [21,22,23]. These pseudogenes are highly homologous to the mRNA encoded by the active gene, with one of the sequenced pseudogenes showing 94% homology with the ASS cDNA [23]. Figure 6 shows the comparison between the cDNA from the active gene and three of the pseudogenes, demonstrating the high degree of homology between the sequences. As can been seen in this figure, most of the differences are single base changes or small insertions and deletions. No major rearrangements have occurred.

Due to the structure and sequence of these processed pseudogenes it has been proposed that these copies arose through a mature mRNA intermediate, that was then reintegrated into the genome. This could have arisen through the action of retroviruses or transposons. Possibly multiple copies arose from a single reverse transcription event followed by duplications and then integration [23].

ASS pseudogenes are not unique to humans, but have also been found in the genomic DNA of chimpanzees. From this evidence and by analysing the mutation rates of the pseudogenes, it has been postulated that the pseudogenes evolved some 10-20 million years ago, before the divergence of the great apes from the hominids [23].

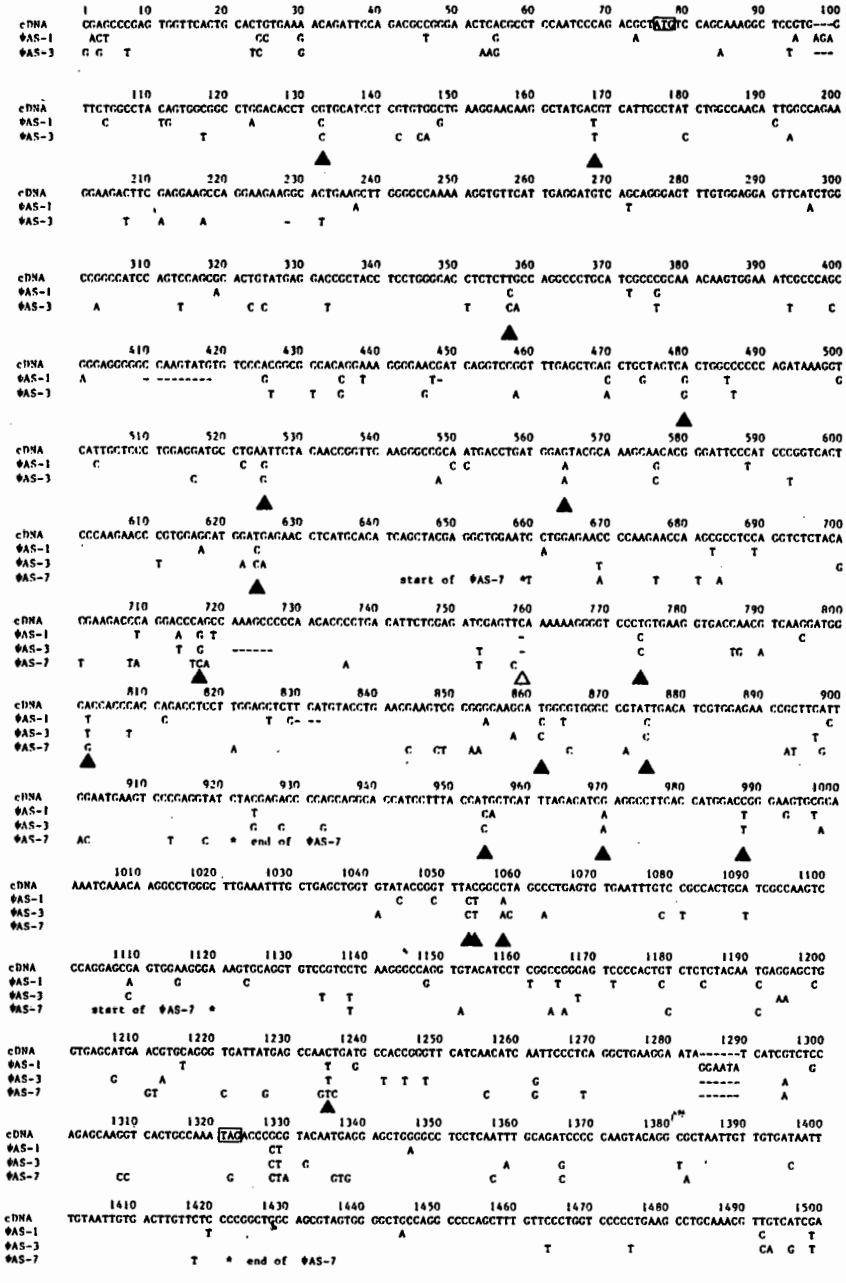

FIGURE 6: A comparison between the nucleotide sequence of human ASS cDNA and human ASS pseudogenes [23].

A comparison of the nucleotide sequence of human ASS cDNA with the sequences of human ASS pseudogenes As-1, AS-3 and AS-7. The cDNA sequence is shown here as well as the bases that differ in the respective pseudogenes. ▲ represents similar base differences between AS-1 and AS-3. Base 3 on this diagram represents base 1 on sequences derived from Genbank.

Strong evidence suggests that there is only one active locus and that none of these other ASS-like genes give rise to the enzyme product. This evidence includes the genetic characteristics of the metabolic disease citrullinemia (ASS deficiency) which is an autosomal recessive disorder, suggesting one active locus [1]. Mutational analysis of cultured fibroblasts with defects in the structural gene, have shown that all the detectable mRNA was derived from one locus [1,9]. Studies on probing for gene duplication have also all been negative [13].

B.iii: Gene regulation.

Two independant mechanisms of ASS gene regulation have been elucidated so far. One is the arginine-mediated repression that has been demonstrated in some cultured cell lines: hamster fibroblasts, human lymphoblasts and the human squamous cell carcinoma cell line RPM1 2650 [1,24,25]. This mechanism is evident by the increase in ASS enzyme activity when the cells are incubated for > 96 hours in arginine-free medium (0.6mM citrulline) [25]. The range of ASS activation in the absence of arginine, is between 3-60 fold depending on the cell type [26].

The other mechanism of regulation of ASS at the gene level is seen in canavanine resistant (CanR) cultured cells. These cells are resistant to the toxic effects of canavanine (an arginine analog) and show different regulatory ASS features from the wild type ASS enzyme. The ASS activity in these cells is 200 fold increased relative to the parental cell

line, this level being on a par with the ASS activity found in normal liver cells [1]. The enzyme activity in CanR cells is also unresponsive to the arginine-mediated repression mentioned above. At this stage it has been postulated that the mechanism that is responsible for increased ASS activity in CanR cells is the same as that in liver cells and may be responsible for the tissue-specific levels of expression in the various cell lines [27].

B.iii.a: Arginine-mediated repression.

It was initially proposed that the increased activity in CanR cells was due to the loss of the arginine-mediated repression seen in other cultured cell lines. This confused the issue when investigators were trying to elucidate the ASS gene regions responsible for the arginine-mediated repression using CanR cells [24,25]. Boyce et al [26] used constructs of the 5'untranslated regions of ASS and the CAT gene (reporter gene) and were able to approximately locate the area responsible for the arginine-negative feed-back mechanism. However, they clearly demonstrated that this element was separate from the element involved in the increased levels of ASS activity in CanR cells.

Since the elucidation of the two separate mechanisms, a number of studies have been performed on the two regulatory mechanisms. With regards to the arginine-mediated repression several facts have emerged: Firstly, the increase in ASS activity in the absence of arginine and the presence of citrulline is not due to citrulline activating the ASS locus,

but is due to arginine-regulation of ASS activity. Secondly, arginine-mediated repression of ASS occurs at the pre-translational level, by controlling ASS mRNA levels and is ASS-specific (does not effect ASL) [26].

Using ASS-CAT promotor fusion constructs, Boyce et al [26] demonstrated that the arginine-regulatory unit was contained in 150bp 5' to the start of exon 1, in the region of the transcriptional start site. This is similar to other systems that are regulated by trans-acting factors, where the binding sites of the trans-acting factors are found within 300bp of the transcriptional start site on the DNA.

Originally, it was proposed that the alternative splicing of exon 2 played a role in this arginine-repression in the different cell types. Exon 2 contains three arginine-codons and it was proposed that by a mechanism of attenuation involving Arg-tRNA, the stability of the ASS could be altered, resulting in the varied ASS activities under different conditions [1,13]. Regulation through tandem codons for a regulatory amino acid is known in bacterial amino acid operons, which are regulated by attenuation and in other regulatory mechanisms that operate in yeast and the SV40 virus [13]. However, Boyce et al showed this idea to be redundant due to the absence of exon 2 in the above mentioned arginine-reactive constructs [26].

A sequence that is more likely to be relevant to arginine repression is TGTGAACGC, at position -137 to -129. This sequence is similar to an element found in promotor regions

of yeast genes that are derepressed during amino acid starvation. Deletion of this sequence in plasmid-constructs resulted in loss of arginine repression [1].

Other regulatory motifs are also found in the 5' untranslated promotor region and these include: TATAA at -30 [18] and multiple GC boxes or hexanucleotide core sequences for SP1 binding. An octomeric sequence of AGAAGTGA at -470 was also identified in ASS and is also present in genes encoding factor VIII and albumin. These sequences are common to house-keeping genes (GC elements) and genes that are expressed in a tissue-specific manner (TATAA and octomeric sequence) [1,16].

B.iii.b: Canavanine - resistance.

CanR cells have been derived from a variety of cell lines. These cells manage to avoid the toxic effects of canavanine by increasing the synthesis of arginine, therby diluting the effects of the arginine analog [26].

The basis of CanR is at present not completely understood. CanR cells express up to 200 fold increased levels of ASS activity and this is directly correlated with an increase in cytoplasmic ASS mRNA [24] rather than to an alteration in ASS anzyme kinetics [28,29]. No major genetic rearrangement has occurred in these cells and gene-duplication as a way of explaining the increase in mRNA levels has also been excluded [29,30]. CanR is a stable feature suggesting the occurence of a genetic mutation [1].

The mechanism that governs the increase in mRNA production in CanR cells acts at the pre-translational level, this could involve increased transcription, increased RNA stability or altered nuclear RNA processing [1].

In RPMI 2650 cells (a squamous carcinaom cell line), where there are as few as three copies of ASS mRNA per cell and the CanR variants express at least 500 copies, Boyce and Freytag [27] showed that the half-life of each set of mRNA species (CanR and parental mRNA) is almost identical (12-24 hours). This result indicates that a change in cytoplasmic mRNA stability is not the mechanism responsible for the CanR, although an increase in the stability of the nuclear ASS RNA cannot be excluded [29]. Transport of ASS RNA from the nucleus to the cytoplasm is also not altered in these mutant cells. These results suggest that the mechanism operating here is either at the level of initiation of transcription, elongation or at some point prior to polyadenylation [27]. it has been reported that changes in elongation rates can affect mRNA stability and therefore affect gene expression [31].

Cellular hybridisation experiments have demonstrated that the CanR mechanism involves a positive trans-acting factor and that increase levels of ASS mRNA are not due to the loss of a repressor [26,27]. This trans-acting factor has been shown to be limiting, ASS specific and operational in the nucleus [32].

CanR cells are not responsive to arginine levels. Two scenarios are possible in the explaination of this finding: One being that the positive trans-acting factor is dominant over the arginine-repression mechanism and the other being that these cells produce enough arginine to become insenstive to the extracellular levels of arginine [26].

The DNA element that binds the positive trans-acting factor does not appear to form part of the ASS promotor, which is where the arginine-repression occurs. Instead it has been proposed that the element is intragenic, occurring within one of the exons [26]. One possibility is that in CanR cells the trans-acting factor increases in efficiency rather than quantity i.e: a change in affinity for the factor. Perhaps the factor recognises a unique DNA secondary structure [30,32].

The importance of establishing the mechanism responsible for the increased ASS activity in CanR cells lies in the fact that these cells show the same level of ASS activity and the high level of ASS mRNA that occurs in liver cells. In fact it has been proposed that the mechanism responsible for the high ASS activity in hepatocytes is the same as that in CanR cells. Further evidence in support of this idea is that a dominant, positive acting factor that operates in the nucleus, has also been shown to be operative in hepatocytes in the production of high levels of cytoplasmic ASS mRNA [27]. The conclusion that CanR/ASS regulation in hepatocytes operates at the post-transcriptional level fits in very well with other regulatory systems operating predominantly or

exclusively in the liver, including regulation of : albumin, transferrin and phosphoenolpyruvate carboxykinase activity [27].

It has been speculated that this mechanism is linked to cell-differentiation and that CanR cells represent a more differentiated cell type than the parental cell line. In other words the mutation that has occurred in these cells has activated a mechanism that forms part of cell-differentiation, probably towards liver differentiation [27].

It is clear that more work needs to be done to specifically locate the mechanism responsible for the increased activity of ASS in hepatocytes and CanR cells. It is important to elucidate this mechanism since it could contribute to the treatment of patients suffering from citrullinemia, which is a disorder resulting from the partial or complete loss of ASS activity.

B.iii.c: 3′ Regulatory sequences.

Using comparisons between the cDNA sequence data of ASS from four mammalian species: murine, rat, bovine and human, two significant regions of homology have been found in the 3′ untranslated region. One is 57bp 3′ to the termination sequence and consists of 74% A+T in a stretch of 42bp. The other occurs 50bp further downstream, with a 50% A+T content. Both of these areas share an almost 100% homolgy between the four species. A+T rich regions have often been associated with rapid turnover of short-lived mRNA species, such as

those involved in the inflammatory response. However, the AUUUA motif that is particularly associated with these mRNAs is not present in ASS. It has also been shown that the 3'untranslated regions may play a role in translational efficiency, as has been shown in β-interferon and others. The fact that these areas are highly conserved suggests that these regions must have some functional importance, however no other genes have been identified so far that have closely related sequences to these found in ASS [16].

1.1.C: CITRULLINEMIA.

C.i: Disease definition.

Citrullinemia is an autosomal recessive disorder caused by the partial or total loss of argininosuccinate synthetase activity. This disease is characterized by an accumulation of citrulline in the blood (at least 40 fold in the plasma), cerebral spinal fluid (100 fold elevation) and in the urine. It is also accompanied by an elevation of the concentration of ammonia in the blood (hyperammonemia) [1].

The classic neonatal form of this disease is characterized by mental retardation, hyperammonemia and early death [11]. The infants seem unaffected at birth, but become irritable and lethargic in the first few days of extrauterine life, showing signs of a depressed neurological state. Convulsions may occur followed by coma and death within the first week of life. Metabolic acidosis, hypoglycemia and hypocalcemia can occur and small microscopic brain lesions from the degradation of nerve cells and myelin are observed. Delayed

myelin formation and the presence of enlarged glial cells with intracellular lipoid accumulation have been seen in some cases [1].

There are several other forms of citrullinemia: subacute, asymptomatic and atypical. These forms are loosely defined and basically reflect the stage of onset of the symptoms, with the subacute type presenting with a gradual onset of neurological symptoms; tremors, seizures, within the first weeks/months of life. Asymptomatic types present with elevated citrulline levels in the relevant fluids, but present no other symptoms, at least in early childhood. The atypical type shows elevated ammonia and citrulline levels, with no apparent liver dysfunction, while slurred speech and delirium may occur without obvious neurological damage [1].

It is interesting to note that the disease shows a male predominance, with a male:female ratio of 10:3 [1].

C.ii: Citrullinemic ASS mutations.

Several assay systems exist for the kinetic analysis of ASS, however they are not frequently used for the analysis of citrullinemic patient cell lines, due to the relatively low level of activity of ASS in normal cultured cells (fibroblasts and lymphocytes) (table 1). However, one kinetic study [13] was performed comparing a normal and citrullinemic liver extract. The results showed a sigmoidal kinetic dependance on the citrulline concentration with an increased Km for citrulline in the patient, when compared to the normal

hyperbolic kinetic dependance in the control ASS. The thermal stability of crude extracts of ASS in the patient was also decreased when compared to the control. The kinetics of ASS in another citrullinemic patient was also studied and revealed an increased Km for citrulline (25-200 fold increase) and aspartate (200 fold) [1]. The normal Km for citrulline in human liver = 0.03mM [1].

Most analyses have been performed at the molecular level, investigating the patient DNA and mRNA. Using molecular biology techniques a full range of mutations have been associated with this disease, ranging from large deletions to single base changes. Table 2 [9] shows some of the mutations that have been identified.

TABLE 2: SUMMARY OF CITRULLINEMIC MUTATIONS

A: Single base changes in ASS cDNA.

ALLELE:	NORMAL:	MUTANT:
G14 -> S	gGC(Gly)	aGC(Ser)
S180 -> N	AgC(Ser)	AaC(Asn)
R157 -> W	CgC(Arg)	CaC(His)
R304 -> W	cGG(Arg)	tGG(Trp)
G324 -> S	gGT(Gly)	aGT(Ser)
R363 -> W	cGG(Arg)	tGG(Trp)
G390 -> R	gGG(Gly)	aGG(Arg)

B: ASS cDNA deletions.

EXON:	DELETION SIZE (bp):
5	189
6	57
7	75
13	132
16	7*

* The result of a splice junction mutation in intron 15 [9].

This molecular heterogeneity is in sharp contrast to many other disorders such as Tay-Sachs disease, cystic fibrosis and Gaucher disease where only a few mutations are responsible for a large proportion of the mutant alleles [9]. The exact frequencies of the various mutations in ASS have not as yet been detailed since large population studies have not been performed.

Due to the presence of a large number of pseudogenes with high percentage homologies to the active gene [23], the Southern-blot technique is not often used to look for gross gene abnormalities. Instead S1-nuclease protection assays [10], mRNA analysis [9] and sequencing of the patient cDNA are preferentially used to characterise patient defects [9].

One of the more common defects is that of abnormal RNA splicing [10] giving rise to abnormal mRNA products. This can arise from single base substitutions within the splice acceptor sites at the ends of introns [8] and often leads to a protein reading frame shift, causing the production of an abnormal protein. The interesting point about these abnormally spliced mRNA molecules is that they are very stable, even if entire exons have been deleted [9].

Single base substitutions have also been found in several citrullinemic patient mRNAs and they are fairly scattered along the length of the mRNA. Table 2 shows some of the single base changes, which are at this stage uninformative with regards to locating key regions of ASS regulation or catalytic activity.

One of the important points to emerge from the analysis of citrullinemic patient cDNA, is that the 3′ terminus of the protein is essential for ASS enzymatic function and is important for maintaining stability of the ASS protein [8].

1.2: INTRODUCTION.

The aim of the experiments in this section of the study, was to establish the cause of the ASS deficiency in the human fibroblast cell line F25. This cell line was established in 1984 from a patient who died within the first week of life, after presenting with the clinical symptoms of citrullinemia. An inital diagnosis of ASS enzyme deficiency was achieved using a citrulline uptake assay which was later confirmed using an assay based on the complementation between ASL deficient fibroblasts and ASS deficient cells [33], the basis of which is discussed in section 1.4. The results of these two assays are shown in figure 9 and 11 respectively and reveal the extent of the ASS enzymatic deficiency that was initially found in the F25 cell line (1-5% of control activity). The purpose of the present study was to establish the cause of this ASS enzyme deficiency at the molecular level.

Mutations that can cause enzyme deficiencies can be characterised at several levels: 1) Protein analysis, which investigates the changes in ezyme kinetics (Km,Vmax) or alterations to the secondary and tertiary structure of the protein. 2) Genetic analysis, to establish the mRNA sequence and consequently the amino acid squence of the protein. the latter allows for the identification for large deletions in the coding region of the gene, point mutations or promotor defects in altered gene expression.

Kinetic analysis of ASS is normally performed on liver extracts, as hepatocytes possess the highest ASS activity. In peripheral tissues, including fibroblasts, the enzyme activity is much lower, 10-100 times lower (table 1) and is therefore more difficult to assay accurately [11]. In the study of F25, the problem of low activity is further compounded by a recorded 1-5% ASS activity of normal (figure 9). It has also been documented that the ASS Vmax values of control fibroblasts vary quite extensively, as seen in table 1 [11], making accurate assessments of patients' results very difficult. Due to these problems, accurate kinetic analysis of the ASS enzyme in the F25 cell line was not performed, although a crude assessment was attempted later in the study. Instead the mutation was investigated at the genetic level.

At the DNA level, mutations associated with citrullinemia can manifest themselves in the form of gene rearrangements or promotor defects that render the gene inactive. At the mRNA level an inactive gene product can result from splicing mutations that result in frame shifts in the transcribed protein. Small deletions, insertions or simply substitutions of nucleic acids in the mRNA transcript coding region may cause incorrect protein folding or alter an essential active site amino acid, which in turn affect the quantity and quality of the protein product.

Southern-blotting, PCR (polymerase chain reaction) and DNA sequencing were utilised in this section of the study to analyse the ASS gene at both the DNA and mRNA levels to locate the mutation responsible for citrullinemia in the F25

cell line.

Crude kinetic experiments were performed on ASS from F25 as already mentioned, and the activation of the ASS promotor in the absence of arginine was also investigated. These metabolic studies were performed to try and gain insight into the consequences of the mutation that was eventually found in the F25 ASS cDNA.

1.3: MATERIALS AND METHODS.

1.3.A: TISSUE CULTURE.

The following fibroblast cell lines were used throughout the ASS study:

F25 = ASS deficient.

F535 = ASS deficient (a cell line derived from the younger brother of F25 as part of this study on citrullinemia)

F199 = ASL deficient.

F500,F527,F488,F489,F699,F688 = control cell lines (no urea-cycle defects).

These cell lines were maintained with Dulbecco's modified Eagles medium (DMEM) (Gibco) supplemented with 10% foetal calf-serum (FCS) (Delta bioproducts) and incubated at 37°C with CO_2 -5% and 90% humidity. The cell culture medium was replaced every four days and the cells were maintained in the absence of antibiotics. Cell cultures were tested on a regular basis for the presence of mycoplasma. At confluency the flasks were trypsinised and the resulting cell suspension split between three flasks.

1.3.B: ARGININOSUCCINATE SYNTHETASE ENZYME ACTIVITY ANALYSIS.

B.i: ^{3}H-phenylalanine - ^{14}C-citrulline dual label uptake.

Confluent flasks were trypsinised and the cell suspension volume increased to 5ml with the addition of BME (Basal medium Eagle) (Flow laboratories) containing 5% FCS and Penicillin(30mg/ml), Streptomycin(50mg/ml) and Neomycin (25mg/ml) (PSN). The cell number of each culture was determined using a coulter counter-Zf (settings: amplification = 2, threshold = 22, aperture current = 2) and 100000 cells aliquoted into 1ml wells (Falcon 24 well plates). Additional BME was added and the cells incubated overnight at 37 °C, 5% CO_2 and 90% humidity.

The medium was removed from each of the wells and replaced with 0.5ml of the following mixtures: 0.5µCi ^{3}H-phenylalanine (55Ci/mmol) (Amersham) and 0.3µCi ^{14}C-citrulline (58mCi/mmol) (Amersham) in 0.5ml medium Z (15mM Na Hepes, 145mM NaCl, 5mM KCl, 1mM $MgSO_4$, 1mM $CaCl_2$,8.3mM glucose (pH 7.3)). The trays were incubated at 37 °C for 4 hours in the absence of CO_2.

After the incubation period the medium was removed and the cells washed 3 times with ice cold 5% trichloro-acetic acid (TCA) to precipitate proteins. The precipitated proteins were dissolved in 500µl 0.1M NaOH and 400µl of the protein solution added to scintillation fluid (Hionic-Fluor) for radioactivity counting. Counts were expressed as cpm/100000 cells. DPM were calculated by taking into account the efficiency of counting ^{3}H and ^{14}C by the Beckman LS 3801

scintillation counter: ^{14}C in ^{14}C window = 72% and ^{3}H in the ^{3}H window = 42%, ^{14}C in ^{3}H window = 18% and the background counts.

Protein determination.

A fraction of the remaining protein solution (80ul) was used to measure the amount of cellular protein present in each well. A solution of 2ml distilled water and 0.4ml Biuret solution (Biorad) [34] was made and 2ml aliquoted into each curvette. A standard protein curve was constructed by measuring the absorbance at 590nm of 80ul of the following BSA (bovine serum albumin) concentrations mixed with the Biuret solution: 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml. These results were graphically represented by absorbance 590nm verses protein concentration on the x-axis. The concentration of the samples (80µl) of interest were determined from their absorbance values according to the standard protein curve. These results give an indication of the variation of cell number between the various wells and can be used to give a more accurate measure of the incorporation of radioactivity into the acid precipitable material when the results are expressed as cpm/µg protein.

B.ii: Complementation assay.

For the complementation assay, 100000 cells of F25 and F199 (ASS deficient and ASL deficient) were co-cultured in 3ml wells and allowed to grow to confluency overnight. As controls, 200000 fibroblasts of F500, F25 and F199 were plated separately into 3ml wells. The overnight BME medium

was replaced with 2ml of medium Z containing 1μCi of ^{14}C-citrulline (58mCi/mmol) (Amersham) and the cells were then incubated at 37°C in the absence of CO_2^- for 6 hours. The medium was removed and the amount of radioactivity accumulated in the cellular precipitate determined as above.

B.iii: Argininosuccinate synthetase activation.

Fibroblasts (400000) of the following cell lines: F500 (control), F25 and F535 were plated into 3ml wells and incubated overnight in BME (5% FCS). The medium was removed and replaced with 3ml of one of the following mixtures:

a) Arginine-free DMEM, 0.6mM L-citrulline, PSN.
b) Arginine-free DMEM, 0.6mM L-citrulline, 5% FCS, PSN.
c) DMEM (0.5mM Arginine), 10% FCS, PSN.

After 48 hours the medium was removed , replaced with 3ml of the same medium and incubated for a further 48 hours (to replace exhausted nutrients and remove waste products). The ASS activity was then assayed by incubating the cells in 1.5ml medium Z containing 0.5μCi ^{14}C-citrulline, for 4 hours. The medium was removed, the cells washed and the amount of radioactivity incorporated into the cellular protein determined as above.

B.iv: Argininosuccinate synthetase kinetic analysis.

Fibroblasts (400000) (F25 and F699) were incubated in Arginine-free DMEM (0.6mM L-citrulline, PSN) for 96 hours (one medium change). The medium was then removed and the

dependance of ASS activity on the concentration of citrulline assayed. The cells were incubated in medium Z containing ^{14}C-citrulline at a constant specific activity of 0.025mCi/mmol (0.5µCi ^{14}C-citrulline / mmol L-citrulline), with the total citrulline concentration being either 20µM, 40µM, 60µM, 80µM or 100µM. The ASS activity was then assessed over a 4 hour time period by measuring the incorporation of ^{14}C into acid precipitable material under the different substrate concentrations.

1.3.C: ARGININOSUCCINATE SYNTHETASE DNA ANALYSIS.

C.I: ASS plasmid amplification and isolation.

The plasmid pAS1 [24] contains a full length copy of the ASS cDNA, inserted at a *PstI* restriction endonuclease site in the pBR322 vector. This plasmid was used as a probe in the Southern-blot analysis of the ASS gene and as a positive control in the development of the PCR technique for the amplification of the mutant ASS mRNA.

E.coli (strain DHalpha) containing the plasmid pAS1 were cultured in the presence of tetracycline (15µg/ml) on agar plates and then 1.5ml overnight cultures of the colonies were established in Luria Broth containing tetracycline (15µg/ml). The plasmid DNA was extracted by the alkaline-lysis method [35,36,37] discussed below: Bacterial cell pellets were resuspended in GTE (50mM glucose, 10mM EDTA, 25mM Tris. HCl (pH 8)) and lysed in the presence of 0.2M NaOH, 1% SDS (sodium dodecyl sulphate). 3M potassium acetate (pH 4.8) was

added to precipitate proteins, host chromosomal DNA and SDS, which were then remove by centrifugation. The supernatant was treated with RNAse (10mg/ml) and the plasmid DNA recovered after phenol/chloroform extraction by ethanol precipitation (2 volumes 100% ethanol at -70 °C for 20 minutes). The final pellet was vacuum dried and dissolved in TE (10mM Tris.HCl (pH 8), 1mM EDTA (pH 8)).

C.ii: Extraction of cellular genomic DNA.

Confluent flasks of cultured fibroblasts were washed with two 3ml washes of saline to remove incubation medium and then incubated in the presence of trypsin for 5 minutes. The dislodged cells were collected and washed again with two washes of saline. The pelleted fibroblasts were resuspended in 0.3ml of digestion buffer (0.1mg/ml proteinase K, 0.5% SDS, 10mM Tris (pH 8), 100mM NaCl, 25mM EDTA (pH 8)) and incubated for 18-24 hours at 55 °C in a shaking incubator (0.3ml digestion buffer per 3x10^7 cells) [38].

Proteins were removed by phenol/chloroform/ isoamyl alcohol extraction [38] and the DNA preferentially precipitated with 0.2 volumes 10M ammonium acetate and 2 volumes 100% ethanol (20 minutes at 70 °C) (RNA requires longer precipitation times). Residual salts were removed from the DNA pellet with a 70% ethanol wash and the final vacuum dried pellet re-dissolved in TE (aided by gentle shaking at 55 °C overnight). Residual RNA was removed by incubation of the samples with DNAse-free RNAse (10mg/ml) for 1 hour, 37 °C and the RNA-free DNA precipitated with ethanol after

phenol/chloroform extraction [38].

C.iii: Southern-blotting.

C.iii.a: Radioactive labelling of the pAS1 probe.

For Southern-blotting purposes, the ASS plasmid probe was radioactively labelled by a random-priming method using random hexamers as primers for the Klenow fragment of DNA polymerase 1 [35]. 10ng-600ng of plasmid DNA was linearised with *BamHI*(10U) in 0.5X KGB buffer (50mM potassium glutamate, 12.5mM Tris.Acetate, 5mM magnesium Acetate, 25mg/ml BSA, 1mM 2-mercaptoethanol), in a reaction volume of 6µl, for 1-2 hours at 37 °C. The fragments were heat denatured by boiling for 10 minutes followed by snap-cooling. Random-hexamers (2µl), 3µl ATG dNTP's (5mM) and 50µCi (5µl) P^{32} -dCTP were added to the denatured, linear DNA (20µl reaction volume) and incubated in the presence of 4U of Klenow for 1 hour at 37 °C to produce the complementary labelled strand. The labelling reaction was halted with the addition of 4µl of 0.1M EDTA.

The labelled probe was separated from unincorporated P^{32}-dCTP by size-eclusion chromotography using a Sephadex G-50 column (3cm)[38].The sample was placed onto the column and fractions eluted with 150µl of TE (pH 8), 15 fractions were collected. 1.5µl of each fraction was added to 4ml scintillation fluid and the radioactivity quantified in a liquid scintillation detector. Figure 7 shows the typical elution profile from the Sephadex G-50 column, with the labelled probe eluting first followed by the unincorporated P^{32}-dCTP. The labelled probe fractions containing the highest radioactivity were pooled and used for Southern-blot analysis.

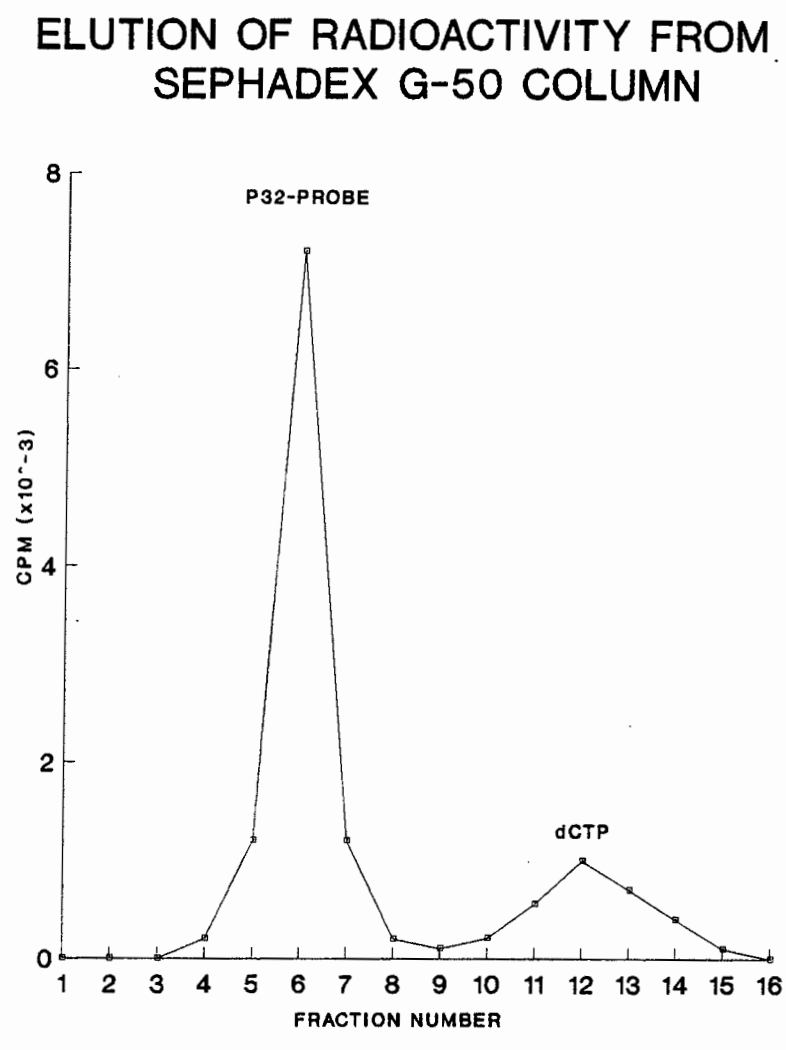

FIGURE 7: Separation of P^{32} labelled DNA probe from unincorporated p^{32}-dCTP.

A random-primed P^{32} labelled pAS1-ASS probe was separated from free P^{32} -dCTP using a Sephadex G-50 column [38]. The random-primed mixture was layered on top of the column and 150µl fractions eluted with TE. 4µl of each fraction was added to 6ml scintillation fluid and the radioactivity content determined (Beckman LS 60001 scintillation counter).

The specific activity of the probe was calculated as follows:

$$\frac{\text{(cpm of pooled fractions) x 100 / 0.4*}}{\text{[DNA - µg]}}$$

* = counting efficiency of the liquid scintillation spectrometer.

The probes that were used for the Southern-blots had a specific activity of between 1x10^7 and 1x10^8 dpm/µg.

C.iii.b: Transfer and hybridisation.

Cellular genomic DNA (10µg) was digested with restriction enzymes *EcoRI*, *HindIII* and *PstI* for 8 hours and electrophoresed on a 1% agarose gel (20x15cm) (Amersham). The alkaline-transfer method was used to transfer the DNA fragments onto a Hybond N+ nitrocellulose membrane [39] over a 16 hour period.

Non-specific binding sites on the membrane were blocked by prehybridisation with 0.25% Blotto (skim milk powder) and 0.06% sodium pyrophosphate (Na-PP), in 0.1% SDS and 6xSSC (0.9M NaCl, 0,09M sodium citrate) for several hours at 65 °C. The labelled ASS plasmid probe was denatured by boiling, added to the prehybridisation solution and then incubated with the membrane for 24 hours at 65 °C.

The non-specifically bound probe was removed from the membrane by a series of washes:

1x 6xSSC, 0.1%SDS, 0.06%Na-PP --- room temp. - 15 mins.
1x 6xSSC, " " --- 65 °C - 15 mins.
1x 3xSSC, " " --- 65 °C - 15 mins.

The membrane was finally vacuum dried and autoradiographed with the aid of intensifying screens. After 2-5 days at -70°C, the film was developed.

1.3.D: ARGININOSUCCINATE SYNTHETASE mRNA ANALYSIS.

D.i: Total RNA extraction.

Total RNA was extracted from confluent fibroblasts using the Single-Step guanidium thiocyanate extraction procedure [38]. Briefly, 1ml of solution D (4M guanidium thiocyanate, 25mM Na citrate (pH 7), 0.1M 2-mecaptoethanol, 0.5% sarcosyl) was added to each flask of cells and the lysed cell components scraped from the base of the flasks. 2M NaAc (pH 4), water-saturated phenol and chloroform/isoamyl alcohol were added sequentially and the solution incubated on ice for 15 minutes. Protein and much of the DNA was removed by centrifugation at 10000g and the RNA precipitated from the aqueous phase with an equal volume of isopropanol and incubation at -70 °C for 30 minutes. The RNA pellet was again treated with solution D (0.3ml) and reprecipitated with an equal volume of isopropanol to ensure the removal of all the protein. The final pellet was washed 3 times with 70% ethanol to remove all traces of guanidium thiocyanate as this interferes with the synthesis of cDNA. The final pellet was dissolved in 200µl water, 10µl removed for quantitation and 500µl 100% ethanol was then added to the rest, for storage at -70 °C. Total RNA was quantified by measuring the absorbance of the diluted 10µl sample at 260nm.

Large flasks ($75cm^2$) of cultured fibroblasts were used in the extraction to maximise yields, giving on average 45µg RNA/flask. Good quality RNA was found to be essential for the synthesis of full length cDNA and to check this, 5µg of total RNA was separated on a 1.2% agarose/formaldehyde gel and stained briefly with ethidium bromide. The quality of the RNA was confirmed by strong, clear 28s and 18s rRNA bands [38]. Quicker confirmation of quality RNA was determined from the 200-300nm scan showing a well-defined peak at 260nm with a 260/280 ratio of greater than 1.8. All solutions were treated with diethylpyrocarbonate (DEPC) (200µl/100ml solution) to destroy RNAse.

D.ii: cDNA synthesis.

ASS mRNA was converted into double-stranded cDNA by a two step process: a) Reverse-transcription, resulting in cDNA-mRNA hybrids, followed by b) the polymerase chain reaction (PCR). PCR allows the synthesis of the second DNA strand and the subsequent amplification of the double-stranded cDNA product.

D.ii.a: Reverse transcription.

The mRNA contained in the total RNA sample was converted into cDNA-mRNA hybrids using AMV reverse transcriptase and oligo dT primers [40,41]. 80U of AMV (Boehringer Mannheim) and 0.5µg oligo dT were used to reverse transcribe 10µg of total RNA. The RNA samples and added oligo dT primers were initially heated to 95°C to ensure complete denaturation and dissolution of the RNA, prior to the addition of the other

additives: 1 x RT buffer (5x = 250mM tris.HCl (pH 8.3), 300mM KCl, 15mM $MgCl_2$) 0.5mM of each dNTP, 5mM dithiothreitol, DEPC treated water, 1µg acetylated BSA, 5U RNAsin and 80U AMV reverse trancriptase (100µl total reaction volume). The reaction was performed at 42°C for 1 hour. Three cycles of this reaction were performed to increase the cDNA-mRNA hybrid yield. At each cycle the hybrids were denatured by heating to 95°C and then 80U of AMV as well as 5U of RNAsin were added and the incubation at 42°C continued.

D.ii.b: Amplification of double-stranded ASS cDNA.

Oligo-dT primed reverse transcription produces mRNA-cDNA hybrids of all the mRNA species in the sample. To produce and amplify ASS double-stranded cDNA specifically, the polymerase chain reaction was used to produce the second strand of the ASS cDNA specifically, with the aid of ASS gene-specific primers. The principles of the PCR reaction are represented in figure 8.

Several sets of ASS specific primers were designed to be utilised in the amplification of the ASS cDNA and these are discussed in more detail in the results section. The basic PCR reaction used in all the reactions was as follows:

<u>100ul reaction volume:</u>

10x reaction buffer (500mM KCl, 100mM Tris.HCl (pH 8.3), $MgCl_2$ 10-70mM), forward and reverse primers (100pmoles of each primer), 0.2mM of each dNTP, DNA template (1fg - 1µg), Taq polymerase (Stratagene) 2.5U / 5U and distilled, filtered water.

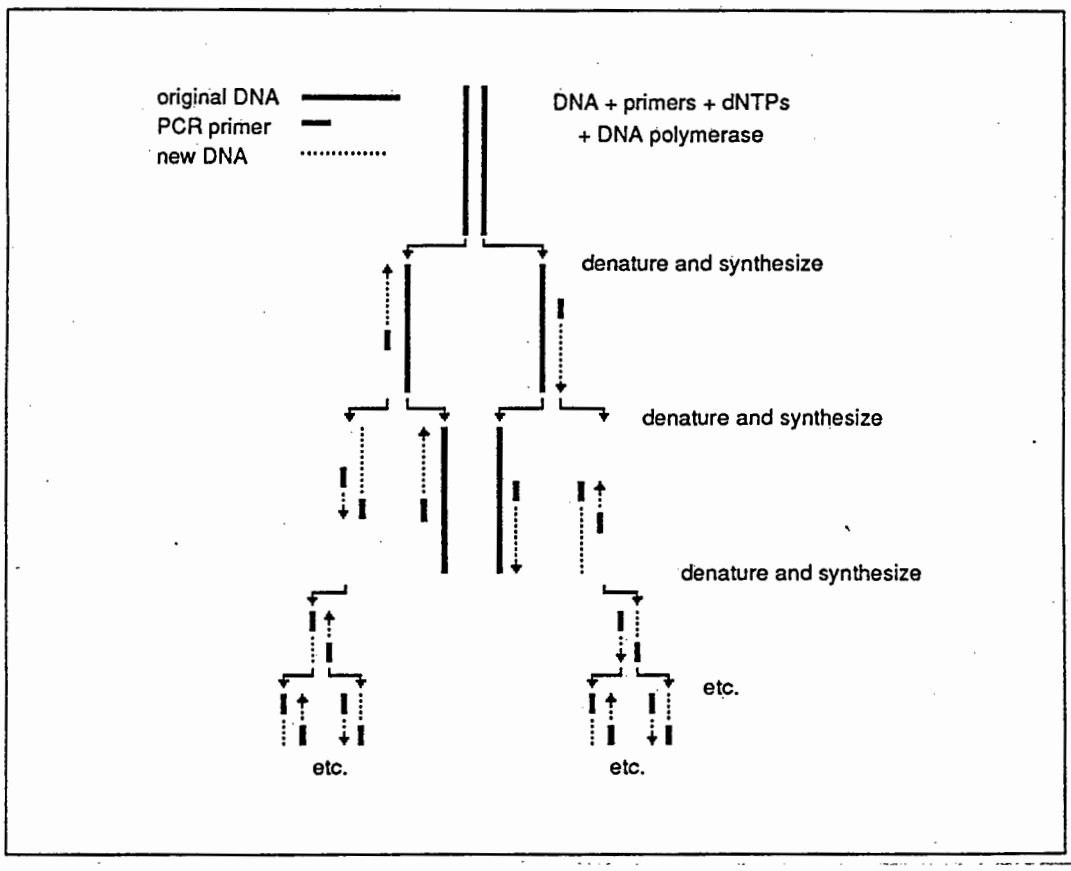

FIGURE 8: The polymerase chain reaction.

A diagrammatic representation of the principles of the polymerase chain reaction (PCR) [38].

The following three step program was implemented in the amplification of the 1307bp fragment of ASS cDNA preceded by an additional Hot-start [42]. The Hot-start involves the addition of all the reagents on ice (except enzyme) followed by the heating of the reaction mixture to > 65 °C before the addition of the enzyme. This minimises non-specific annealing of the primers and helps to prevent primer-dimer formation.

1) 95 °C -1.5 minutes DENATURATION STEP

Add enzyme and mineral oil

2)	55 °C -1 minute	ANNEALING	39 cycles
	72 °C -7 minutes	EXTENSION	
	93 °C -1 minute	DENATURATION	
3)	55 °C -1 minute	ANNEALING	
	72 °C -10 minutes	EXTENSION	

The three-step program used in the amplification of the three 500bp overlapping ASS cDNA fragments was as follows:

1) 95 °C -1.5 minutes

2)	42 °C or 48 °C or 50 °C - 1 minute	39 cycles
	72 °C - 2 minutes	
	93 °C - 1 minute	
3)	42 °C or 48 °C or 50 °C - 1 minute	
	72 °C - 10 minutes	

The products of each PCR amplification were assessed on a 2% agarose (Amersham) gel or a 4% wide range low-melting point agarose gel (NEN products), stained with ethidium bromide and visualised under UV.

To establish the optimised magnesium concentration for each set of primers, a magnesium titration was performed. This involved the amplification of the template at various

magnesium concentrations: 1 - 6mM, to establish the most suitable concentration.

D.iii: Purification of PCR products.

PCR products were separated from unincorporated primers, primer-dimers, free dNTPs and non-specific products by two methods: a) "Magic PCR preps" [43] (Promega) purification from low melting point agarose, or b) "Qiaex" [44] (Qiagen) extraction from normal agarose.

D.iii.a: "Magic prep".

The "Magic prep" purification system [43] allowed for the rapid purification of PCR products that had been excised from low-melting point agarose (NEN products). The agarose fragment was melted at 70 °C and then mixed with an ion-exchange resin that binds DNA present in the agarose (through weak ionic interactions). The resin was loaded onto a small column, the agarose and ethidium eluted with a quick 80% isopropanol wash and the DNA eluted in 50µl of TE [43].When smaller volumes were required the DNA was precipitated with 1/10th volume 3M Na acetate (pH 5.2) and 2 volumes 100% ethanol (-70 °C for 20 minutes) and then washed with 70% ethanol. The DNA prepared in this way was used for cloning and subsequent reamplification of PCR fragments.

D.iii.b: "Qiaex".

Larger PCR products that were separated on normal agarose gels (due to the separation limitations of the available low-melting point agarose), were purified from gel slices

using the "Qiaex" purification kit from Qiagen [44]. This involved the binding of DNA to silica beads, the removal of agarose and ethidium and the elution of the DNA fragments with TE. The 1% agarose gel fragment was solubilised with the addition of QX1 (3M NaI, 4M $NaClO_4$, 10mM Tris.HCl(pH 7), 10mM sodium thiosulphate) and the Qiaex bead suspension followed by incubation at 50 °C for 10 minutes.The DNA-Qiaex suspension was centrifuged and the pellet washed with QX2 (8M $NaClO_4$, 10mM Tris.HCl (pH 7)) to remove the agarose and contaminating dyes and QX3 (70% ethanol, 100mM NaCl, 10mM Tris.HCl (pH 7.5)) to remove the sodium perchlorate. The pellet was thoroughly dried and the DNA pellet eluted from the resin by incubation with TE at room temperature for 5 minutes. This elution was repeated to increase the recovery yield (60%).

D.iv: Cloning of cDNA PCR products.

Fibroblast cDNA amplified through PCR, was cloned into a plasmid vector and then sequenced to establish if the ASS mutation occured at the level of the mRNA. Cloning involves several steps: enzymatic manipulation of vector and insert, transfection of competent cells and selection of recombinants.

D.iv.a: Blunt-end ligation.

The cloning method used was "blunt-end" ligation [45] and was performed as follows: The purified PCR product was blunt-ended ("polished") with the Klenow fragment of *E.coli* DNA polymerase 1 and the addition of 0.55mM dNTPs and 5mM Mg^{2+} at 37 °C for 1 hour. The mixture was then denatured at 65 °C to

inactivate the Klenow fragment.

The plasmid vector pUC18 DNA was linearised with *SmaI* (12U/µg plasmid DNA), which produces blunt-ended fragments. The "polished PCR fragment and the plasmid vector were then mixed in a proportion of 5:1 (500ng/100ng) and incubated with T7 ligase overnight at room temperature to ligate the two fragments together. *SmaI* was also included in the ligation reaction to prevent the re-ligation of the "empty" plasmid [45]. This does not effect the ligation of insert and vector however, as this ligation does not regenerate a *SmaI* site.

D.iv.b: Transfection of competent cells.

Competent *E.coli* cells (strain DH5 alpha) were prepared [46] and stored at -70°C until needed, at which point they were thawed on wet ice. 100µl of cells were incubated on ice in the presence of 8µl of ligation mixture for 45 minutes, heat shocked at 37°C for 45 seconds and further incubated at 37°C for 1 hour in Luria Broth. 100-200µl of cells were then plated onto agar plates, containing 50µg/ml Ampicillin and coated with 40µl of X-gal (5-Brom-4-chlor-3-indolyl-B-D -galactopyranosid) (20mg/ml) (dissolved in dimethylformamide) and 4ul IPTG (isopropyl-B-D-thiogalactopyranosid) (200mg/ml in water), and incubated at 37°C overnight [35,38].

D.iv.c: Selection of recombinant clones.

pUC18 possesses two genes of importance in the selection of recombinants: An ampicillin resistance gene, which allows only those cells that carry the plasmid to grow in the presence of ampicillin, and the α-fragment of the β-galactosidase gene. This gene fragment allows bacterial cells containing the plasmid to cleave X-gal into galactose and X, which is a blue dye. The *SmaI* site lies on the plasmid lies within the α-fragment and thus if an insert is ligated at this site, the galactosidase gene is disrupted and the cells cannot cleave X-gal, producing white colonies. However, if the plasmid religates without the insert, the cells produce blue colonies [35,38].

1.5ml overnight cultures were established from white colonies on the amp-X-gal-IPTG plates and the alkaline-lysis method was used to extract plasmid DNA from the cultures [36]. A quick size test was performed to screen for plasmids containing inserts. Samples were evaluated on a 1% agarose gel and those plasmids that showed a 25-50% size increase were recultured and the DNA purified [36]. Contaminating RNA was removed by incubation with RNAse (10mg/ml), phenol/chloroform extraction and reprecipitation with ethanol. The plasmid DNA was then precipitated specifically by the addition of 6.5% PEG-8000 and 0.4mM NaCl to the dried DNA pellet. After incubation on ice for several hours, the precipitate was collected by centrifugation, ethanol washed and dissolved in TE (20µl). These purified samples were

digested with *EcoRI* and *PstI* (4U of each in 1x KGB) to excise the inserts and analysed on a 1% agarose gel for the presence of the correctly sized insert fragment. Purified DNA from insert positive colonies was then further analysed in the following sequencing reactions.

D.v: Sequencing.

Cloned ASS PCR products were sequenced using the Pharmacia T7 sequencing kit that utilises the Sanger-dideoxy method of sequencing.

The PEG/NaCl purified DNA (20µl) from the positive clones was denatured by the addition of 2µl of a solution of 2N NaOH and 2mM EDTA. The NaOH was then neutralised with 8µl 1M Tris.HCl (pH 4.5) and the denatured DNA precipitated with 1/10 volume 3M Na acetate and 2 volumes 100% ethanol (20 minutes at -70 °C). The pellet was washed with 70% ethanol and vacuum-dried, and then the insert annealed to a sequencing primer by heating 2µl primer (10pmoles), DNA, 2µl annealing buffer (1M Tris.HCl (pH 7.6), 100mM $MgCl_2$, 160mM DTT) and 10µl distilled water to 65 °C, then allowing the solution to cool slowly to room temperature [37].

Following the annealing reaction, the annealed primer was extended in a brief labelling reaction by T7 DNA polymerase, to incorporate dATP-S^{35} into the first few nucleotides of the second strand. The reaction was initiated with the addition of 3µl labelling mix-dATP (dCTP, dGTP, dTTP-1.375mM, 333.5mM NaCl) and 1µl dATP-S^{35} (5µCi) on ice followed by 2U of T7 sequenase, at which point the samples were incubated at room

temperature for 2.5 minutes to allow the labelling reaction to proceed. After 2.5 minutes, the labelling reactions were terminated by the addition of 4.5µl of labelling reaction to each of 4 tubes: G A T C, containing 2.5µl of the respective dideoxy nucleotide mixes. These termination reactions were incubated at 37 °C for a further 5 minutes and the reactions halted with the addition of 5µl stop solution (0.3% Bromophenol Blue, 0.3% Xylene Cyanol, 10mM EDTA (pH 7.5), 97.5% deionised formamide).

The products of chain termination were then denatured at 95 °C for 3 minutes and electrophoresed (4 adjacent lanes) on a 6% acrylamide, 8M urea denaturing gel for several hours. The gel was run at 75W, fixed in 5% acetic acid/10% methanol/0.5% glycerol to remove the urea, dried under vacuum at 80 °C and exposed to B-max x-ray film (Amersham).

1.4: RESULTS.

1.4.A: ENZYME ANALYSIS.

Preliminary diagnostic tests on the F25 patient revealed elevated ammonia and citrulline levels in the urine and plasma. This finding suggested a dificiency in either the ASS or ASL enzymes. Two experiments were primarily performed on this cell line to confirm the diagnosis of citrullinemia i.e: ASS deficiency, a dual-label uptake and a complementation assay. These experiments were repeated in this study to confirm the diagnosis before proceeding to molecular biology studies on ASS.

Firstly, a dual-label (^{3}H-phenylalanine and ^{14}C-citrulline) uptake experiment was performed on the cultured fibroblasts. When ASS and ASL are both operating optimally, the fibroblasts are able to convert ^{14}C-citrulline into ^{14}C-arginine, which is then incorporated into ^{14}C-protein at the same rate as controls. The purpose of the second label, ^{3}H-phenylalanine, is to control for variation in cell number and cell viability, since all cells should incorporate ^{3}H-phenylalanine at the same rate regardless of any urea cycle defects. The ratio of formation of ^{14}C: ^{3}H protein gives a more accurate representation of the utilisation of citrulline since it adjusts for cell differences.

F25, three control cell lines (F500, F489, F488), an ASL deficient cell line (F199) and F535

(which is a cell line derived from the affected sibling of F25), were used in the initial dual label experiment. Fibroblasts were incubated in medium Z containing ^{3}H-phenylalanine and ^{14}C-citrulline for 4 hours and the amount of radioactivity incorporated into acid precipitable material was then assessed. The incubations were performed in medium Z as this medium is arginine free, which forces the cells to utilise the labelled amino acids for protein synthesis and therefore increases the amount of label incorporated into cellular protein.

Figure 9A shows the amount of ^{3}H-protein formed by each cell line, reflecting that all the cells formed similar levels of ^{3}H-protein. This indicates that the cells were healthy and that there were no major differences in cell number between the various cell lines. Figure 9B shows the amount of ^{14}C-protein formed reflecting the utilisation of citrulline and quite clearly shows the poor use of citrulline by F25 when compared to the controls. In fact a 20 fold decrease can be seen between this cell line and the controls. Again when looking at the ratio's of ^{14}C-protein/^{3}H-protein, (figure 9C) which adjusts for the slight variation in cell number, it is clear that F25 has a deficiency in citrulline uptake or citrulline metabolism. The ratio for a normal cell line is 0.05, while F25 has a ratio of 0.003. It must be noted that there is a large variation in the utilisation of citrulline by controls, when different control cell lines and experiments are compared (incorporation of 2000 to 38000 ^{14}C-cpm into ^{14}C-protein figures 9 and 23).

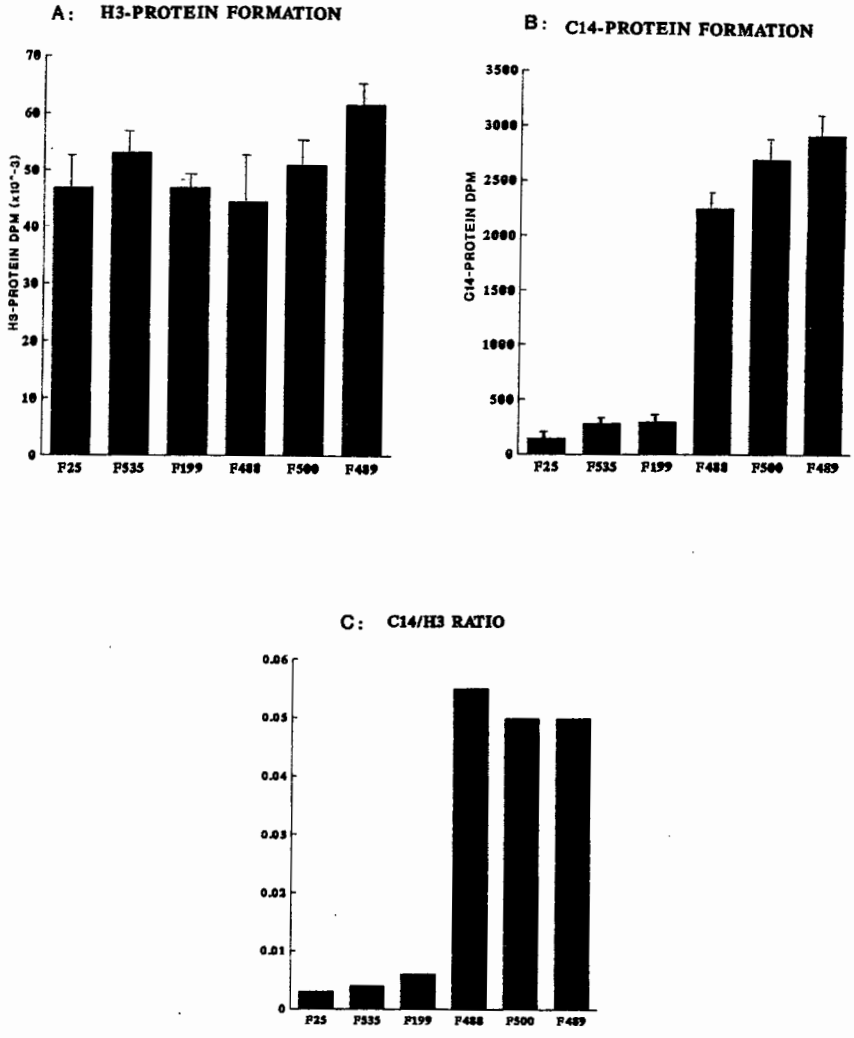

FIGURE 9: Dual-label uptake assay: The determination of ASS / ASL activity.

Fibroblasts (100000 cells) were incubated in 0.5ml medium Z containing 0.5µCi ^{3}H-phenylalanine and 0.3µCi ^{14}C-citrulline for 4 hours. The medium was removed and the cellular proteins precipitated with 5% TCA. The acid precipitable material was dissolved in 0.5ml 0.1M NaOH and 400µl counted to determine the quantity of ^{3}H and ^{14}C-protein formed. F25/F535 = citrullinemic patients, F199 = ASL deficient, F500, F489, F488 = controls (no urea cycle defects). The error bars represent the range of results seen in the triplicate wells.

However, F25 always remained within the range of 1-5% of the controls. F535 also shows very low utilisation of citrulline as was expected, since citrullinemia is an inherited metabolic disorder and so the mutation in both cell lines should be the same.

At this stage however, it was not possible to determine from the experiment whether the defect in F25 was with the ASS or ASL enzymes, as can be seen with the behaviour of F199, the ASL deficient cell line in figure 9B. This cell line gives almost identical results to F25 in the utilisation of ^{14}C-citrulline, due to its inability to convert argininosuccinate to arginine.

After establishing a defect in citrulline metabolism, a second experiment, a complementation assay, was performed to investigate which enzyme was defective. This assay was developed by Davidson et al [33] and relies on cellular communication between cells in the exchange of substrates. They demonstrated that ASS deficient cells and ASL deficient cells could complement each other in the utilisation of citrulline. This was shown to occur through the formation of gap-junctions between the cell types, when grown in co-culture. These junctions allowed the co-culture to successfully use citrulline for the production of arginine (see figure 10).

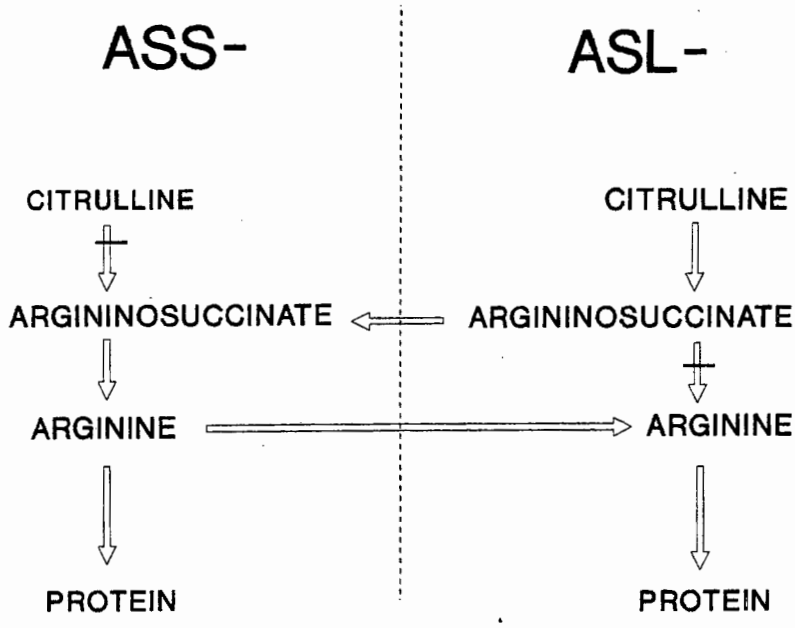

FIGURE 10: The principles of the ASS / ASL complementation assay.

Fibroblasts are able to communicate through gap junctions, allowing the exchange of metabolites between cells. Using these gap junctions (-----) ASS and ASL deficient cells are able to complement each other through the exchange of argininosuccinate and arginine, to allow both cell types to utilise citrulline for arginine synthesis.

To establish if a cell line is ASS deficient, specimen cells would be co-cultured with ASL deficient cells and the utilisation of ^{14}C-citrulline assessed. If formation of ^{14}C-protein by the co-culture was comparable with the control, then complementation had occurred and the specimen cells were ASS deficient.

F25 and F199 (ASL deficient) were incubated separately and as a co-culture in the presence of ^{14}C-citrulline. Figure 11 shows the results, reflecting the inability of these two cell lines to metabolise citrulline independantly, while when in co-culture they can convert the same amount of ^{14}C-citrulline into ^{14}C-protein as the control cell line. The ability of these two cell types to co-operate indicates that the defect in F25 lies in the ASS enzyme rather than an ASL deficiency.

1.4.B: DNA ANALYSIS: SOUTHERN-BLOTTING.

After the confirmation that ASS was defective in the F25 cell line, the first step in the identification of the ASS mutation at the gene level, was to check for any gross abnormalities in gene structure i.e: rearrangements, insertions or deletions. DNA from the patient cell lines F25 and F535, as well as from a control fibroblast cell line, F527, were digested with *PstI* and *EcoRI* restriction endonucleases and Southern-blotted.

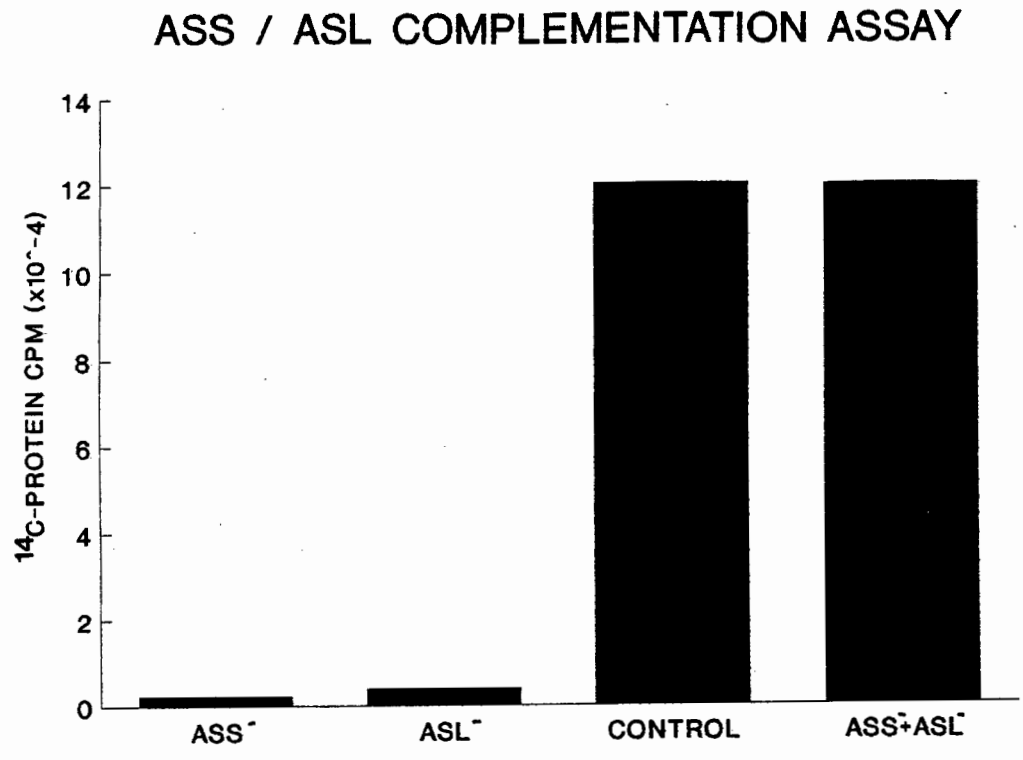

FIGURE 11: ASS / ASL complementation assay.

F25 (ASS deficient), F199 (ASL deficient) and F500 (control) fibroblasts (200000 cells) were incubated for 4 hours in medium Z containing 1 μCi ^{14}C-citrulline to assay their ASS and ASL activities (^{14}C-citrulline->^{14}C-arginine->^{14}C-protein). F25 and F199 (100000 cells of each) were co-cultured and incubated in the above medium to assess their combined ASS/ASL functions. The cellular protein fractions were collected and the ^{14}C-cpm in these fractions recorded.

Several attempts were made to achieve a reasonable Southern-blot. Problems encountered included: RNA contamination, which interfered with restriction enzyme digests and led to an over-estimation of the starting material; Incorrect stringency of washing; Low specific activity of the radioactive probe, caused by too much initial DNA. Figure 12 shows the best result.

Although a number of differences in the restriction banding patterns between control and the mutant cell lines can be seen, conclusions are difficult to draw from these results as the picture is complicated by the presence of 14 pseudogenes. These pseudogenes areintronless and show up to 93% homology with the coding region of the active ASS (figure 6) [25]. It is because of this homology that the Southern-blot is difficult to interpret, since the differences highlighted in figure 12 may reflect mutations in the pseudogenes rather than the active ASS gene itself.

1.4.C: mRNA ANALYSIS.

C.i: PCR - cDNA analysis.

To avoid the problems with the ASS pseudogenes, it was decided to approach the problem from a different angle, that being the analysis of the coding region of ASS, by investigating the ASS mRNA. Fortunately the pseudogenes are not transcribed so they cannot interfere in this analysis [23]. mRNA analysis allows one to address the following questions: 1) Is a transcript produced from the gene ? 2)Is it of the expected size? 3) Is it of the correct quantity?

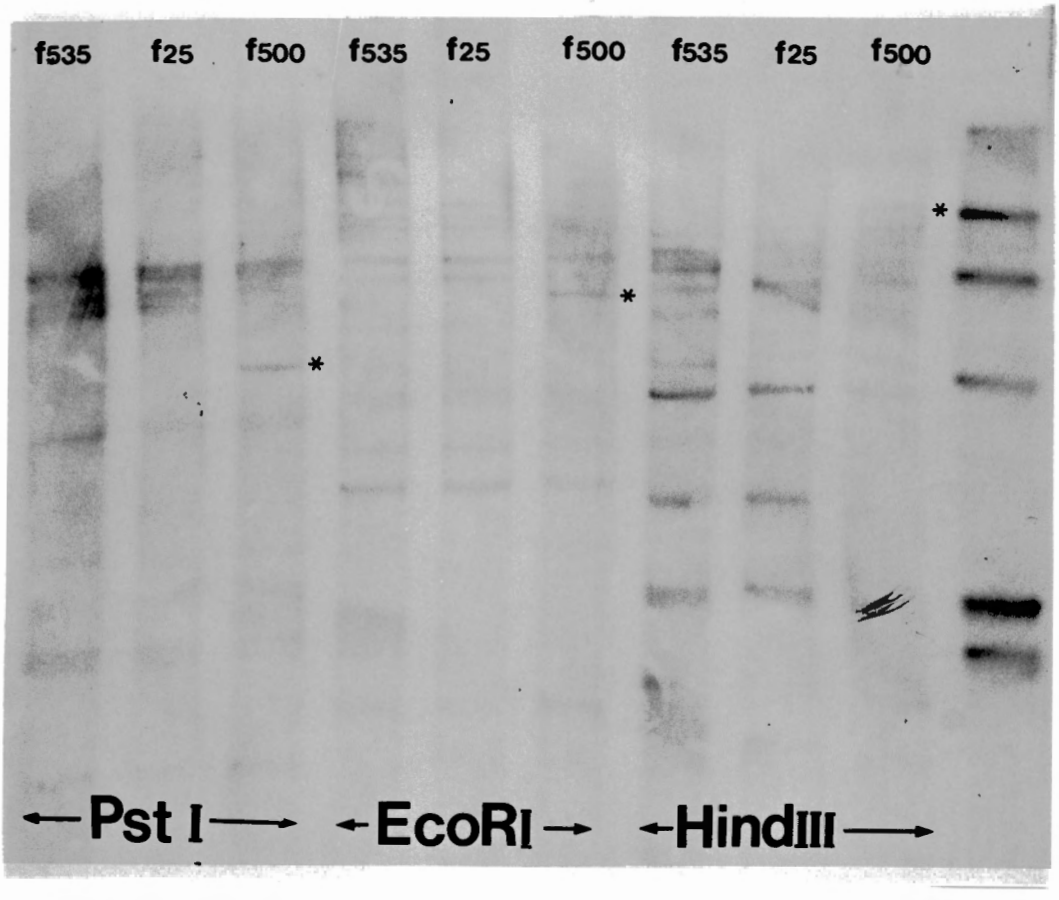

FIGURE 12: ASS DNA analysis - Southern-blot.

Genomic DNA from F25, F535 and f500 (control) was digested with *PstI, HindIII* and *EcoRI* restriction enzymes, blotted onto Hybond N+ and probed with P^{32} labelled pAS1-ASS DNA (10^7 dpm/µg). Observed differences in the restriction patterns of control ASS (F500) and the citrullinemic DNA (F25 and F535) are indicated by * symbols.

4) Is the sequence of the mRNA as expected? The most convenient way of investigating the nature of a particular mRNA is to look at the cDNA copy, a DNA copy of the mRNA, which is much more stable and more readily manipulated than mRNA. This involves the production of cDNA-mRNA hybrids to the total mRNA and then the production and amplification of a specific double-stranded cDNA (specific mRNA), with the use of specific primers in a polymerase chain reaction (PCR). From here the size and sequence of the mRNA can be analysed.

cDNA-mRNA hybrids were created for the following cell lines: F500-control, F25 and F535. The amplification of ASS specific cDNA was then performed using the following sets of ASS specific primers:

PRIMER 1A-22mer 5'-3'=GAAAACAGATTCCAGACGCCGG (P1A)

PRIMER 1B-18mer 5'-3'=CGCCTCCAATCCCAGACG (P1B)

PRIMER 2-22mer 5'-3'=GGGGATCTGCAAATTGAGGAGG (P2)

PRIMER 3-20mer 5'-3'=TGTAGAATTCAGGCATCCTC (P3)

PRIMER 4-20mer 5'-3'=ATTGGAATGAAGTCCCGAGG (P4)

PRIMER 5-18mer 5'-3'=CCACAGGAAAGGGGAACG (P5)

PRIMER 6-18mer 5'-3'=GGTGCCTGCTGGGGTCTC (P6)

Figure 13 shows the binding positions of these primers and the fragment sizes that they produce , when used in appropriate PCR reactions.

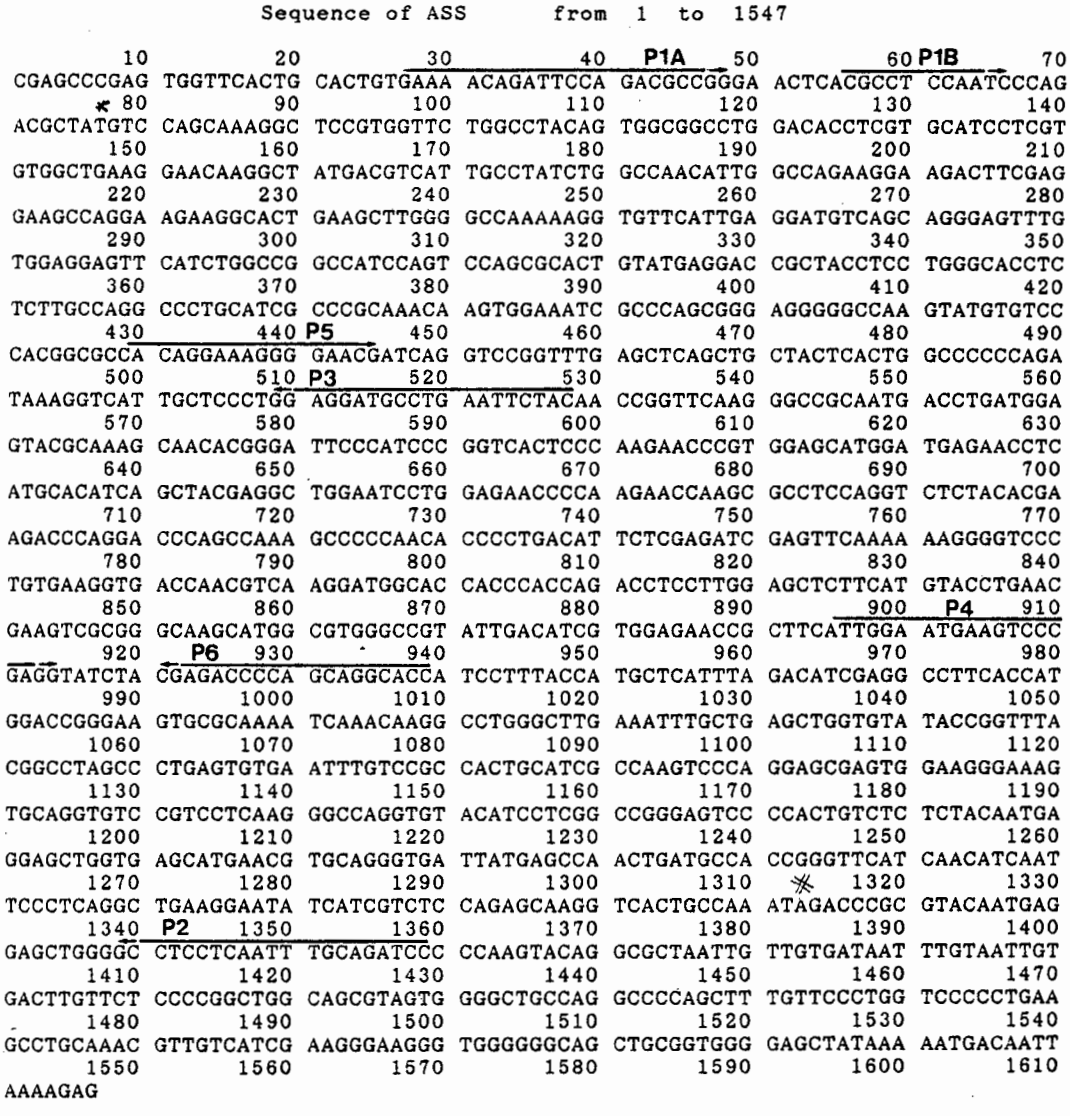

FIGURE 13: Positions of the ASS-specific PCR / sequencing primers.

Diagrammatic representation of the relative positions of the primers used in the amplification of ASS fragments from pAS1 and cellular ASS cDNA. P1A - P2 = 1334bp, P1B - P2 = 1307bp, P1B - P3 = 460bp, P2 - P4 = 480bp, P5 - P6 = 510bp. * = ASS translation start codon, # = termination codon.

C.i.a: Amplification of pAS1 ASS insert.

Due to the low level of expression of ASS in peripheral tissues compared to hepatocytes [1,11] (lower cytoplasmic levels of mRNA), as well as the report that some cell types contain as few as three ASS mRNA molecules per cell [27], it was necessary to optimise the PCR conditions to amplify ASS cDNA from a very small starting concentration. In order to achieve this, the plasmid pAS1 was used as a template in the preliminary studies of PCR optimisation. As mentioned earlier this plasmid contains a complete copy of the ASS mRNA inserted into a pUC18 vector.

PCR program design.

Several factors had to be taken into consideration when designing the PCR program. Firstly, a fragment of at least 1236bp needed to be amplified (coding region of ASS [1]) for complete analysis, and secondly the ASS mRNA/cDNA template was expected to be rare among the mRNA species.

The program that was used to amplify the complete ASS coding region as well as additional 5' sequence (1334bp), consisted of 40 cycles of the following: denaturation for 1 minute at 93 °C, 1 minute at 55 °C to anneal the primers and 7 minute extensions at 72 °C. 2-3 minute extension times are normally used to amplify 400-700bp fragments, so 7 minutes ensures the 1334bp product is completed.

A step program of 15 cycles of 5 minute extensions followed by 15 cycles at 6 minute extensions, then 10 cycles at 7 minutes was also attempted, but was found to be inadequate

(extensive DNA smearing was produced on agarose gels). The step program takes into account the decrease in Taq polymerase enzyme activity after extended incubations at high temperatures [47], allowing longer extension times as the enzyme activity decreases. The previously mentioned program of 40 cycles of 7 minute extensions was however, found to be more successful. The decrease in enzyme activity was compensated for by the initial addition of 2.5U of Taq polymerase (Stratagene), followed by a further 2.5U at 20 cycles.

For the formation of the shorter 400-500bp products discussed below, a shorter extension time of 2 minutes was chosen and additional enzyme was not added. However, 40 cycles was still performed due to the expected rarity of the template.

PCR reactions used to amplify the 1307bp and the 3 500bp fragments were initiated with a Hot-start, so as to limit the formation of primer-dimers [42], and all the reactions were completed with a final extension of 10-15 minutes to ensure that all the products were completely extended.

It is important to note that all PCR reactions (plasmid and cellular) were monitored for contamination, by amplification of a blank reaction mix per PCR reaction. This blank contained all the PCR components excluding DNA template, and thus should not produce any amplified product unless one of the components is contaminated. PCR positive reactions were only recorded when the blank was negative i.e: no contamination detected.

Amplification of pAS1 ASS fragment 1334bp/1307bp.

Initially an attempt was made to amplify the entire coding region of the ASS cDNA, 1334bp, using PRIMER 1A and PRIMER 2. Using an initial target template concentration of 0.9ng, a magnesium titration was performed to determine the optimal concentration necessary for the PCR reaction. Magnesium concentrations of 1 and 2mM were found to be the most effective in the amplification of the plasmid insert, when using 40 cycles, 55 °C annealing temperature, 7 minute extensions and 5U of Taq polymerase (2.5U Taq, 20 cycles - 2.5U Taq, 20cycles) (results not shown).

To test the sensitivity of this amplification, further dilutions of the plasmid were made and used as PCR templates. 0.09ng-90fg template concentrations were examined. Bands of the correct size (1334bp) were amplified from a starting concentration of 0.09ng to 0.9pg but no lower. This result implies that the limit of amplification and detection using this method and primer pair is amplification from 6x10^5 molecules of target sequence (1334bp). Taking into account that as few as three mRNA molecules of ASS mRNA may be present per cell [27], that RNA is extracted from approximately 10^6 cells and the amounts of RNA used in the cDNA and PCR reaction, the sensitivity needs to be lower than 10^4 molecules.

Although the bands were visible, band intensity decreased with the more dilute samples and smearing became more intense (figure 14).

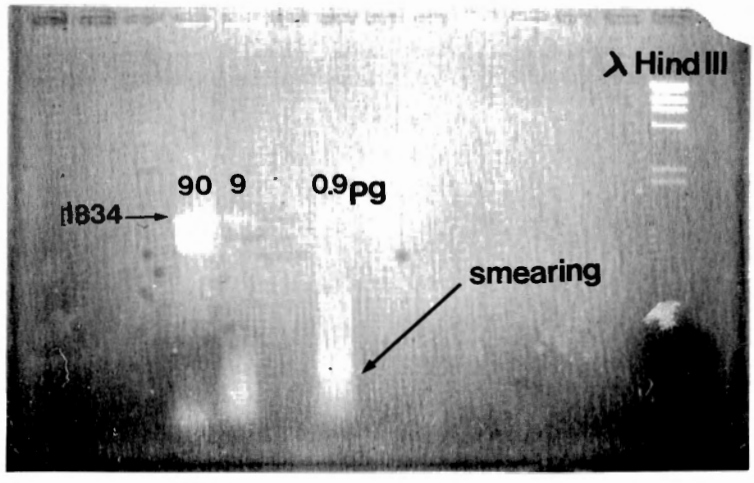

FIGURE 14: Amplification of pAS1-ASS: 1334bp, using P1A and P2.

ASS cDNA from pAS1 was amplified using primers P1A and P2 (1334bp). [Mg] = 1mM, 55 °C annealing temperature, 7 minute extensions, 40 cycles, 2.5U Taq polymerase / 20 cycles. The amplifications from 90pg - 0.9pg of ASS cDNA are shown here and demonstrate the increasing smearing as the initial template concentration is decreased.

Several possibilities were investigated for the reasons behind the smearing and poor yield: contamination in the buffers, contaminated primers, incorrect dNTP concentration, enzyme overloading, incorrect annealing temperature, differing buffer requirements. All these possibilities were eliminated as the reasons behind the smearing.

Originally these primers were designed without the aid of computer programs for primer design, so when the "OLIGO" computer program became available the structure of the two primers was examined in more detail to establish any detrimental complementarity. Figure 15 shows the results of this analysis. Self-annealing of PRIMER 1A, as well as the annealing between PRIMER 1A and PRIMER 2 would probably result in the formation of primer-dimers. This information could readily explain the smearing mentioned earlier, as it probably represents primer-dimer formation and the concatamerization of these products. Due to the abundance of primers in the reaction mixture, primer-dimers will be preferentially formed when very dilute amounts of target DNA are present [48]. This would in turn explain explain the present sensitivity limitations.

To test this hypothesis and try to improve the PCR sensitivity, new primers were designed that had minimal primer-dimer formation, possessed no self-complementarity and had compatible annealing temeratures: PRIMER 1B and PRIMER 2 (fragment size: 1307bp). As well as the alteration of primer structure, a Hot-start [42] procedure was adopted to minimise formation of primer-dimers.

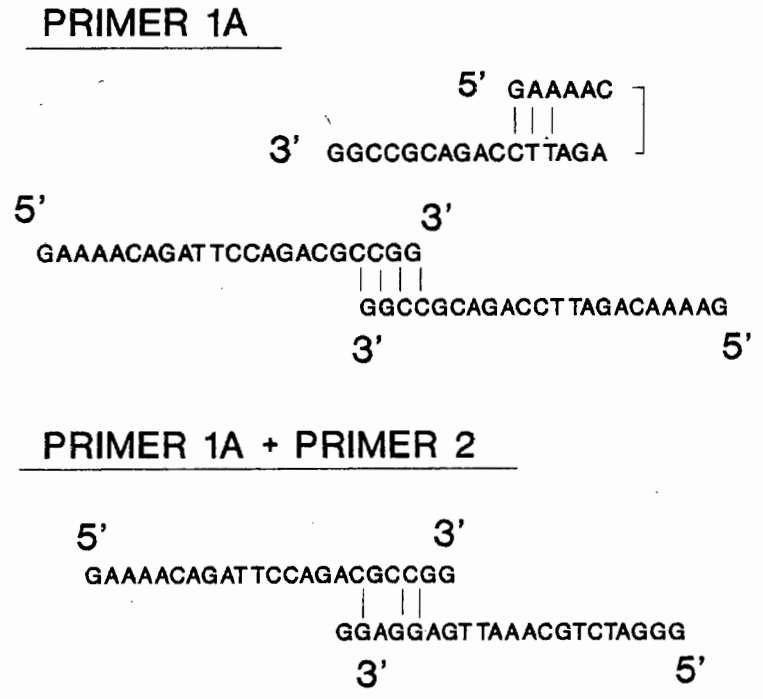

FIGURE 15: Primer-dimer formation.

"OLIGO" analysis of P1A and P2, showing the complementarity between P1A and P2 and the self-complementarity within P1A that may lead to the formation of primer-dimers. The formation of significant amounts of primers-dimers lowers the efficiency of a PCR reaction and is generally avoided.

Although the two primers show no complementarity, it has been postulated that Taq polymerase can still form primer-dimers from the two primers, by stabilizing the juxtaposition of the primers, allowing extension to occur [48]. This will occur in dilute template solutions, so it is necessary to limit the formation of these dimers as much as possible. The Hot-start method involves the addition of polymerase, Mg^{2+} or primers only after the rest of the PCR cocktail has been heated to greater than 70 °C. This prevents the formation of non-specific annealing, by allowing primer extension only at higher temperatures [42,48].

A magnesium titration using the new primer pair - PRIMER 1B and PRIMER 2 (DNA template concentration of 0.9ng)(40 cycles, 55 °C annealing, 7 minute extensions and additional 2.5U Taq at 20 cycles), revealed two magnesium optima: 1 and 5mM (figure 16). Target concentrations of 0.09ng-0.09fg were then used as templates for these primers. Figure 17A shows the results, reflecting the successful amplification of a clear band from a starting concentration of 9fg. This figure represents approximately 6x10^3 molecules of target DNA. This should be sensitive enough to detect any cDNA if it is produced in the F25 cell line.

To establish the absolute lower limits of this amplification, 0.1µCi P^{32}-dCTP was added to the PCR mix, to enable newly synthesized strands to be radioactively labelled, allowing for a more sensitive means of detection.

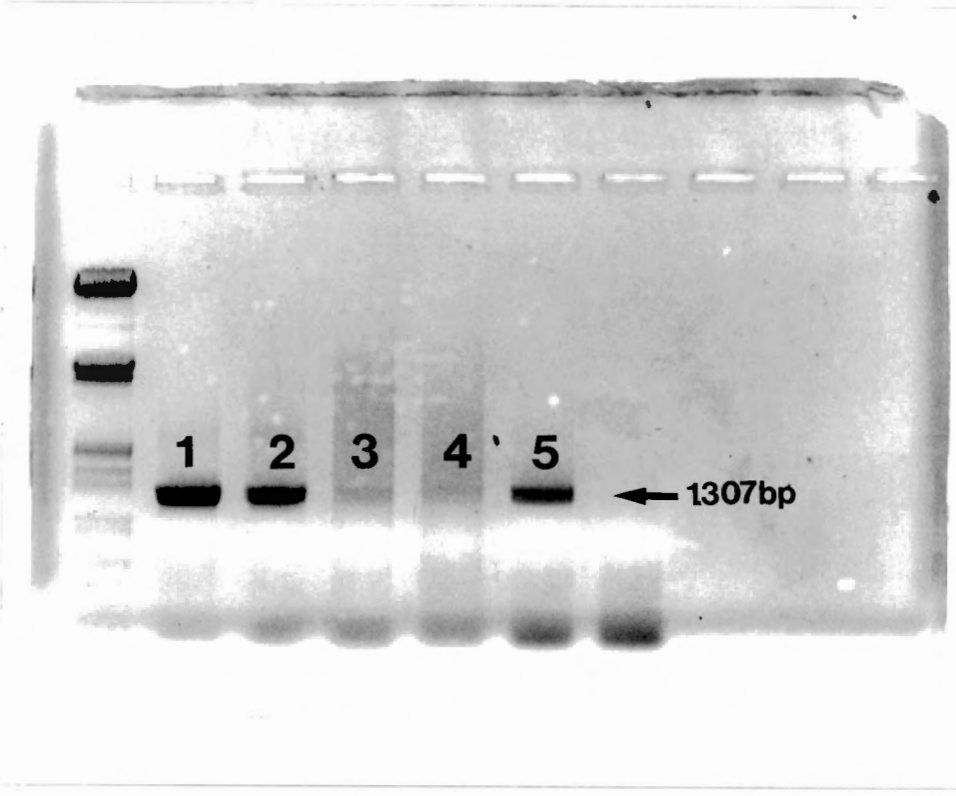

FIGURE 16: Magnesium optima for the amplification of pAS1-ASS: 1307bp.

A magnesium titration for the amplification of 1307bp of ASS cDNA from pAS1 using P1B and P2. [Mg] = 1 - 5mM, 55 °C annealing temperature, 7 minute extensions at 72 °C, 40 cycles, 2.5U Taq polymerase (Stratagene) / 20 PCR cycles. A Hot-start was also performed.

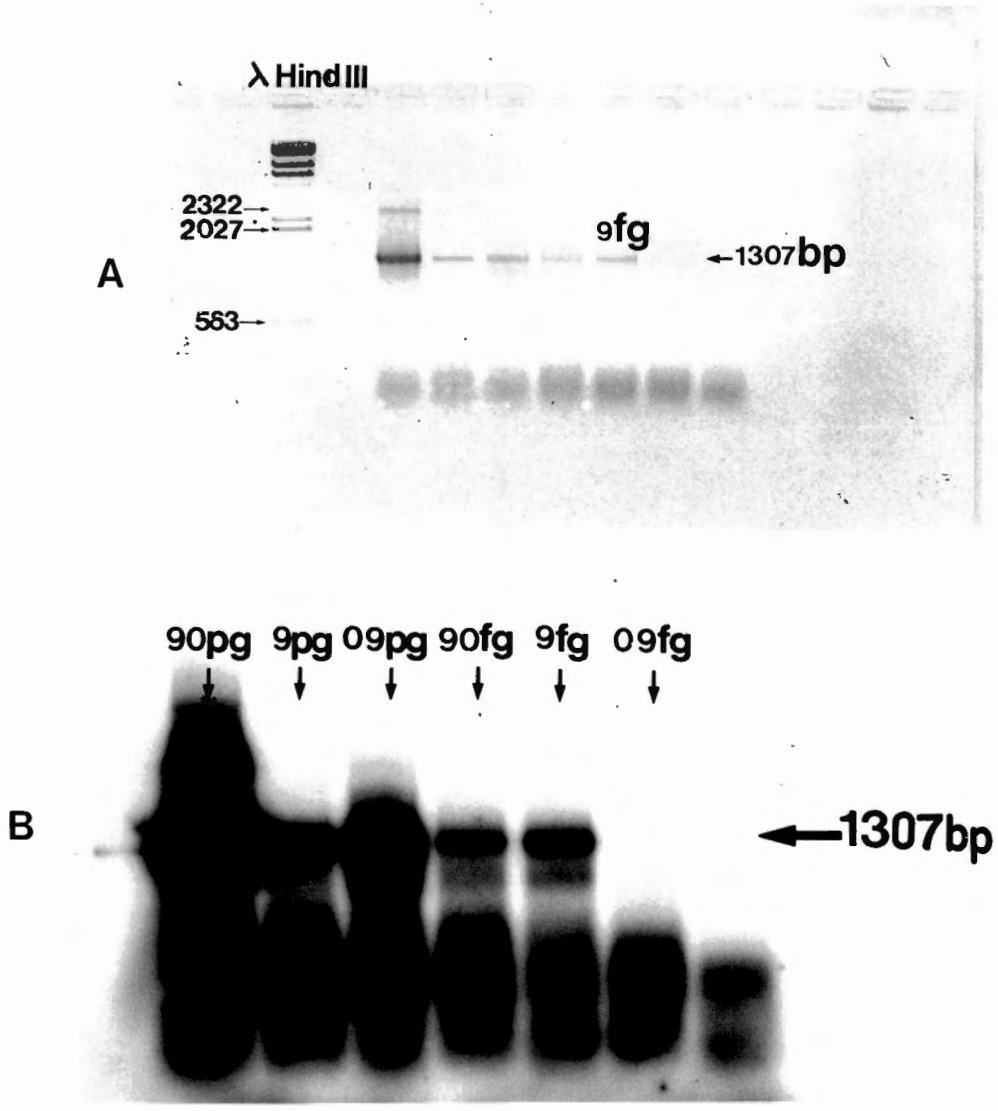

FIGURE 17: PCR sensitivity.

Amplification of 1307bp of ASS from pAS1 using P1B and P2, from initial DNA concentrations of 0.09ng - 9fg. [Mg] = 1mM, 55 °C annealing temperature, 7 minute extensions, 40 cycles, 2.5U Taq polymerase / 20 cycles, Hot-start, and 0.1μCi p^{32} -dCTP was included to improve detection sensitivity. **A)** An ethidium stained agarose gel of the PCR products. **B)** An autoradiograph of the same gel showing the successful amplification from ASS DNA concentrations in the 0.09ng - 0.9fg range.

The samples were electrophoresed and the gel autoradiographed. Figure 17B shows the result, indicating a faint PCR band of the expected size (1307bp) amplified from a starting concentartion of 0.9fg - 6x 10^2 molecules of ASS cDNA!

Amplification of pAS1 ASS fragments: 460-510bp.

Due to the length of the ASS cDNA and the initial difficulty with the amplification of the total cDNA from the cell lines and the plasmid, it was decided to amplify shorter, overlapping fragments of the ASS cDNA. The cDNA coding region was divided into three fragments of +/- 500bp each using primers P1B+P3, P2+P4, P5+P6 shown in figure 13.

Again optimal conditions were established for each set of primers and the optimal conditions are shown in table 3:

TABLE 3: PLASMID PCR SPECIFICATIONS.

PRIMER PAIR:	ANNEALING TEMP:	No. CYCLES:	[Mg2+ mM]:	FRAGMENT SIZE(bp):
P1B + P3	42°C	40	2,3,4	460
P2 + P4	48°C	40	2	480
P5 + P6	50°C	40	3	510

All reactions were performed using the reaction profile of 40 cycles, 2.5U Taq polymerase and 2 minute extension times as described in the methods section. Strong clear bands were observed with each subset and the various fragment sizes are shown in figure 18. The sensitivity also allowed amplification from 9fg of initial template DNA.

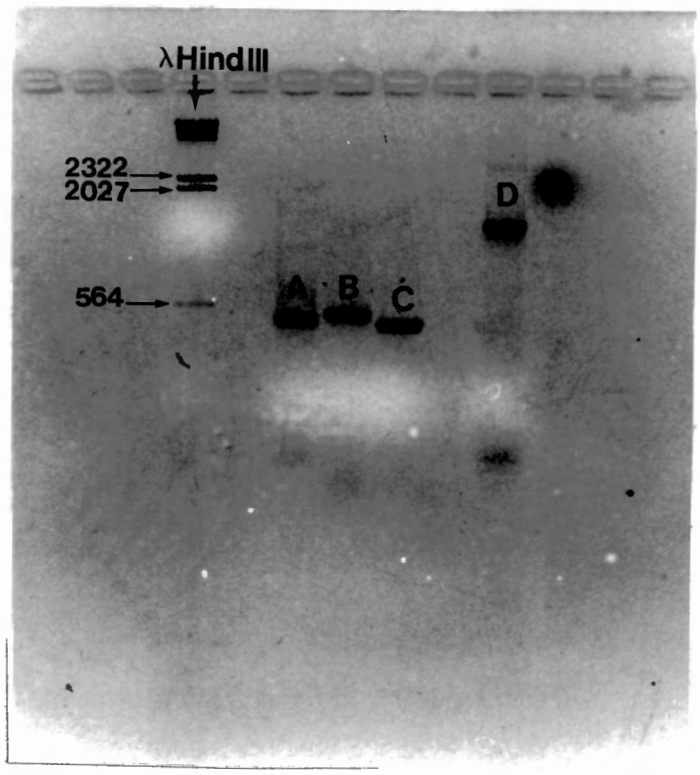

FIGURE 18: Fragmented ASS amplification from pAS1.

Amplification of fragmented ASS cDNA from the ASS cDNA insert of pAS1. **A** = P2-P4: 480bp, **B** = P5-P6: 510bp, **C** = P1-P3: 460bp, **D** = P1B-P2: 1307bp. Refer to table 3 for the details of these PCR reactions.

After establishing the optimal conditions for the amplification of the various plasmid fragments, it was important to establish if these conditions would be altered in the presence of the elements from the reverse trancription mix. This is relevant as the reverse transcriptase mix contains dNTPs that could readily affect the PCR reaction. To test this possibility, a plasmid dilution was made in the reverse transcriptase mix and an aliquot used for the PCR reaction P2+P4. The results showed a shift in the magnesium requirements, with the optimum shifting from 2mM to 3mM and 4mM. This factor was taken into consideration when amplifying cellular cDNA from reverse transcription mixes, using the higher magnesium optima for the PCR reactions.

C.i.b: Amplification of cellular cDNA.

The initial aim was to amplify the entire ASS cDNA of the patient and control fibroblast cell lines and then compare their sequences. However due to the problems with the initial set of primers PRIMER 1A and PRIMER 2, it was decided to amplify the three overlapping fragments, as mentioned above.

Fragmented cellular ASS cDNA: 460-510bp.

Reverse transcribed RNA (1µg) from F25, F535 and F500 (control) were used as templates for the PCR reactions as described in the plasmid section using PRIMER1B-PRIMER3, PRIMER5-PRIMER6 and PRIMER2-PRIMER4. The results are shown in figure 19A and show the successful amplification of the three PCR fragments in all three cell lines. The expected sizes of

510bp (P5+P6), 460bp (P2+P4) and 480bp (P1+P3) were obtained in all three cell lines.

The results of these amplifications provide several useful pieces of information about the F25, F535 mutation. Those being that ASS mRNA is produced in the citrullinemic cell lines and it is of the expected size, indicating that no major deletions or insertions are present in this ASS mRNA.

The three PCR fragments produced for each cell line were used in the cloning and sequencing reactions discussed in section c.ii.

Amplification of cellular ASS cDNA: 1307bp.

After the synthesis of a newly designed PRIMER1B, synthesis of full length cDNA from the three fibroblast cell lines was attempted. Using the optimal conditions for 1307bp plasmid amplification (P1B and P2 (100pmoles of each), 40 cycles, 55 °C annealing, 7 minute extension time, enzyme top-up (2.5U) at 20 cycles) F25,F535 and F500 (control) cDNA was used as the PCR template. cDNA samples were initially denatured at 95 °C for 2 minutes and snapped cooled on ice prior to addition to the PCR reaction to ensure complete denaturation of the cDNA-mRNA hybrids. Figure 19B gives the results, showing the successful amplification of the 1307bp fragment in the control and F25 cell lines. The 1307bp fragment was also successfully amplified from F535 cDNA. This result confirms the earlier result of the presence of a full length coding region in the ASS deficient cells (F25, F535), with the absence of any major insertions or deletions.

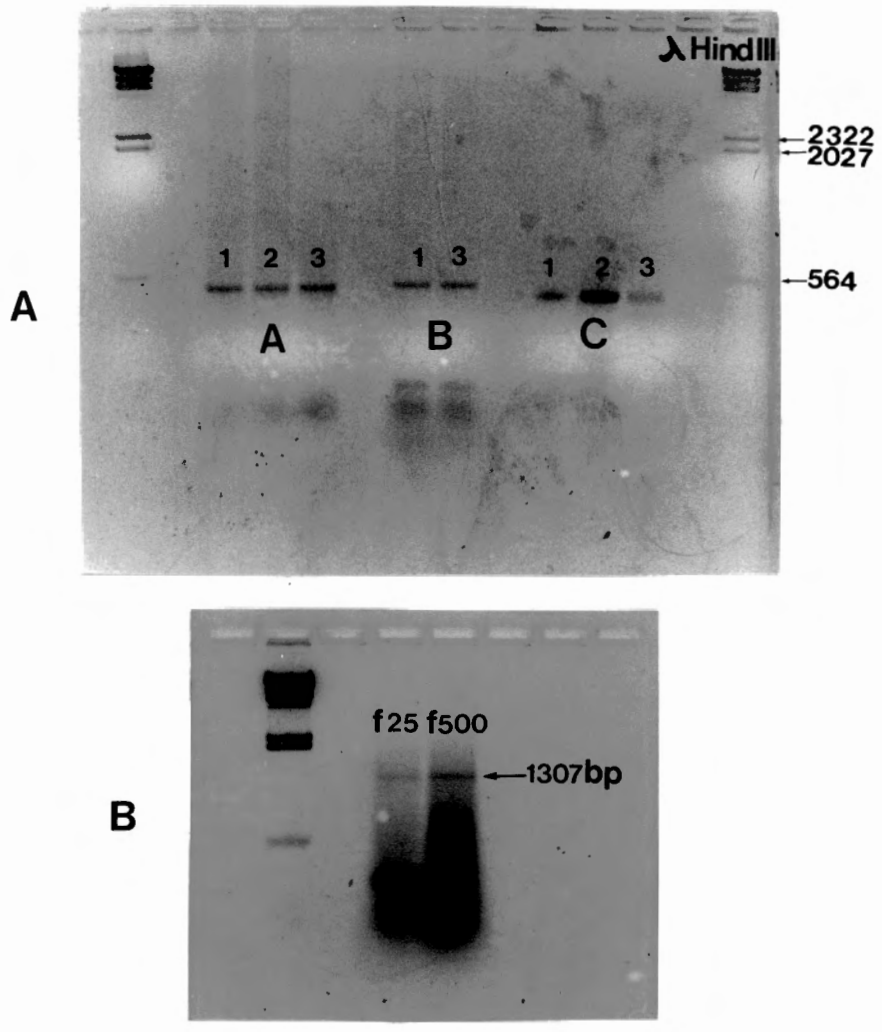

FIGURE 19: ASS amplification from cellular cDNA.

ASS cDNA was amplified from 2 citrullinemic patient cell lines (F25 and F535) and a control cell line (F500). **(A)** represents fragmented ASS amplification where A = P2-P4: 480bp fragment, B = P5-P6: 510bp and C = P1-P3: 460bp fragment. 1 = F25, 2 = F535, 3 = F500. **(B)** shows the amplification of 1307bp of ASS cDNA from F25 and F500. The cDNA solutions were primarily denatured at 95°C for 5 minutes prior to addition to the PCR reaction which was initiated with a Hot-start. The following PCR conditions were used: [Mg] = 1mM, 55°C annealing temperature, 7 minute extensions, 40 cycles, 2.5U Taq polymerase (Stratagene)/20 cycles.

Unfortunately, these results give no indication of the relative amounts of mRNA produced in these cells.

C.ii: Cloning and sequencing.

C.ii.a: Cloning.

PCR products can be sequenced directly or cloned and then sequenced. Cloning has an advantage in that large amounts of material can be generated for sequencing purposes from small quantities of starting material. The disadvantage of cloning is that a number of clones must be sequenced to distinguish a true mutation from a random Taq polymerase error. The cloning route was chosen for the sequencing of the three overlapping PCR fragments synthesised from F25.

SmaI "blunt-end" ligation was chosen as the method of inserting the PCR fragment into the pUC18 vector [45]. One of the general disadvantages of "blunt-end" ligation, is that it tends to result in a relatively high plasmid religation background level. The *SmaI* method lowers the percentage of self-religated vectors by the inclusion of *SmaI* in the ligation mix , to keep the vector "open". It can however, only be used if the PCR insert fragment does not possess an internal *SmaI* site. Further refinements such as removing the 5′ phosphate groups from the vector or increasing the insert:vector ratio, can be performed to further reduce the high background levels.

With regards to the cloning of the three PCR fragments of F25, it was found that when the insert:plasmid ratio was 5:1, 5 positive clones out of 15 white colonies were produced.

Although the background was quite high, enough clones were produced for sequencing purposes, so no refinements were made to the cloning procedure. The following controls were used to assess the method: cell competancy check, *SmaI* efficiency in preventing plasmid religation and efficiency of ligase to religate the plasmid in the absence of *SmaI*. Using these controls, it was found that all stages of the cloning experiment were operating effectively.

Initial screening of white colonies was performed by looking for shifts in the size of the intact plasmid on gel electrophoresis, as compared to insert negative plasmid, as can be seen in figure 20A. These provisional positives were digested with *EcoRI* and *PstI* to cut out the insert from the multiple cloning site of the plasmid vector and the fragments separated on a 2% agarose gel. Positive colonies were taken as those that contained the 460bp, 480bp or 510bp fragments (figure 20B).

C.ii.b: Sequencing.

Three of the positive clones from each separate cloning experiment for F25 were sequenced to ensure that any detected mutations were not Taq polymerase or sequencing errors. The PCR fragments were sequenced in both directions using their respective PCR primers.

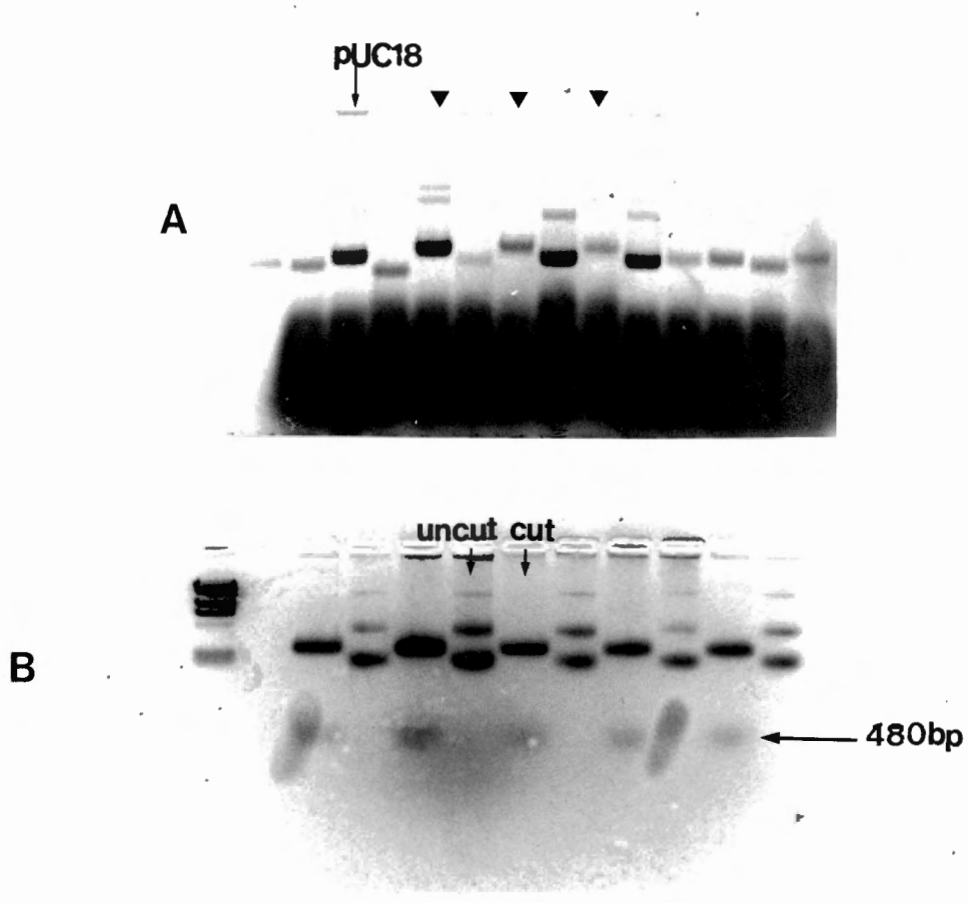

FIGURE 20: Selection of ASS recombinant plasmids.

ASS PCR products were cloned into pUC18 vectors and amplified in competent DH5 alpha *E.coli* cells. Plasmid extracts were prepared by alkaline-lysis [38,39] and then analysed for the presence of the ASS inserts. (A) = plasmid extracts prepared from white colonies (blue-white selection) from the cloning of F25 ASS P2-P4 fragment into pUC18. Plasmids containing inserts showed size shifts on a 1% agarose gel (▼). (B) = plasmids showing size shifts were digested with *PstI* and *EcoRI* to confirm the presence of the 480bp fragment.

The first 900bp of the ASS cDNA from F25 i.e: P1-P3 and P5-P6 fragments showed no sequence divergence from the published human sequence available from Genbank [13]. However, the fragment P2-P4 revealed five base changes, as shown below:

```
          1040                        1064
reference:  TATACCGGTTTACGGCCTAGCCT
F25       :  ......A....CT...AC.....
```

These base changes then result in the alteration of four consecutive amino acids:

```
             320                      330
reference: A E L V Y T G L R P S P E
F25      : . . . . . . . S F W H . . .
```

These results were shown in all three clones of the P2-P4 fragment, indicating that the changes are unlikely to be Taq polymerase errors. It is very unusual to have so many base changes in one area, so to confirm these results the P2-P4 fragment of F535 was also cloned and three clones sequenced. The sequences again revealed the same five base changes, showing that the original result was not a sequencing artifact.

In order to understand the significance of these mutations, the F25 / F535 mutant amino acid sequence was compared to several other ASS sequences available on Genbank: human, mouse and bovine. The following interesting result was found:

```
          320                    330
Human : A E L V Y T G L R P S P E
F25   : . . . . . . . S F W H . . .
Mouse : . . . . . . . G F W H . . .
Bovine: . . . . . . . G F W H . . .
```

This comparison showed that the mutant sequence showed more homology to the mouse and bovine sequences rather than to the documented human sequence [13]. This finding is very unusual and suggests that the human ASS sequence on Genbank is incorrect . To investigate this hypothesis , the P2-P4 PCR fragment from F500 , a control fibroblast cell line , was cloned and three clones sequenced. This "normal" sequence was then compared to the Genbank sequence:

```
          1040                        1064
reference:  TATACCGGTTTACGGCCTAGCCT
F500      :  ..........CT...AC.....
```

This result demonstrates that the sequence on Genbank is not representative of a normal human ASS cDNA sequence. Taking this into account, the F25 mutations were reassesed by comparison with our control fibroblast cell line F500. The results are shown in figure 21 , which represents the autoradiograph of the two sequences, and summarised below:

```
                    1045
                     |
                     ↓
F500(control): TATACCGGTTTACGGCCTAGCCT
F25          : ......A................

  324      324
GLYCINE -> SERINE
```

This G->A transition at 1045bp in the F25 ASS cDNA causes the amino acid change: Gly -> Ser at position 324 in the F25 subunit protein sequence. This mutation is not a Taq polymerase error or a sequencing artifact and represents a true ASS mutation present in the mRNA coding regions of both F25 and F535.

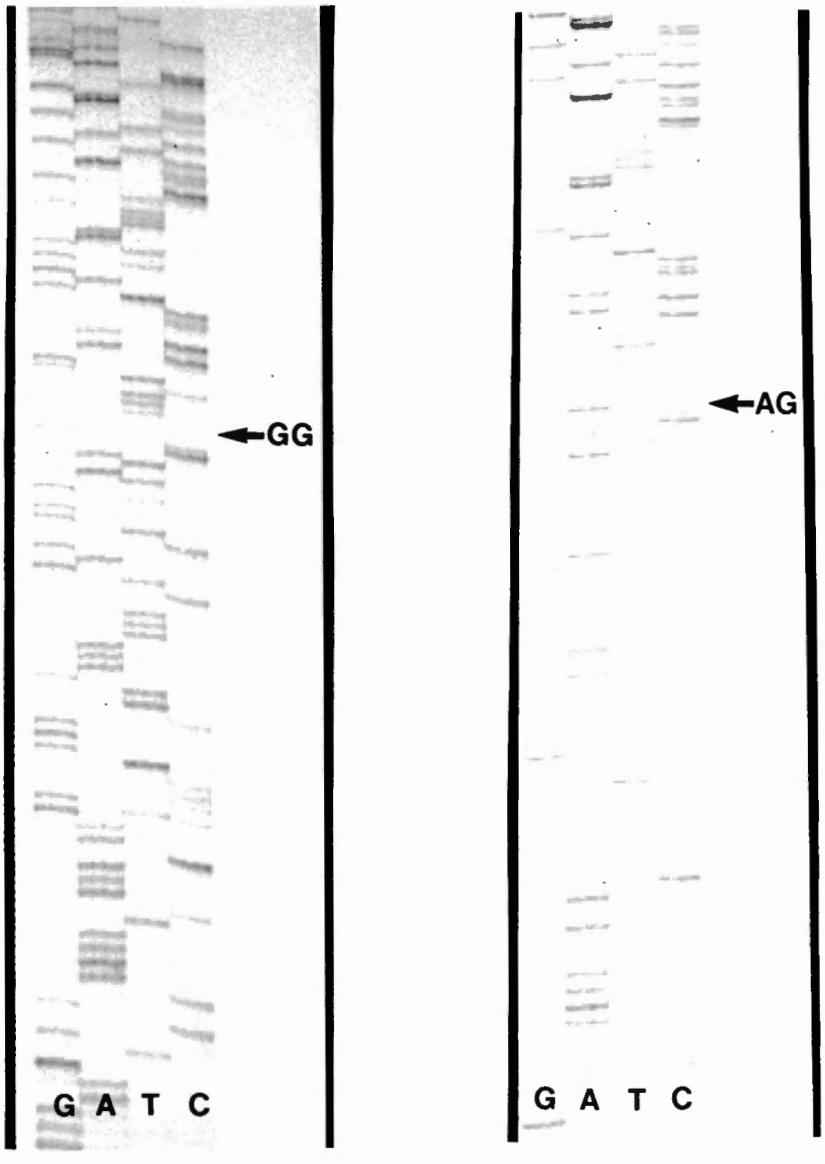

FIGURE 21: Sequence comparison of F25 (ASS deficient) and F500 (control).

The three overlapping ASS cDNA PCR fragments from F25 and F500 were sequenced using P2, P3, P4, P5 and P6 primers and their sequences compared. A single base difference between F25 and F500 was found at position 1045bp, which represented a G->A transition mutation in the F25 ASS cDNA.

1.4.D: ARGININOSUCCINATE SYNTHETASE ACTIVATION.

The molecular experiments have answered several questions regarding the mutation in the F25 citrullinemic cell line i.e: an ASS mRNA transcript is produced in these cells and it is of the expected size, however the coding region of this transcript contains a single point mutation (position 1045bp), resulting in a Gly->Ser amino acid change in the subunit protein sequence.

However, this may not be the only mutation responsible for the ASS deficiency in F25. At present no information has been gathered regarding the amount of ASS mRNA produced i.e: is the promotor defective ?

As mentioned in the review, it has been reported that in some cultured cell lines ASS is regulated at the transcription level by extracellular arginine concentrations . It is known that arginine acts at the ASS promotor of these cells to depress the levels of cytoplasmic ASS mRNA produced. When arginine is removed from the medium the levels of cytoplasmic ASS mRNA increase 360 fold depending upon the cell type. At present this phenomenon has not been demonstrated in human fibroblasts. To investigate the presence of this regulatory mechanism in cultured fibroblasts and to investigate the possibility of a defect in the promotor region of ASS in F25, the activation of ASS in the absence of arginine was researched.

F25, F535 and control fibroblast cell lines F500, F699 and F688 were incubated in the presence of arginine-free (0.6mM citrulline) medium, 0.05mM arginine (0.6mM citrulline) or 0.6mM arginine medium for 96 hours [25] (medium was changed at 48 hours to replenish glucose levels and remove waste products) in an attempt to activate transcription from the ASS promotor. The medium was removed and the cells incubated in the presence of medium Z (arginine-free) for 20 minutes to allow for the metabolism of any intracellular arginine. The cells were then incubated in the presence of ^{14}C-citrulline (medium Z) to study the effects of pre-incubation (96 hours) on the activity of the ASS enzyme in the various cell lines. Figure 22 gives the results of this experiment.

The histogram represents the amount of ^{14}C-radioactivity incorporated/400ug of cellular protein, rather than per cell number, as it was found that cell protein levels varied significantly between the different media, in patients and the controls after the 3-step experiment. This variation could have been a result of two factors, one being that due to the inability of the ASS and ASL enzymes to co-operatively produce enough arginine for protein production in the absence of extracellular arginine, cellular proteins were being degraded to allow the production of essential proteins. The other possibility being that significant cell death was occuring in the wells free of extracellular arginine, again because ASS and ASL cannot synthesize the necessary quantity of arginine from citrulline for survival.

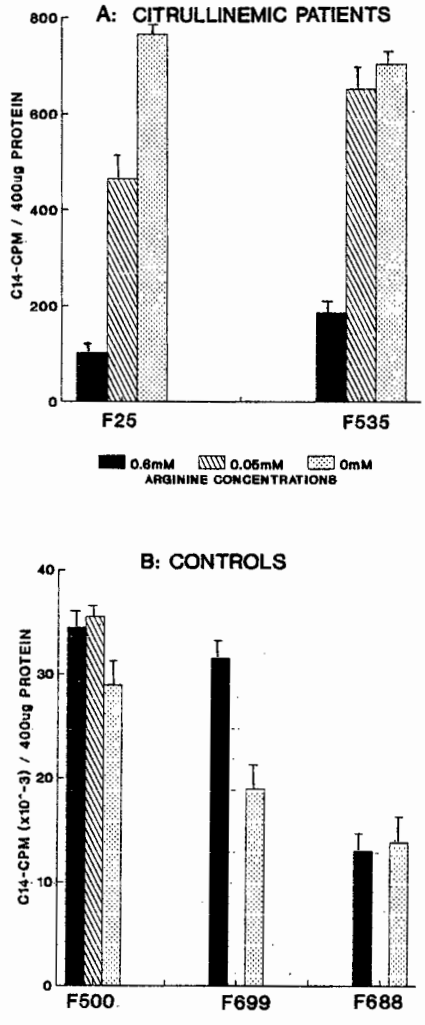

FIGURE 22: ASS activation.

F25, F535 (citrullinemic cell lines) as well as F500, F699 and F688 (controls) were pre-incubated in medium containing varying amounts of arginine (0, 0.05, 0.5mM) for 96 hours. After the incubation period the ASS activity was assayed by measuring the incorporation of 0.5µCi ^{14}C-citrulline into ^{14}C-protein over a 4 hour period in medium z. The cellular acid precipitable material was collected (0.5ml) and the radioactivity content determined. The results were expressed as ^{14}C-cpm/400µg protein to correct for observed differences in cell numbers. The error bars indicate the range of results shown in the triplicate wells.

To investigate the first possibility, the media from the 48 hour and 96 hour incubations was analysed on the amino acid analyser to look for differences in the levels of amino acids. Significant protein degradation should result in an increased level of certain amino acids.

However, no such differences were noted between the 3 different media after the 48 hour incubation periods. Upon closer examination of the cells during the actual ASS activation experiment, a notable decrease in cell number was evident in the arginine-free medium. Cell death probably explains the drop in cell number more accurately. This must be kept in mind when investigating ASS mRNA levels.

Figure 22 shows that control fibroblasts are relatively unresponsive to extracellular arginine levels in the regulation of ASS activity. This result has also been shown in several other repeat experiments, with ASS activity only increasing between 1-1.5 fold in the presence of 0.05mM arginine and decreasing by up to 0.5 fold in the complete absence of arginine. The decrease may be a reflection of the lack of growth factors that would normally be supplied in the foetal calf serum.

The citrullinemic cell lines show very different results, with F25 showing a very definite 7 fold increase in ASS activity after incubation in arginine-free medium for 96 hour, and with a 4 fold increase in the presence of 0.05mM arginine (5%FCS). These results are mimicked to a large extent in the F535 line, showing a 6 fold increase in the

absence of arginine. These results have also been repeated and although results vary, F25 shows definite increases in ASS activity when incubated in the absence of arginine: 5-15 fold increase. The variation is probably due to experimental variation in the utilisation of citrulline by cultured fibroblasts as reported by Baumgarten [49]. These results could represent several possibilities : 1) In light of reported evidence that ASS promotor activity is regulated by arginine in other cultured cell lines [1,24,25,26], there could be a promotor defect in the F25 and F535 cell lines, making them sensitive to the arginine regulation. 2) The other possibility is that this reflects an isotopic dilution effect, with intracellular arginine diluting the ^{14}C-Arginine formed by ASS from ^{14}C-citrulline, leading to lower ^{14}C-protein incorporation results in the cells incubated for 96 hour in media containing 0.6mM or 0.05mM arginine.

As a test for this activation hypothesis , the ratio of ^{14}C-citrulline : ^{3}H-arginine uptake was measured after the different incubation conditions to assess any dilution effects in both the F25 and F500 cell lines (labels were incubated in separate wells). If true activation was occuring in F25, the ^{14}C:^{3}H ratio should be expected to increase 5-15 fold in the cells incubated for 96 hour in the absence of arginine, reflecting the increased ASS enzyme activity. However, identical ^{14}C:^{3}H ratios were found in cells fed in the presence and absence of arginine for 96 hour. The "activation" shown in F25 and F535 (figure 23) therefore probably relects an isotopic dilution effect of unlabelled

arginine on ^{14}C-arginine, rather than true ASS transcription activation.

No obvious dilution effect was seen in the control fibroblasts. This can however be explained by the ASS activity in these cells. 0.6mM L-citrulline is included in the arginine-free incubation medium, which enables control cell ASS/ASL activity to produce significant intracellular arginine levels over the 96 hour period. The arginine pool size in these cells must be very similar to that of cells incubated in the presence of arginine (96 hours) as no significant isotopic dilution effect is observed. In the citrullinemic cell lines F25 / F535, the 1-5% residual ASS activity is presumably unable to compensate for the decrease in the intracellular arginine pool over the 96 hour period, leading to the pronounced dilution effect when comparing cells incubated in arginine-free to 0.5mM arginine medium.

1.4.E: KINETIC ANALYSIS OF ARGININOSUCCINATE SYNTHETASE.

Although the enzyme kinetics were not originally studied, due to the very low levels of ASS activity in the citrullinemic cell lines, the increased ^{14}C-citrulline (5-15 fold) incorporation found in these cells after intracellular arginine depletion (96 hours in arginine-free medium), made it possible to study citrulline metabolism more readily. Thus an attempt was made to study the kinetics of ASS in a control and citrullinemic F25 cell lines. Ideally, cell-free assays are performed to accurately assess the parameters of an

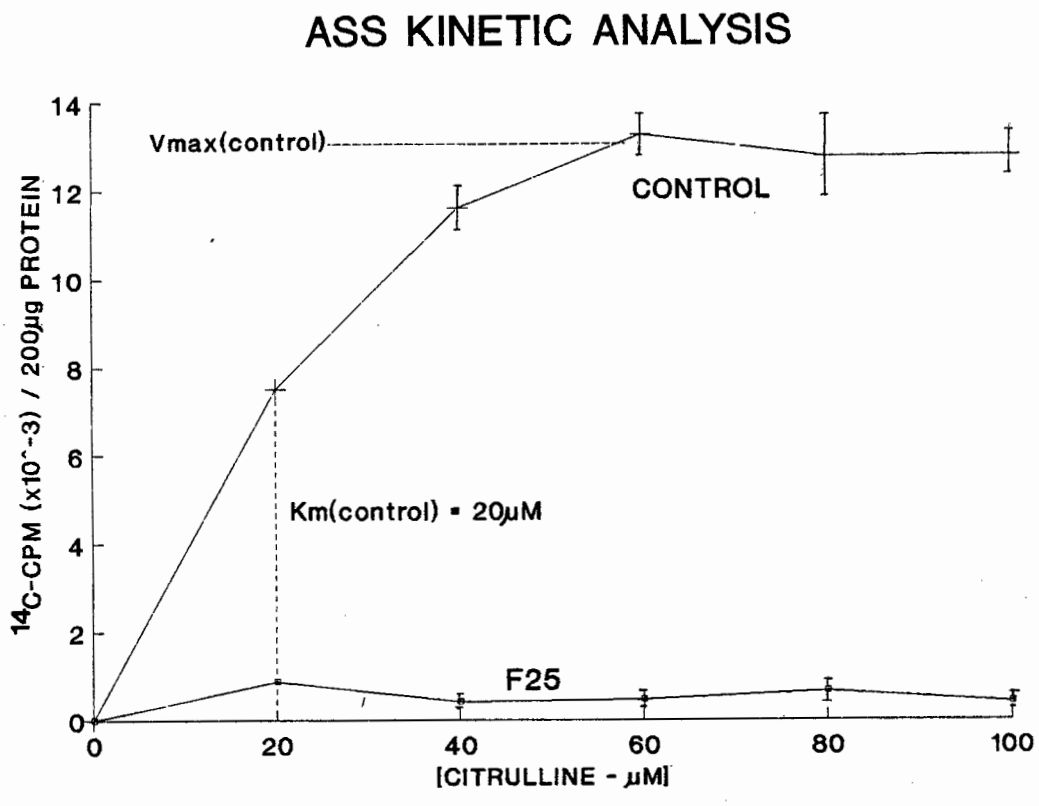

Figure 23: ASS kinetic analysis.

The kinetic parameters (Vmax and Km) of the ASS enzyme from F25 (citrullinemic patient) and F699 (control) were analysed by measuring ASS activity a citrulline concentration range of 10-100µM in a whole cell assay. ASS activity was measured by determining the incorporation of ^{14}C-citrulline (constant specific activity) of 25µCi/mmol) into ^{14}C-protein over a 4 hour period, in the presence of the unlabelled L-citrulline. The error bars represent the range of results seen in the triplicate wells.

This would bring the Km for citrulline into the range of 4-6mM, which was not covered in the present study. The result obtained in figure 23 would be compatible with either an increase in Km or a decrease in Vmax, and there is insufficient activity in these cells to be able to perform an accurate discrimination between these two possibilities.

1.5: DISCUSSION.

1.5.A: GLYCINE -> SERINE MUTATION.

Little is known about the amino acid residues involved in the catalytic activity of ASS, therefore analysis of kinetic mutants may help to elucidate the nature of the active site. For this reason the nature of the mutation responsible for the ASS deficiency in the F25 cell line, from a citrullinemic patient, was investigated.

A genetic analysis of the mutation responsible for the reduced ASS activity (1-5%) in the F25 cell line was performed. This involved the investigation of possible genetic rearrangements at the DNA level, and the analysis of the coding region of the active ASS gene (q34 chromosome 9). The results of these studies showed that a mRNA transcript was produced from the ASS locus in the abnormal cell line, and the coding region was of the correct size. This indicates that no abnormal RNA splicing was occurring. Upon analysis of this coding region, a single point mutation was found at position 1045bp, a G->A transition which results in an amino acid change Gly->Ser at position 324 in the subunit protein sequence.

The possibility that perhaps a contaminating nuclear pseudogene was actually sequenced was eliminated due to the fact that only one base mismatch was detected. ASS pseudogenes show a very high homology with the ASS mRNA, as can been seen from figure 6 other point mutations

would also be expected. This G->A transition was not the result of a Taq polymerase error or a sequencing artifact since the same mutation was found on sequencing 6 separate clones. Instead it represents a true mutation in the coding region of the ASS cDNA in both the F25 and F535 cell lines.

The consequences of this mutation on the catalytic activity of ASS are difficult to determine exactly as little is known about the tertiary structure of the protein or the active site amino acids. Some analysis of the protein has been performed by the comparison of the primary structure between various species (figure 5) [18].Several areas of homology have been determined through this analysis and two of these have the properties of possible substrate-binding sites, however glycine 324 is not located in any of these regions. It is important to note that glycine 324 is highly conserved between species, indicating that it may have an important role in ASS protein function. The importance of this glycine residue was further confirmed by the observation of a Gly->Ser 324 mutation in three other unrelated cases of citrullinemia, in a study performed by Kobayashi et al [9] that investigated 53 cases of the disease.

Glycine is a small, neutral amino acid that is important in protein folding, as it is easily accommodated in turns and protein interiors. Serine on the other hand is hydrophilic and would thus be most commonly found on the surface of proteins. This change in hydrophobicity and size could interfere with the folding of the protein, altering the active site itself or the ability of the subunits to form a

tetramer. The single ASS subunits possess some activity [1], so an inability of the subunits to form a tetramer may be a possible explanation for the residual activity in F25. A change in protein folding may also effect protein stability, decreasing the half life of the ASS enzyme.

Limited kinetic analysis of ASS was performed on both control and F25 cell lines (figure 23). The kinetic curve of the control gave a typical Michaelis-Menten reaction curve with an approximate Km of 20μM, which is in close agreement with that reported for liver [1]. However, F25 gave no interpretable curve, which would be consistent with F25 representing a Km mutant, although the results were in no way conclusive. It is possible that the Gly->Ser mutation may have decreased the enzyme affinity for citrulline, thus markedly reducing enzyme activity under normal physiological concentrations of citrulline. More informative results on the kinetics of this enzyme in F25 could be obtained from the analysis of purified ASS from this patient. The whole cell studies performed here only give an indication of the kinetic parameters, they are by no means ideal.

This mutation alone may not completely explain the decrease in activity of ASS in F25. Citrullinemia is a rare autosomal recessive disorder, thus requiring both ASS alleles to be mutated. Homozygosity is unusual in the case of rare disorders unless consanguinity has occurred earlier in the family history. The more likely situation is that of compound heterozygosity, different mutations on each ASS allele. This has been found to be the case in most of the studies

performed on citrullinemic patients [9].

Regarding the situation with F25, the results may suggest either a homozygous mutation or compound heterozygosity. Due to the presence of the G-A mutation in effectively six clones (three from F25, three from F535) it is likely that at this locus the mutation is homozygous. However, it is possible that only one allele is actually synthesised in these mutant cells, that being the one containing the G-A mutation. In this case the patient would be a compound heterozygote, with the other mutation representing a "promotor" defect on the other allele, where the amount or stability of ASS mRNA is affected.

It has been reported that in some cultured cell lines, ASS activity is regulated by an arginine-mediated negative feed back mechanism [24,25,26]. This regulation occurs at the pre-translation level and involves the control of ASS mRNA levels. The DNA element responsible for the mediation of the arginine-repression of ASS activity has been shown to occur within 150bp upstream from the transcription start-site i.e: within the promotor region [26]. In lymphoblasts, HeLa and KB cells a 3-60 fold increase in ASS activity is noted when the cells are incubated in the absence of arginine [26], with the increase directly linked to an increase in ASS mRNA levels.

An attempt was made to demonstrate this regulatory pathway in fibroblasts and to observe any abnormalities in the citrullinemic cell lines, by looking for ASS activation after incubation in arginine-free medium for 96 hour [25]. No ASS

activation was noted for control cell lines, however 5-7 fold activation was seen in the citrullinemic cell lines (figure 23). This result would have been consistent with a promotor defect resulting in the increased sensitivity of th ASS promotor to arginine, as is observed in other cultured cells [24,25,26]. However, this apparent increase in ASS activity was found to be due to an isotopic dilution effect of intracellular arginine pools on ^{14}C-protein formation, rather than true ASS activation. Thus according to the experiments performed in this study, the ASS promotor in fibroblasts is unresponsive to exogenous arginine levels and no evidence was collected regarding a promotor defect in F25. A more accurate means of assessing arginine repression of transcription from the ASS promotor, would be to study the levels of ASS mRNA produced when the cells are incubated in the presence and absence of arginine.

To elucidate the nature of the F25/F535 defect more clearly, several other studies need to be performed: The genetics of the parents must be analysed to establish if the affected children are homozygotes or compound heterozygotes. Levels of ASS mRNA produced in F25 must also be ascertained to establish if there is a promotor defect in these cells. This can be achieved by Northern-blotting or radioactive PCR techniques. The exact consequences of the Gly->Ser mutation in F25 can only be determined by a combination of experiments to establish the mutations effects on substrate binding, protein folding and subunit association.

1.5.B: GENBANK ERROR.

During the investigation of the citrullinemia mutation, it was found that the human ASS sequence [13] deposited in the Genbank database possessed a set of 4 incorrect bases. This conclusion was derived in two ways, one being that a sequence of a control fibroblast cell line differed from the Genbamk human sequence and the other being that the amino acid sequence of the Genbank human entry differed significantly from the sequence of other species (section 1.4.C.ii.b).

This result can be interpreted in terms of the discovery that the cells that were originally sequenced by O'Brien et al [13] were actually mutant cells. The human cells were CanR RPM1 2650 cells, utilised because of their increased expression of ASS mRNA, making it easier for cDNA prepartion, cloning and sequencing. As mentioned in the review on ASS, the mechanism of CanR has been shown to have similarites to the regulatory mechanism responsible for the high ASS activity in hepatocytes [27]. This mechanism may also be involved in the tissue-specific expression of ASS activity. Through the research on CanR cells more information about this possible ASS regulation mechanism is being investigated. At this stage, it is known that CanR results in increased levels of ASS cytoplasmic mRNA, that the mechanism operates at the pre-translational level and that a dominant positive trans-acting factor is involved [1,27,30,32]. It is not certain where this trans-acting

factor binds and it has been postulated that perhaps the sequence is present in one of the exons [26].

It is the information that the sequence involved in CanR and high activity in liver cells may be intragenic, that makes the "mistake" in the Genbank sequence very relevant. Perhaps these 4 base changes occur in the DNA element involved in this regualtory mechanism and are responsible for the increased ASS expression in these CanR cells. Before any further speculation was made in this respect, a thorough literature search produced a paper [9] published some 10 years after the human ASS sequence was originally published. This paper by the original authors admitted that the 4 base changes identified in this thesis were actually the result of a "sequencing error". However, it is hard to understand how O'Brien et al [13] could have made such a mistake in reading the sequence, since they sequenced three separate clones. It is therefore imperative that the original CanR clones are re-sequenced before these results are ignored.

SECTION 2

INVESTIGATION OF THE NITRIC OXIDE PATHWAY IN FIBROBLASTS.

2.1 REVIEW: THE NITRIC OXIDE PATHWAY.

2.1.A: INTRODUCTION.

The nitric oxide pathway represents the following reaction:

$$\text{L-arginine} \xrightarrow[\text{nitric oxide synthase}]{} \text{L-citrulline} + NO^{\cdot}$$

This reaction was only discovered six years ago in 1987 [50], when it was found that the elusive endothelium relaxing factor, involved in relaxation of vascular smooth muscle cells, was in fact the unstable, free radical nitric oxide (NO) [51,52]. Since this finding, NO·has been shown to be involved in many aspects of mammalian physiology from host immune defense mechanisms to neuronal stimulation. Nitric oxide is the smallest molecule, lightest and first gas known to act as a biological messenger in mammals [53].

The detailed nature of the production of NO· from L-arginine is shown in figure 24 [54] and demonstrates the unusual metabolic route, although still speculative at this stage. Another aspect of nitric oxide is that it was found to be an intermediate in the production of nitrates in the mammalian system [55]. Reduction of nitrates to nitrites is involved in the production of nitrosamines which are potent carcinogens [55,56], thus an understanding of the NO· pathway may have implications in understanding the process of carcinogenesis.

The key behind the diversity of functions of this gas is the enzyme that produces it: nitric oxide synthase (NOS). It has been demonstrated that there are at least two isoforms of this enzyme: constitutive (cNOS) and inducible (iNOS).

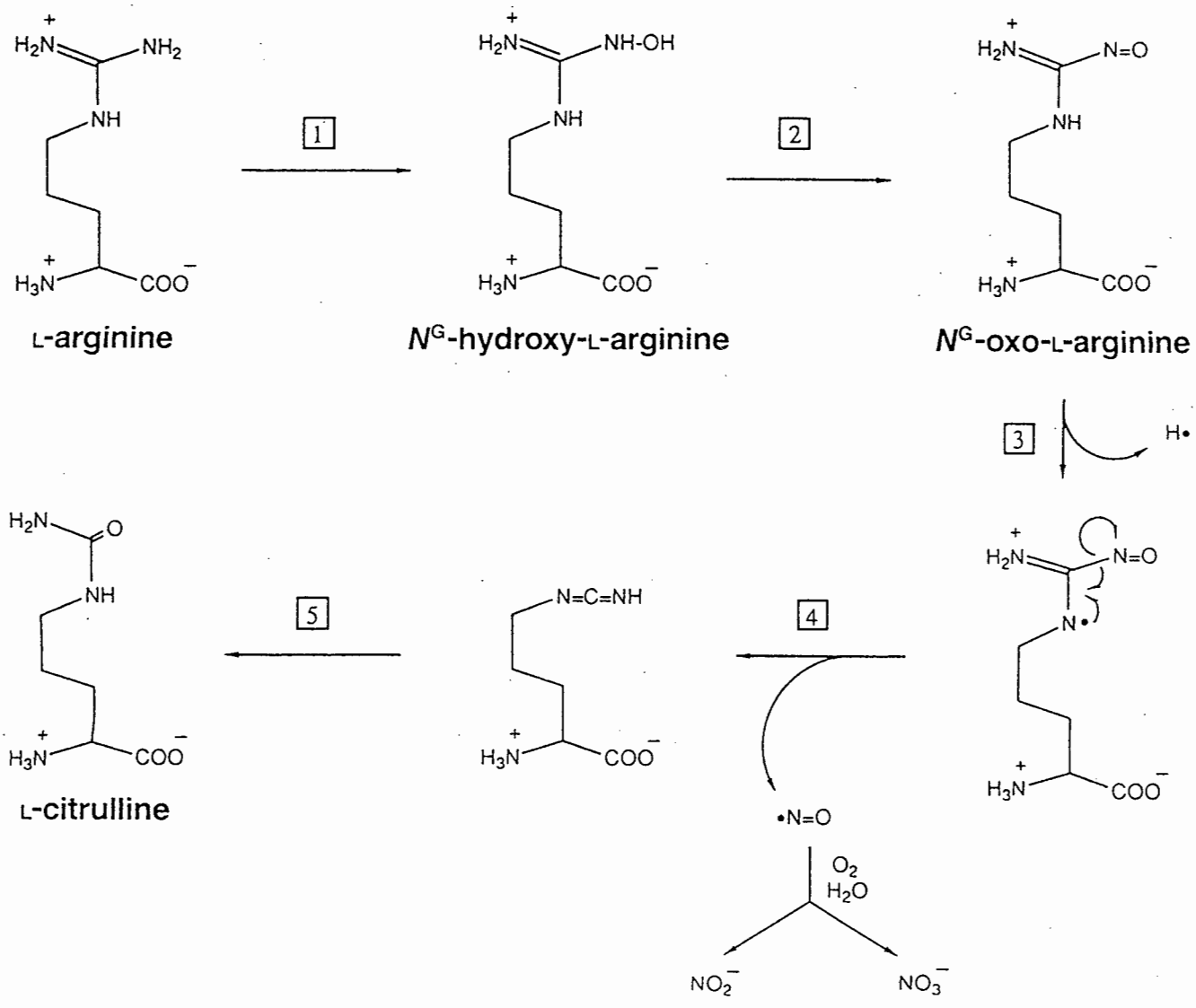

FIGURE 24: The metabolic route proposed for the formation of NO˙ from L-arginine by nitric oxide synthase [55].

A five step (1-5) reaction scheme for the conversion of L-arginine to NO˙, nitrate, nitrite and citrulline by nitric oxide synthase.

The specific distribution of the two isoenzymes and the different mechanisms of regulation allow NO· to fulfill such varied functions. Both isoenzymes catalyse the same reaction, both are dioxygenases, cytosolic and utilise the same cofactors: tetrahydrobiopterin (BH_4), NADPH, FAD and FMN [57,58,59,60]. However, they differ extensively in their dependance on Ca^{2+} and in the amount of nitric oxide that they produce. The two enzymes share a 51% amino acid sequence homology [60], differing largely in the region of the predicted calmodulin binding site [61]. The subunits of the inducible form are slightly smaller in size (125-135kDa) to that of the constitutive enzyme (150-155kDa) and appear to be active in the dimeric form rather than monomers (cNOS) [58].

The use of both FAD and FMN as co-factors is unusual, with only one other mammalian enzyme in this category: NADPH-cytochrome P-450 oxireductase [61]. The FAD and FMN cofactors of this enzyme (P-450) allow the one-electron reduction of hemoproteins by stabilizing the resultant radical, formed within the oxireductase. It is possible that NOS utilises a similar mechanism in the synthesis of NO·. Another similarity between P-450 and NOS is the presence of ironprotoporphyrin lX, with cysteine as the ferric (111) iron ligand [62]. An interesting point that deserves further investigation relates to one of the bi-products of the P-450 reaction - carbon monoxide (CO). One of the major functions of P-450 is the donation of electrons to the heme-oxygenase enzyme which breaks down heme. When P-450 donates electrons

to heme-oxygenase CO is liberated. Evidence has been gathered [63] that supports the notion that CO is also a neuronal messenger, involved in the regulation of cyclic GMP levels. Thus the structural similarity between P-450 and NOS may refect their involvement in the production of a new class of neurotransmitters.

2.1.B: CONSTITUTIVE NITRIC OXIDE SYNTHASE.

The constitutive form of NOS has been detected in vascular endothelial cells and cerebellar neurons and is dependant upon Ca^{2+} (100-500nM) [60], calmodulin and oxygen activity [64]. The constitutive form is a rapid response enzyme involved in signal transduction, producing small bursts of $NO^{\cdot}$ in response to changes in Ca^{2+} concentrations. The $NO^{\cdot}$ produced by this enzyme acts as an unusual neurotransmitter [65], involved in the stimulation of guanylate cyclase activity [51]. Most neurotransmitters are amino acids or peptides, which are stored in specific vessicles and on release interact with specific receptors on the surface of cells [63]. $NO^{\cdot}$ operates very differently, simply diffusing from one cell to another once synthesized in the cytoplasm [53,66].

B.i: Endothelial cells.

In vascular endothelial cells $NO^{\cdot}$ is involved in vascular muscle relaxation [60]. Blood vessels are dilated by neurotransmitters such as acetylcholine and norepinephine, that cause the muscle layer to relax or contract. As mentioned above, neurotransmitters normally act through

receptors on the surface of muscle cells, However, no such receptors were found for acetylcholine. Acetylcholine receptors were in fact found on the surface of vascular endothelial cells that line the vascular muscle cells [63]. The mechanism of action was found to involve another neurotransmitter: NO$^{\cdot}$ (refer to figure 25) [60,63,67]. When acetylcholine binds to the receptors on the endothelial cells it causes an influx of Ca^{2+} into the cell, which binds to calmodulin. This complex in turn binds to cNOS and activates it [63] to produce small amounts of NO$^{\cdot}$ in the presence of oxygen [67]. Due to the fact that this enzyme is constitutively produced, a rapid response of NO$^{\cdot}$ can be produced upon activation.

NO$^{\cdot}$ is lipid soluble and quickly diffuses across all membranes to the smooth muscle cells [64]. Here the NO$^{\cdot}$ free radical binds to the heme co-factor (Fe-thiol centre) of guanylate cyclase, causing a structural change that activates the enzyme more than 50 fold [60,62,63]. Guanylate cyclase then causes an increase in cGMP levels which results in the relaxation of the vascular smooth muscle cells, and an overall decrease in blood pressure [60,62,63]. NO$^{\cdot}$ has also been shown to operate as a neurotransmitter in the relaxation of smooth muscle cells in the gastrointestinal tract, the air passages [53] and in the relaxation response of the urethra [68].

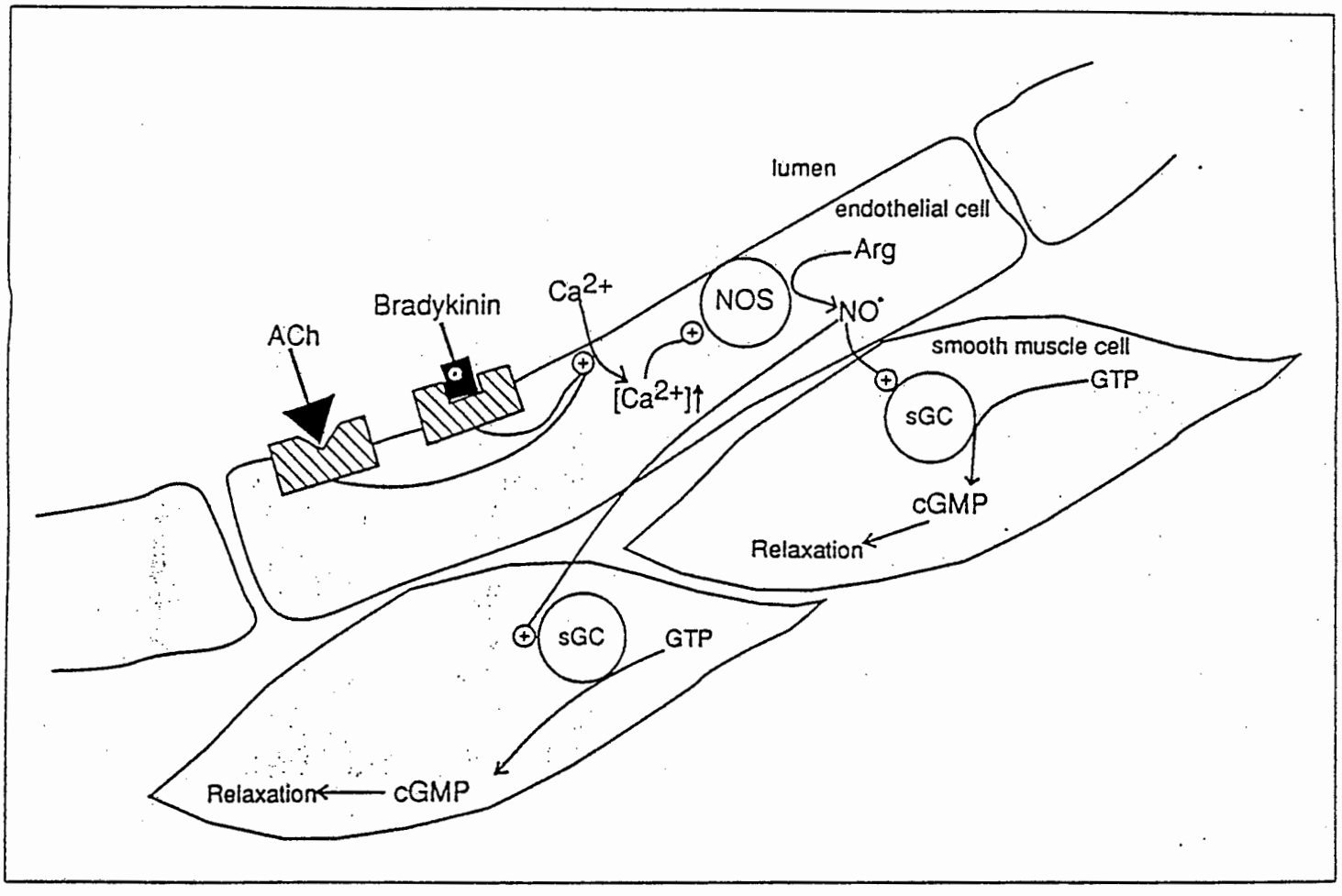

FIGURE 25: The synthesis and action of nitric oxide in endothelial cells [60].

The signal transduction pathway of endothelium-dependant vascular relaxation. ACh = acetylcholine, cGMP = cyclic GMP, NOS = nitric oxide synthase, sGC = soluable guanylate cyclase, (+) = stimulation.

It is interesting to note that the drugs that are used to treat angina (nitroglycerine and its derivatives) act by being metabolised to $NO^{\bullet}$, which then causes the dilation of blood vessels [55,69,70,71].

B.ii: Neurons.

In cerebellar neurons the excitory neurotransmitter glutamate has also been shown to function through the production of $NO^{\bullet}$ [63]. The effects of glutamate are mediated through several types of activated channels, one of which is a N-methyl-D-aspartate (NMDA) receptor [72]. This receptor controls a post-synaptic, voltage-dependant slow calcium channel. When glutamate binds at these receptors (NMDA) (see figure 26), it causes the opening of the calcium channels. The calcium binds to calmodulin, which in turn activates cNOS. The $NO^{\bullet}$ that is produced activates guanylate cyclase [63].

NMDA-type receptors have been implicated in the triggering of long-term potentiation (LTP) (the strengthening of synapses through use) which is proposed to be an important memory mechanism. Because NMDA receptor activation is involved in both LTP and $NO^{\bullet}$ production, the possibility of $NO^{\bullet}$ being involved in this memory mechanism was investigated [70]. LTP involves the post-synaptic neuron signalling the pre-synaptic neuron to release more neurotransmitter. The intracellular retrograde messenger needed to convey this message was found to be $NO^{\bullet}$ [70].

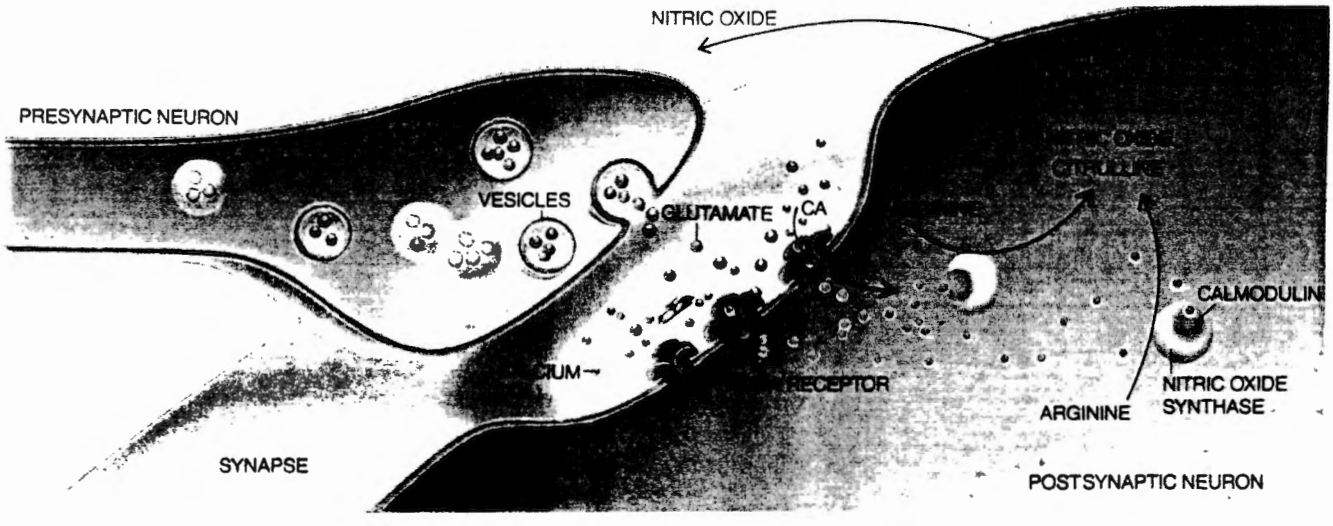

FIGURE 26: Nitric oxide production in neurons [63].

Neuronal nitric oxide is synthesised and released in response to the binding of glutamate to the NMDA receptor. Binding causes the influx of calcium ions into the neuron, this calcium binds to calmodulin which in turn activates cNOS to produce $NO^{\bullet}$.

B.iii: Other cNOS locations.

cNOS has been located in the pituitary gland, in neurons that synthesize and release vasopressin and oxytocin [63,73]. In the adrenal gland it has been found in neurons that stimulate the release of adrenalin from adrenal cells [63]. In light of these observations it has been proposed that $NO^{\cdot}$ is involved in the control of hormone activity.

Constitutive, Ca^{2+} dependant NOS has also been reported in the brain [9,63] with the majority of the cNOS being located in the neurons [63].

Mast cells have been implicated in inflammation, allergy, parasitic infection as well as other processes. Bissonnette et al [74] showed that at least 34% of the cytotoxicity of peritoneal mast cells can be attributed to the production of $NO^{\cdot}$. $NO^{\cdot}$ production by these cells does not require protein synthesis and has more similarity to endothelial cell $NO^{\cdot}$ synthesis (involves cNOS) than to macrophage $NO^{\cdot}$ production (involves iNOS, see below). Due to this similarity and the fact that mast cells are often found near nerve termini and vasculature, it has been proposed that mast cells may also participate in the vascular changes that occur during pathological responses.

2.1.C: INDUCIBLE NITRIC OXIDE SYNTHASE.

This isoenzyme (iNOS) is inducible at the gene level, with transcription being activated in response to cytokine stimulation [60,61,71]. Several hours of cytokine stimulation are necessary to activate the Ca^{2+} independent iNOS

[55,61,63], however once this protein has been induced it produces large amounts of $NO^{\cdot}$. iNOS has been detected in macrophages [54,58,61], neutrophils [71] and smooth muscle cells [74,75,76].

The $NO^{\cdot}$ that is produced by iNOS participates in the host immune defense system against invading organisms and tumour cells. iNOS was first discovered in macrophages where it is responsible for some of the cytotoxic effects of these cells. Since then it has also been shown to exist in a variety of other cells where it supposedly operates in a similar way. The production of $NO^{\cdot}$ metabolites (nitrates and nitrites) upon cytokine stimulation has been shown to occur in EMT6 and TA3 murine adenocarcinomas cell lines [54], murine fibroblasts [77], rat hepatocytes, rat kupffer cells [69], endothelial cells [78], and leukocytes [50].

When micro-organisms enter the body, lymphoblasts are activated by accessory cells presenting foreign antigens [50] . In response they produce cytokines such as γ-interferon, which activate the macrophages to attack the micro-organisms see figure 27 [71]. Macrophages are further stimulated by interferon in conjunction with other molecules such as tumour necrosis factor (TNF) and lipopolysaccharide (LPS) [55]. By binding to the macrophage cell surface γ-interferon activates the transcription of iNOS and results in the production of large amounts of $NO^{\cdot}$. The exact method of transcription induction by these cytokines is at present unknown, although protein kinase C has been directly linked to this signalling process [79].

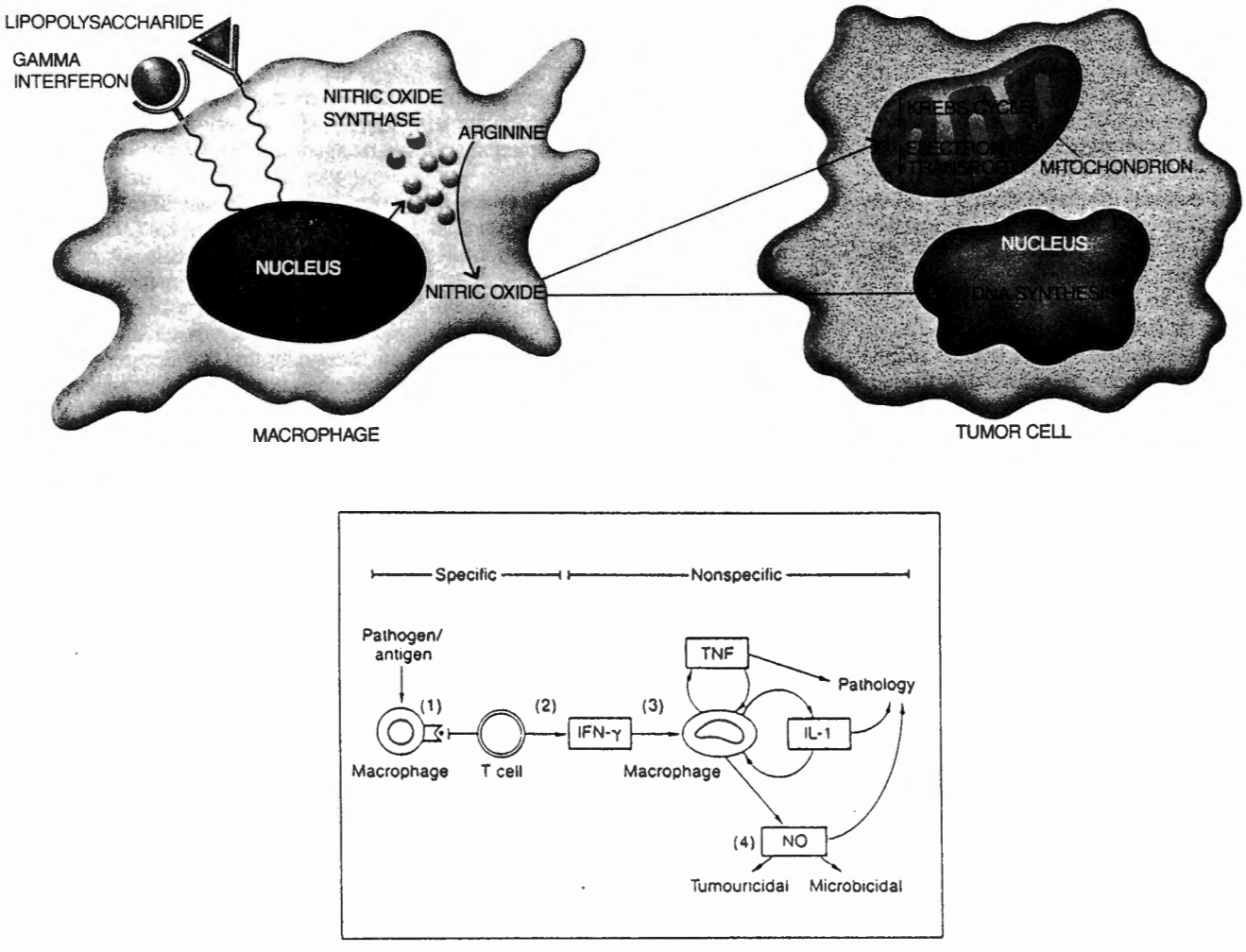

FIGURE 27: Nitric oxide involvement in non-specific host immune defence mechanisms [50,63].

When microorganisms enter the system, lymphocytes are stimulated to produce cytokines such as interleukin (IL-1), tumour necrosis factor (TNF) and gamma-interferon (γ-IFN). These molecules induce the transcription of iNOS in the macrophages. The nitric oxide synthesised by this enzyme diffuses out of the macrophages into microorganisms and tumour cells. It inhibits cell growth by binding to Fe-thiol centres of enzymes involved in DNA replication and energy production.

It has been demonstrated that ɣ-interferon stimulates the activity of guanosine-triphosphate cyclohydrolase 1 (5-100 fold increase) [80], which catalyses the key step in the synthesis of BH_4 [71,77,81]. BH_4 has been shown to be an essential co-factor of the inducible isoenzyme of NOS, less so in the case of the constitutive form ,which shows a variable 2-9 fold activation in the presence of this co-factor [57,59,60,73] . In the case of cNOS, Wolff and Datto [73] suggested that some cNOS sources may retain a tightly bound form of BH_4, which confers activity in the absence of exogenous BH_4 , explaining the variability in results.

It has been reported that BH_4 participates in the initial step of the L-arginine ---> $NO^{\cdot}$ reaction , resulting in the N-hydroxylation of arginine [77]. Some controversy surrounds the involvement of BH_4 in this enzymatic reaction, with some groups reporting that BH_4 participates directly [73] and others suggesting that its effect is indirect (in the case of cerebrallar cNOS) [58]. It is possible that BH_4 stimulates NOS activity by an allosteric mechanism [73].

It has also been reported that BH_4 acts as a co-factor of indoleamine 2,3dioxygenase (IDO), which is also strongly induced by cytokine stimulation [82]. IDO is involved in the degradation of the essential amino acid tryptophan and is thought to play some role in cytokine induced host defense mechanisms. How the actual NOS gene is activated to synthesize NOS mRNA upon cytokine stimulation still remains a mystery, although IDO and BH

may be involved.

Other than human macrophages the stimulation of BH_4 synthesis by γ-interferon and LPS has been shown to occur in human fibroblasts [82], murine fibroblasts [77,81] and a number of human tumor cell lines [80]. In murine fibroblasts this increase in BH_4 synthesis has recently been directly linked to the production of $NO^{\cdot}$ from L-arginine [77]. It is not known if in other human cell lines an increase in pteridine synthesis by cytokines results in the production of $NO^{\cdot}$.

Returning to the inducible $NO^{\cdot}$ response, the $NO^{\cdot}$ that is produced by the iNOS diffuses out of the macrophages and binds to Fe-thiol centres of the bacterial/tumor cell enzymes, causing the formation of iron-dinitrosyl-dithiolate complexes [62]. $NO^{\cdot}$ only has a half-life of 6-30 seconds [50,64] as it reacts with oxygen to form nitrate and nitrite [59,63], thus the target cells have to be fairly close to be affected. In binding to the Fe-S group $NO^{\cdot}$ alters the 3-dimensional structure of the active site and prevents enzyme activity. Enzymes that are normally affected are those involved in DNA replication and mitochondrial respiration (complex 1 and 11, aconitase and ribonucleotide reductase) [83]. Depending upon the activity of the affected enzyme, either the bacterial/tumor cell growth will be slowed or the cell will die (figure 27) [51].

At the site of infection available arginine is quickly converted to NO$^{\cdot}$ and citrulline as a consequence of macrophage stimulation. However, further arginine that enters the site is then kept limiting by the action of arginase so as to prevent the prolonged synthesis of NO$^{\cdot}$. This control mechanism is necessary as large amounts of NO$^{\cdot}$ damage cells in a non-specific manner, preventing the healing process. In this respect NO$^{\cdot}$ production has also been linked to the inhibition of platelet aggregation [63,71,75,84]. The control of the NO$^{\cdot}$ pathway thus hinges around the control of available arginine, requiring a delicate balance between the ARG -> ORN and ARG -> NO$^{\cdot}$ reactions of arginine metabolism. It has been estimated that in activated macrophages, 70% of Larginine is metabolised by arginase and the remaining 30% by the NO$^{\cdot}$ route [7].

In addition to the above cytotoxic effects, NO$^{\cdot}$ reacts with superoxide to form peroxynitrite, which once protonated forms nitrogen dioxide and the hydroxyl radical. These radicals are extremely cytotoxic and may also be involved in combatting invading organisms [64].

2.1.D: REGULATION OF NOS.

The cytotoxic effects of macrophages have been shown to be highly L-arginine dependant, requiring an extracellular concentration of greater than 0.25mM arginine for maximal activity (Km = 37μM) [7]. Bogle et al [7] demonstrated that an increase in the transport of L-arginine into the macrophage occured upon macrophage activation and that

arginine availability was rate-limiting in the production of $NO^{\cdot}$. This arginine dependance has also been shown in smooth muscle cells' iNOS [76]. These results suggest a regulatory step, independant of direct NOS regulation and that selective inhibition of cellular arginine uptake may be a mechanism of reducing macrophage cytotoxicity. L-lysine and L-ornithine have been shown to directly inhibit NOS activity at concentrations higher than 5mM [7].

Castillo et al [56] recently showed that there exists significant compartmentalisation of arginine metabolism within the whole body. There was shown to be a definite barrier between the hepatocytes and the plasma, as well as compartmentalisation within the liver itself (associated with urea cycle enzyme distribution). The functional importance of this compartmented metabolism of arginine has not been elucidated, but it may play a role in the activity and regulation of the $NO^{\cdot}$-pathway. This also has relevance to NOS inhibitors as the compartmentalisation may effect their efficiencies.

Helper-T-lymphocytes TH1 and TH2 produce different lymphokines that often act antagonistically. TH1 produces IFN-γ and IL-2, while TH2 synthesises IL10, IL-4 and IL-5. IFN-γ inhibits proliferation of TH2 cells, while IL-4 and IL-10 inhibit IFN-γ secretion. It has also been shown that IL-4 and IL-10 affect the cytotoxic effects of macrophages, by preventing the induction of $NO^{\cdot}$ production by IFN-γ , when cells are pretreated with these lymphokines [85].

What makes the situation of NOS regulation more complex is that one cell type may contain both types of NOS (iNOS and cNOS), producing $NO^{\cdot}$ for very different functions i.e: endothelial cells [60,78] and macrophages [86]. This presumably requires complex regulatory mechanisms. N-nitro-L-arginine, N-amino-L-arginine and N-methyl-L-arginine have been shown to reversibly inhibit NOS [68]. However, cNOS is most effectively inhibited by N-nitro-arginine, while iNOS is preferentially inhibited by the methyl and amino derivatives [66,87]. Using these various inhibitors one would be able to selectively affect one isoform without seriously affecting the production of $NO^{\cdot}$ by the other isoenzyme.

2.1.E: OTHER NOS ISOFORMS.

The classification of two isoforms of NOS made it easier to understand the complex roles of $NO^{\cdot}$ as a neurotransmitter and an immune defense mechanism. These enzymes are sufficiently different to explain how they can be regulated independantly, especially with regards to Ca^{2+} dependancy. However, as NOS is being isolated from other sources, it appears that the situation is more complex. Evans et al [88] purified a cytokine-inducible form of NOS from rat liver (predominantly in hepatocytes) and showed that although it is not dependant upon Ca^{2+} , it is stimulated in the presence of calmodulin, which is very unusual [88]. The $NO^{\cdot}$ produced by this enzyme seems to be involved in the suppression of protein synthesis and protection of the liver against damage [88,89].

Although the cNOS enzyme found in endothelial cells operates in the same way as the neuronal cNOS, it has been shown that these two enzymes are distinct isoenzymes [88], being generated from different genes. Neuronal cNOS (nNOS) is totally cytoplasmic, while endothelial cNOS (eNOS) is often associated with membranes and has slightly smaller subunits [59,86]. It has now been found that iNOS, nNOS and eNOS are derived from independant genes that form part of a gene family [86].

2.1.F: NITRIC OXIDE AND DISEASE.

It has already been mentioned that the production of nitric oxide has to be tightly controlled as large amounts produce detrimental effects. In fact several neuronal diseases have been attributed to defects in the $NO^{\cdot}$ pathway i.e : Huntington's and Alzheimers diseases [63].

One way that neuronal damage is caused is by the prolonged activation of NMDA receptors, which causes an excessive influx of calcium resulting in the production of large amounts of $NO^{\cdot}$. The action of $NO^{\cdot}$ in turn results in a continual high level of cGMP, which appears to eventually result in neuronal damage. Strangely enough it has been shown that the neurons that contain the NOS are protected while the adjacent neurons are damaged [63].

Due to this new understanding of the role of nitric oxide in neuron activity , drugs that inhibit the $NO^{\cdot}$ pathway such as N-monomethyl-L-arginine (L-NMMA) are now being used to treat strokes [63].

It has been proposed that $NO^{\cdot}$ may also be involved in cerebral injury when ischemic or hypoxic brain tissue is reperfused with oxygen, see figure 28. Ischemia depolarises the neuronal membranes causing the release of glutamate from synaptic vesicles. This release activates the influx of calcium into the cells, which in turn activates an oxygen-dependant NOS. This enzyme synthesizes $NO^{\cdot}$ from arginine when oxygen is readmitted to the brain during reperfusion. Ischemia also causes the induction of superoxide production by xanthine oxidase. Superoxide then reacts with $NO^{\cdot}$ to form the peroxynitrite anion ($ONOO^-$). $ONOO^-$ is fairly stable, enabling it to diffuse over several cell diameters, however once protonated it decomposes to form two very destructive cytotoxic oxidants: nitrogen dioxide and the hydroxyl radical, which result in cerebral damage [60,64]. The formation of these radicals is shown below:

$$NO^{\cdot} + O_2 \quad \text{----->} \quad ONOO^-$$

$$ONOO^- + H^+ \quad \text{----->} \quad ONOOH$$

$$ONOOH \quad \text{----->} \quad NO_2 + {}^{\cdot}OH$$

$$NO_2 + {}^{\cdot}OH \quad \text{----->} \quad NO_3^- + H^+$$

However, peroxynitrite can also cause damage by reacting with sulfhydryl groups [90] and metal ions to form a potent nitrating agent [64]. From the evidence of the reaction of superoxide with $NO^{\cdot}$ it is possible that these two molecules are involved in other pathological processes such as sepsis and inflammation.

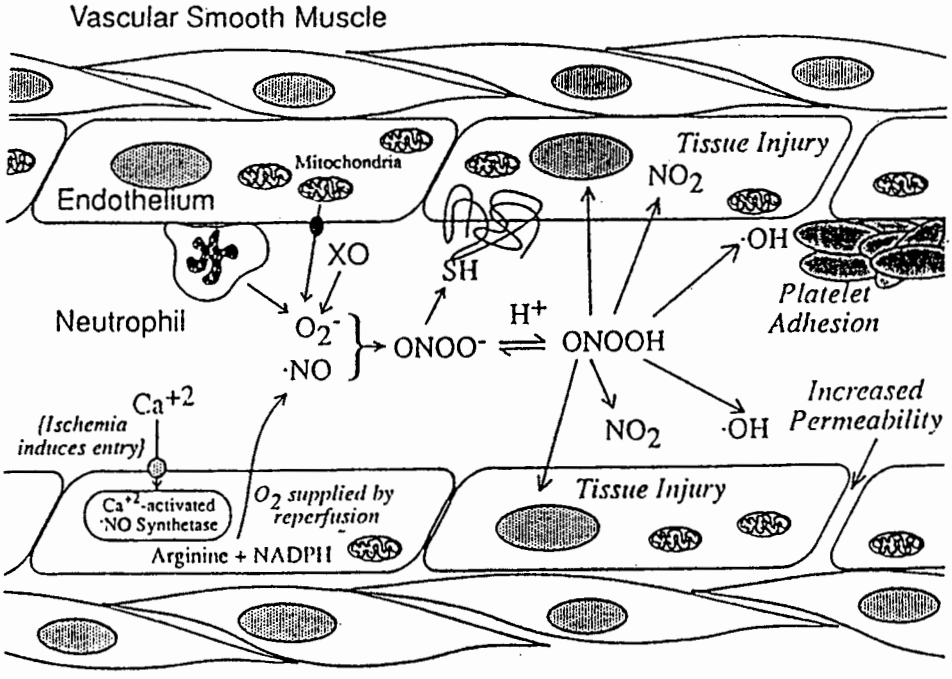

FIGURE 28: Superoxide-mediated cerebral injury after reperfusion [64].

Ischemia allows calcium to enter into the endothelial cells, which stimulates cNOS to produce NO at maximal rates when the tissue is reperfused with oxygen. However, when the tissue is reoxygenated superoxide (O_2-) may also be produced by mitochondria, neutrophils and xanthine oxidase (XO). Superoxide and NO· react to form peryoxnitrite anion (ONOOH) that decomposes to form deadly hydroxyl radicals (·OH) once protonated.

Interestingly, it has been suggested that $NO^{\cdot}$ may be produced to protect cells from superoxide mediated damage. Rubanyi et al [67] found that $NO^{\cdot}$ decreased the reduction of cytochrome C by the superoxide produced in leukocytes. This reduction was found to be from the direct reaction between $NO^{\cdot}$ and $O_2^{\cdot-}$. Perhaps under conditions where large amounts of superoxide are produced, $NO^{\cdot}$ is synthesised to act as an oxygen free-radical scavenger. This aspect obviously needs more research to establish if $NO^{\cdot}$ is friend or foe!

In type 1 diabetes, it was generally assumed that the cytokines IL-1 and TNF were directly responsible for the destruction of islet cells. However, Kroncke et al [91] have since shown that $NO^{\cdot}$ produced by cytokineactivated macrophages and endothelial cells, is in fact responsible. This evidence supports the idea that $NO^{\cdot}$ may injure normal cells and contribute to inflammatory tissue destruction.

Septic shock results in many hospital deaths . It is associated with the over-production of $NO^{\cdot}$, by the inducible NOS [58,60] in cells including endothelial cells and smooth muscle cells [76]. A substantial fall in blood pressure occurs when large amounts of $NO^{\cdot}$ are produced in response to a severe bacterial infection. It has been demonstrated that administration of nitric oxide synthase inhibitors can normalise the blood pressure. Interleukin-2 treatment is often used to treat some forms of cancer, however it stimulates the production of $NO^{\cdot}$, which can result in life threatening low blood pressure. To avoid this side-affect IL-2 is given in conjunction with $NO^{\cdot}$ inhibitors, allowing

IL-2 to act on the cancer cells, without the hypotensive side-effects [53].

It is accepted that nitrite, under acidic conditions, can deaminate purines, pyrimidines and various forms of RNA and DNA. These reactions occur through the protonation of NO_2^- to form N_2O_3, an electrophilic nitrosating agent [92]. Nguyen et al [92] demonstrated that $NO^{\cdot}$ in combination with O_2^- can mimick the effects of NO_2^- and damage and mutate mammalian DNA. $NO^{\cdot}$ was found to induce DNA strand breaks and cause the deamination of guanine. This would result in a high percentage of G.C-->A.T transitions in cells exposed to high concentrations of $NO^{\cdot}$. With relation to cancer formation two types of mutations in the P53 gene have been associated with cancer. These are 1) transitions at CpG dinucleotides in colon cancer and 2) G.C-->T.A transversions in liver and lung cancer. These mutations can be explained in terms of exposure to $NO^{\cdot}$ as a result of the inflammatory response, or bacterial infection.

2.1.G: REACTIONS OF NITRIC OXIDE.

At this point the cytotoxic and neurological effects of nitric oxide have been attributed to $NO^{\cdot}$ itself, however due to its reactivity it has been proposed that other $NO^{\cdot}$ reaction products may be the biologically active molecules [62]. A summary of the possible reactive products is given below in figure 29.

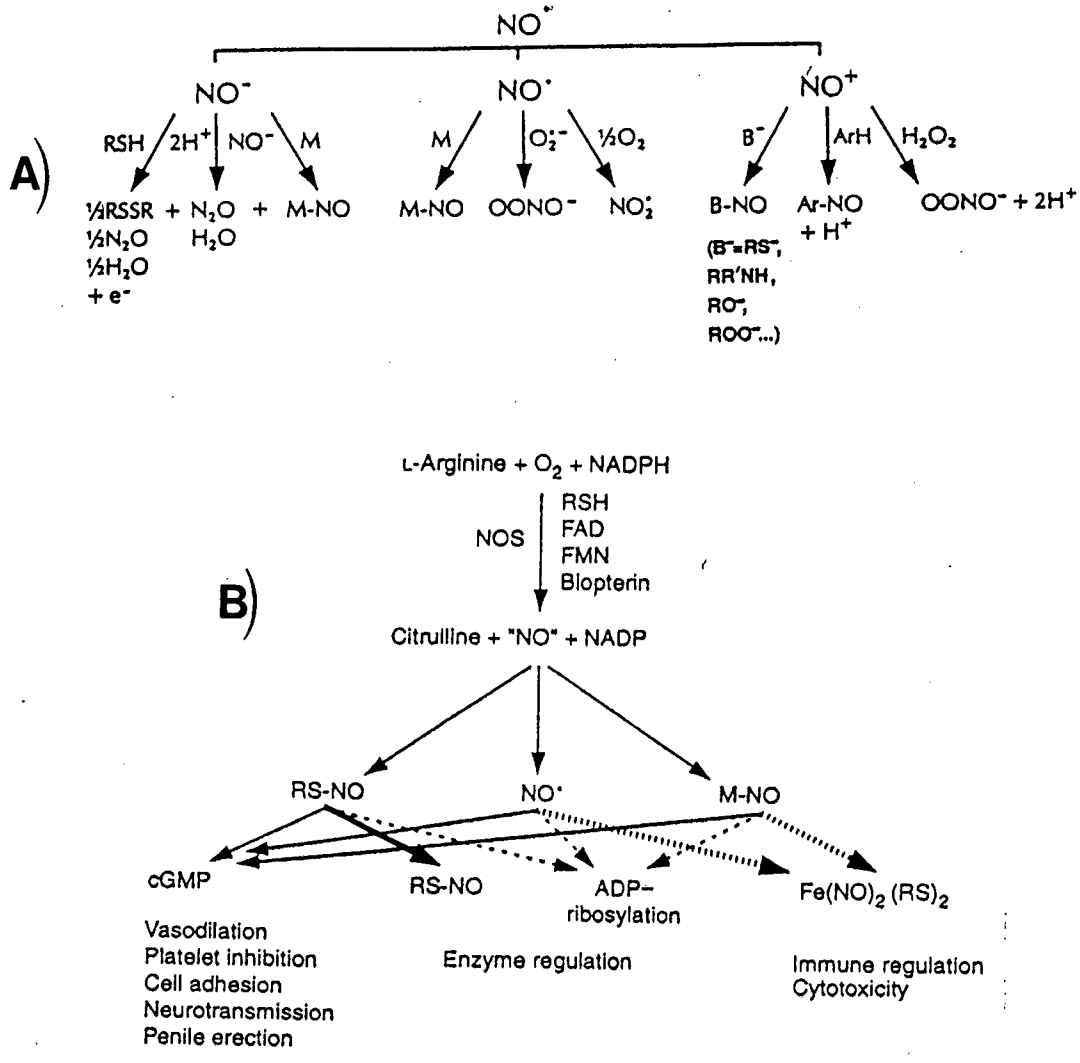

FIGURE 29: Summary of the chemistry of the interrelated forms of NO• of potential biological significance [62].

A) Redox-interrelated forms of NO•. The primary reactions of NO• involve oxygen, superoxide and redox metals (M). NO⁻ reacts with metals and sulphydryls (SH) . Nitrosation reactions involve electrophilic aromatic substitutions (Ar) and addition to bases (B). **B)** Summary of NO• biochemistry. The reaction products of NO• that allow NO• to fulfill it's functions as a neurotransmitter and a host immune defence mechanism.

The redox state of $NO^{\cdot}$ seems to influence its affinity for different target functional groups i.e: $NO^{\cdot}$ reacts at the heme site of hemoglobin, while NO^{+} reacts with intramolecular amine and sulfhydryl centres and NO^{-} at sulphydryl centres. At this stage it is not exactly clear if $NO^{\cdot}$, NO^{+} or NO^{-} is involved in the activation of guanylate cyclase, or what functional group is attacked [62].

Although most research has been performed on mammalian models, it is possible that $NO^{\cdot}$ functions in other systems as well. The elusive endothelium relaxing factor (EDRF) has been demonstrated in reptiles and the key enzyme in the reactivity of $NO^{\cdot}$, guanylate cyclase, has also been found in squid giant axons. It is possible that the $NO^{\cdot}$ pathway has an early evolutionary origin [51].

2.2 INTRODUCTION: NITRIC OXIDE PRODUCTION, A NOVEL APPROACH TO STUDY NITRIC OXIDE PRODUCTION IN FIBROBLASTS.

$NO^{\bullet}$ is fast becoming an integral part of cellular biology and the understanding of its effects essential to the elucidation of the mechanisms underlying many diseases. The $NO^{\bullet}$ story is not an easy one to understand, with many isoforms of NOS that are widely distributed among different cell types and $NO^{\bullet}$ itself demonstrating a varity of biological roles.

Many studies on $NO^{\bullet}$ production have been performed using cell free extracts of NOS [54,57,73,88], measuring $NO^{\bullet}$ production under in vitro conditions. More recently whole cell assay systems have been utilised [7,78,85], including studies on macrophages and a variety of human tumor cell lines. In both these systems NOS enzyme activity has been measured in a number of ways. NOS activity can be measured indirectly, by measuring the formation of nitrates and nitrites from $NO^{\bullet}$ [7,78,85] or somewhat more directly by measuring the formation of citrulline from arginine (since citrulline and $NO^{\bullet}$ are produced in equimolar quantities [63]) under various conditions [54,59,73,88]. The problem with whole cell assay systems, when analysisng $NO^{\bullet}$ production by the formation of citrulline, is that cells that contain ASS are capable of metabolising the citrulline, making quantitation difficult.

The citrullinemic cell-line F25 has an almost complete loss of ASS activity (1-5% of controls) making it a good model for studying the production of NO· in fibroblasts. Fibroblasts contain several enzymes of the urea cycle: arginase, ASS, ASL and carbamyl phosphate synthetase [1,6]. However, ornithine tanscarbamylase activity has not been reported in fibroblasts, which implies that fibroblasts are unable to produce citrulline from arginine or ornithine. Inducible NOS activity can then be detected in fibroblasts by the formation of citrulline from arginine after the required cytokine induction. In citrullinemic fibroblasts (F25) the lack of ASS activity will allow this citrulline to accumulate, making it easy to detect.

So far, NO· production has not been directly demonstrated in human fibroblasts although some evidence suggests that NO· synthesis is possible. Firstly, WernerFelmayer et al [77] reported that BH_4 synthesis in murine fibroblasts after treatment with cytokines , is directly linked to the production of NO· Secondly BH_4, which is a cofactor of iNOS, has also been shown to be synthesized in response to interferon stimulation in human fibroblasts [82]. No clear function had previously been found for cytokine-induced BH_4 synthesis in mammalian cells and it is possible that it is mainly produced as a co-factor for the production of NO· [77]. Finally , fibroblasts show similarites to macrophages in their ability to increase both IDO and BH_4 synthesis in the presence of a

combination of cytokines (γ-interferon, LPS and TNF) and it has been proposed that fibroblasts may in fact participate in the general immune response [82].

Fibroblasts are readily accessible for diagnostic purposes and the demonstration of $NO^{\bullet}$ production in these cells may allow for the development of assay systems for $NO^{\bullet}$ production defects.

The purpose of this study was to use the F25 model to investigate the production of $NO^{\bullet}$ by iNOS in fibroblasts, in response to cytokine induction. The chosen method of investigation of $NO^{\bullet}$ production was the analysis of ^{14}C-citrulline formation from ^{14}C-arginine. This demanded an efficient method of separation of citrulline, arginine, ornithine and urea, all of which may be formed in fibroblasts from the metabolism of arginine. Section 2A deals with the development of the various separation techniques, while section 2B investigates the preliminary tests required to develop the nitric oxide detection assay in fibroblasts.

SECTION 2A: EVALUATION OF AMINO ACID SEPARATION TECHNIQUES.

2.3: INTRODUCTION.

To investigate the $NO^{\cdot}$ pathway in fibroblasts, it was necessary to study the utilisation of arginine by these cells. The assay for $NO^{\cdot}$ detection involved the incubation of fibroblasts with radioactively labelled arginine (^{3}H, ^{14}C), preparation of cellular supernatants after the incubation period and then the analysis of the radiolabelled amino acid products of metabolism.

There are several techniques that can be used to separate and quantify the amino acid products: HPLC, thin-layer chromatography and ion-exchange chromatography. These methods separate amino acids according to size and differences in the properties of their side chains. The most sensitive separation method is the HPLC system. However, a suitable HPLC system was not initially available and so the suitability of thin-layer chromatography and ion-exchange chromatography was investigated. Upon the aquisition of appropriate HPLC columns, the optimal separation of radioactive amino acid metabolites on these columns was also investigated.

2.4: MATERIALS AND METHODS.

2.4.A: ION-EXCHANGE CHROMATOGRAPHY.

Ion-exchange chromatography is performed using resins such as Dowex-50 and separates amino acids under the influence of gravity rather than high pressure. Cation-exchange resins (Dowex-50) used in ion-exchange chromatography separate amino

acids according to their net charge differences at a specific pH. Cation-exchange resins possess negatively charged groups that bind the postively charged groups of the amino acids. The strength with which the amino acids bind to the resin depends upon the pKa value and the number of charged amino groups [93].

Amino acid mixtures are made acidic, so that the amino acids are optimally positively charged, loaded on to the column and then eluted with a basic solution. The acidic amino acids are eluted first followed by neutral and then basic amino acids.

The procedure that was used in this study was a modification from the separation technique used by Windmueller and Spaeth [2]. 1, 2 or 2.5mls of prepared Dowex-50 (Biorad) analytical grade cation-exchange resin (washed several times with distilled water) was loaded into a column of either 0.7cm or 0.5cm in diameter.

Amino acid mixtures were prepared as follows:

1ml Basal Medium Eagles (BME) containing:

1µCi ^{3}H-arginine (Amersham, 19Ci/mmol) and/or
1µCi ^{14}C-citrulline (Amersham, 58mCi/mmol)
1µCi ^{14}C-ornithine (Amersham, 208mCi/mmol)

0.5ml of the solution was adjusted to pH2 with 0.1M HCl.

The prepared amino acid solutions were loaded onto the Dowex-50 columns and eluted with ammonia solution (25%) (BDH). Fractions were collected in 1ml aliquots. The relative positions of the eluted amino acids were determined by measuring the radioactivity in each fraction. 100µl of each fraction was first neutralised with glacial acetic acid, to

prevent the chemoluminescence that occurs in alkaline solutions, then added to 6ml scintillation fluid (Packard Hionic-Fluor) and the radioactivity quantified in a scintillation spectrometer (Beckman LS 3801). The cpm values were converted to dpm by adjusting the values for background counts and the counting efficiency of the scintillation counter (^{3}H in ^{3}H window = 42%, ^{14}C in ^{14}C window = 72%, ^{14}C in ^{3}H window = 18%).

2.4.B: THIN-LAYER CHROMATOGRAPHY: SILICA PLATES.

Thin-layer chromatography is a technique that separates amino acids according to their mobilities in organic solvents. It relies on the separation of amino acids between two phases: stationary phase (thin layer of water on a solid support i.e: silica or cellulose) and a mobile organic phase. The amino acids are placed at the bottom of the plate and as the organic solvent moves across the plate, so the amino acids are separated by partition between aqueous and organic phases. Hydrophilic amino acids remain in the stationary aqueous phase, whereas more hydrophobic amino acids partition preferentially in the organic solution and move rapidly up the plate with the solvent front. In this way amino acids are separated according to the hydrophobic nature of their side chains [93].

The amino acids of interest in this study: citrulline, arginine and ornithine have basic side chains which, according to Pataki [94], are best separated using two-dimensional chromatography. This involves the separation

of an amino acid mixture in one dimension with one type of organic solvent (propanol), followed by the rotation of the plates and separation of similar amino acids using another solvent (phenol), see figure 30. In this way amino acids with similar properties are more successfully separated.

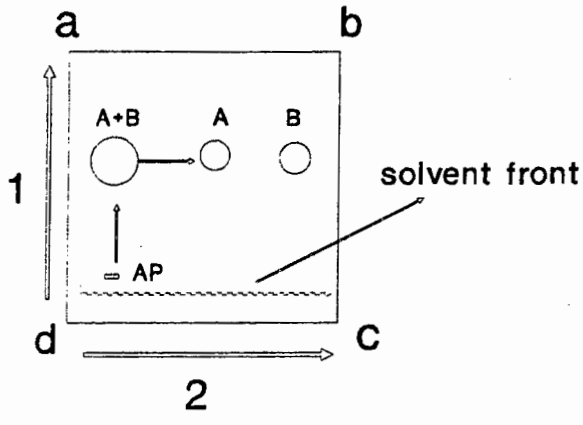

FIGURE 30: Two-dimensional thin-layer chromotography.
Samples are placed at the bottom of the silica plate and separated in the first dimension with the movement of the first solution. The plates are air dried and placed in the second solvent after being rotated 90°. Amino acids with similar partition coefficients in solvent 1 may then be separated as the second solvent front progresses. AP = application point, A and B = amino acids.

Standard amino acid solutions : ornithine, arginine , citrulline and argininosuccinate (2.5mM in 0.1M HCl) were neutralised before use with an equal volume of 0.1M NaOH and then 5-10µl of the samples were applied to the corner of a 10 x 10cm silica plate (Merck 60F254). The amino acids were separated in the first dimension with propanol:water = 7:3 (v/v) and in the second dimension with phenol:water = 75:25

(w/v). The plates were air-dried between solvent changes and developed with ninhydrin (0.2g / 100ml). The separation of radioactive standards (^{14}C) was visualised by autoradiography of the silica plates using hyper-film-Mp (Amersham).

Radioactivity yields of individual amino acids were determined by the removal of the ninhydrin stained spot of interest from the silica plate and dissolving it in 6ml scintillation fluid (Packard Hionic-Fluor). The radioactivity content of the spot was then determined as in section 2.4.A, taking into account the quenching effects of ninhydrin and silica.

2.4.C: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC).

With HPLC, samples are driven through an ion-exchange column under intense hydrostatic pressure of 5000-10000 pounds per square inch. Under these conditions amino acids are separated with high resolution. The high pressure contributes to short extension times and hence little diffusion. HPLC methods are reproducible and easily automated [93].

The two columns that were used in this study were a C18-reverse-phase column and a cation-exchange column. The cation-exchange system operates by separating amino acids according to charge differences . Reverse-phase columns operate slightly differently. The column packing material is coated with hydrophobic hydrocarbon chains (C18), a solution of octane-sulphonate is then run through the column. The octane interacts with the C18 via hydrophobic interactions, while the sulphonate interacts with the charged amino acid

side chains.

C.i: Reverse-phase: octane-sulphonate / acetonitrile.

The method that was used for the separation of urea cycle amino acids was a modification of the method used by Blachier et al [95]. A mixture of two buffers was used to separate citrulline , arginine , ornithine , urea and argininosuccinate [96]:

Buffer A: 0.1M Na Acetate, 10mM octane sulphonate (pH 5.5).

Buffer B: 0.2M Na Acetate (pH 4.5): Acetonitrile 10:3, 10mM octane sulphonate (Sigma).

The following program was implemented:

Flow rate: 1ml/min

Time(min):		
0	100% A	0% B
30	60% A	40% B
42	0% A	100% B
47	100% A	0% B
53	END	

Samples were adjusted to pH7 and 100ul volumes loaded onto the column. The HPLC program was run on a Beckman 126 System Gold HPLC system, using a Beckman C18-ultrasphere column (5u, 4.6mm x 25cm).

C.ii: Cation exchange: Li-A / Li-R system.

An abbreviated version of the separation program used on the amino acid analyser (Beckman 6300) was used in some cases to definitively separate citrulline and urea . The cation-exchange column (Beckman system 7300 / 6300, P / N 338050) was maintained at 67°C and equilibrated with Li-A (1% lithium citrate, 0.5% lithium chloride, 0.5% hydrogen chloride, 98% water) for 8-10 minutes prior to the loading

of samples (100µl). The samples were diluted and acidified in Li-S dilution buffer (1% lithium citrate, 1% thiodiglycol, 0.7% HCl, 0.5% benzoic acid, 96.8% water) to pH 2.2 before loading. The sample components were separated by an isocratic gradient of 100% Li-A for 35 minutes at a flow rate of 0.3ml/minute, and the column rejuvenated by two minutes of Li-R (1% LiOH, 99% water).

In both HPLC separation systems, individual radioactive amino acid peaks were located on a radio-chromatography detector (Flo-one/Beta, series A-100), attached in series to the HPLC. FloScint IV (Packard Radiomatic) scintillation fluid was used in a ratio of 4:1 in this procedure.

C.iii: Quench curves.

The quenching effects of the solutions used in the two HPLC systems were evaluated to ensure that the radiochemical detector was sensitive enough and to enable cpm -> dpm conversions where necessary.

In the case of the cation-exchange column (Li-A), the system operates as an isocratic gradient system and thus a static quenching effect could be measured [97]. The radiochemical detector cell was filled with scintillation fluid and Li-A in a 4:1 ratio. The flow was stopped and the background counts determined . The cell was then filled with scintillation / Li-A fluid, to which 10000 dpm/ml of ^{14}C label had been added, after which the flow was again halted and the counts / minute determined over several minutes. The efficiency of the radiochemical detector was determined as

follows:

$$E = \frac{\text{average } ^{14}C \text{ counts/minute}}{\text{dpm/cell } (= 2.5 \times 10000)} \times 100$$

For the reverse-phase system (octane-sulphonate), a gradient of buffer A and B was used to separate the amino acids, therefore a quench curve needed to be established to evaluate the effects of the concentration gradient on the efficiency of the radiochemical detector [97]. 8000dpm/ml ^{14}C was added to the scintillation fluid. The HPLC system was then run in conjunction with the "spiked" scintillation fluid and the cpm at each time point determined . The radiochemical detector "efficiency curve" program calculated the dpm at each time point, taking into account the HPLC flow rate, cell size, scintillation flow rate and dpm/ml scintillation fluid. The efficiency at each time point was then calculated as follows:

$$E = \frac{\text{cpm}}{\text{dpm}} \times 100$$

2.5: RESULTS.

2.5.A: ION-EXCHANGE CHROMATOGRAPHY.

Citrulline and arginine were used for the initial optimisation studies of this separation technique, as they represent the greatest charge variation between the amino acids of interest.

The best separation result using this method was obtained using a 2ml (7mm diameter) Dowex-50 cation-exchange column. The results are shown in figure 31A. Arginine and citrulline were separated sufficiently, however the other amino acids (i.e : ornithine) were found to elute together with these peaks, making it difficult to successfully analyse separate amino acids.

Several attempts were made to improve the quality of separation. The effects of the diameter and height of the column (1 - 2.5ml, 5 - 7mm diameter), as well as the effects of the grade quality of the eluting ammonium hydroxide solution were investigated. These effects were monitored by investigating the elution of citrulline and the results of changing these parameters are shown in figure 31B. Firstly, these results show the variation in the elution position of citrulline when the column parameters were altered. Secondly, it was found that a high quality grade ammonium hydroxide solution was necessary for high recovery yields and sharp amino acid peaks. Taking these results into account, a 2ml (7mm) column was found to be the most effective, resulting in a sharp citrulline peak with recovery yields of 97%.

ION-EXCHANGE CHROMOTOGRAPHY

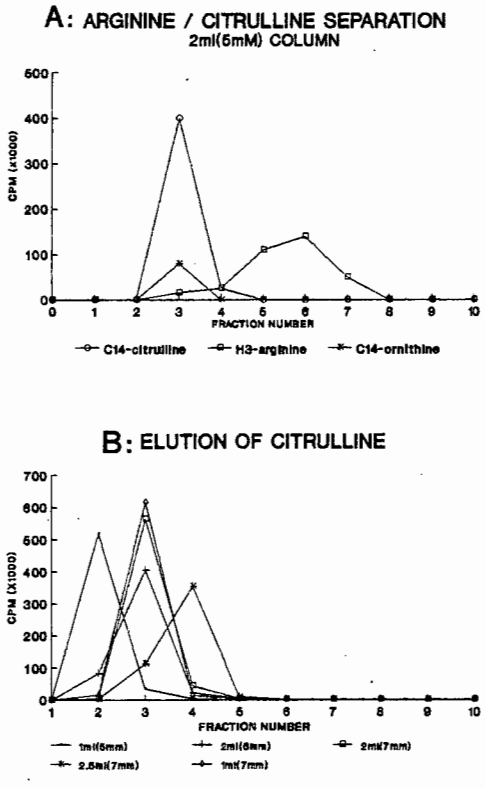

FIGURE 31: Ion-exchange chromatography, separation of amino acid standards.

A) Arginine (^{3}H) and citrulline (^{14}C) were separated on a 2ml (5mM diameter) Dowex-50 column. The sample mix was loaded on top of the column and 1ml fractions eluted with 25% ammonia solution. 100µl of each fraction was counted to locate the relative positions of the amino acids. The elution position of ^{14}C-ornithine is also shown. **B)** The effects of column volume and diameter on the elution position of ^{14}C-citrulline were evaluated.

2.5.B: THIN-LAYER CHROMATOGRAPHY.

The separation of arginine, citrulline and ornithine on silica thin-layer chromotography plates is shown in figure 32 and demonstrates the efficient separation of these amino acids using this two-dimensional system. Argininosuccinate was found to migrate as two separate species on either side of arginine, figure 32 shows the approximate positions.

Regarding the recovery yields from the silica plates, it was found that silica in combination with ninhydrin exerts a quenching effect on the radioactive emission of the isotopes. It was found that counting efficiency was reduced by 34% in the presence of these components. Taking this quenching effect into account, as well as the efficiency of the scintillation counter, the yield of ^{14}C-citrulline from the silica spot was found to be 75% (^{3}H yield = 78%). Losses were due to loss of silica particles in the removal of the ninhydrin stained amino acid spots, residual radioactivity remaining at the point of application and possible contaminants or degradation products in the isotope stock solutions, that elute differently during two-dimensional chromatography.

Although this method gave good separation of the amino acids, it was time-consuming and sample volumes had to be reduced to 5-10µl to allow for effective separation. Inaccuracies arise easily when dealing with such small volumes.

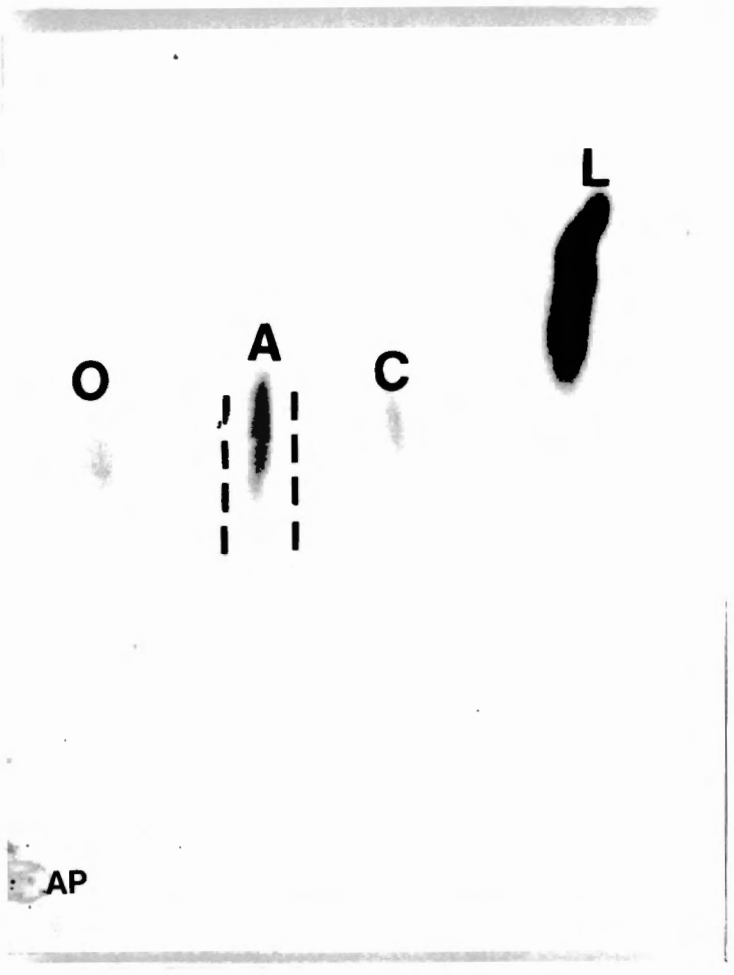

FIGURE 32: Thin-layer chromatography, separation of amino acid standards.

Arginine (A), citrulline (C) , ornithine (O), leucine (L) and argininosuccinate (-----) were separated on silica chromatography plates using two-dimensional chromatography. Amino acid mixtures were separated in the first dimension with propanol:water (7:3 v/v) and phenol:water (75:25 w/v) in the second dimension. Plates were air-dried and stained with ninhydrin. AP = application point.

2.5.C: HPLC.

C.i: Reverse-phase octane-sulphonate.

This method [95] was previously used for the analysis of urea cycle intermediates by Blachier et al and was therefore chosen as a suitable method in this study. Radioactive labelled citrulline,ornithine and arginine were found to be efficiently separated using the recommended conditions [95]: Buffer A = pH 4.5, Buffer B = 4.5, which is illustrated in figure 33. However, when argininosuccinate and urea were analysed, these conditions were found to be inadequate in the separation of these components from the afore mentioned amino acids.

In solution argininosuccinate can exist in three forms A, B and C. A is the non-cyclic form, while B and C are cyclic anhydrides that are formed at high temperatures and under acidic conditions [98]. By varying temerature and pH, it is possible to change one form of argininosuccinate into another, although no method is 100% effective and two forms will always be present. Under alkaline conditions A and B forms are favoured [98]. Labelled argininosuccinate was not available for use, so its elution position was detected by separation of unlabelled argininosuccinate (15mM) from L-citrulline (15mM), with fractions collected at 30 second intervals and the positions of the two components visualised by reaction with an equal volume of ninhydrin (0.2g/ml). Figure 34B shows the poor separation achieved under the recommended conditions (buffer A = pH 4.5). Figure 34A, C and D show the results of changing the pH of buffer A in an

attempt to improve the separation. These results show that under more alkaline conditions (>pH 6), two forms of argininosuccinate are separated. These probably represent the A and B forms, with the A eluting first. Separation of the various forms of argininosuccinate was not favourable as the B form co-elutes with citrulline, so a pH of 5.5 for buffer A was chosen as the most effective system for separating argininosuccinate and citrulline.

The pH change of buffer A from 4.5-5.5 had no effect on the relative positions of citrulline, arginine and ornithine, although the retention times altered slightly: citrulline = 3.4 minutes, ornithine = 5.8-6.2 minutes and arginine = 26.5 minutes. Figure 35 shows a typical profile of these standards. Using this method the cpm in each major amino acid peak represented 90-97% of the radioactivity loaded on the column.

One criticism of the method of Blachier et al [95] was that they were unable to separate urea from citrulline, which is a major metabolite of arginine metabolism. To investigate this separation, unlabelled urea (7mM) was eluted on the reverse-phase system (buffer A = pH 5.5) and its position determined by ninhydrin staining. It was found to elute before citrulline in fraction 7 (30 second fraction collection), with citrulline in fraction 8 and argininosuccinate in fraction 6. Upon the acquisition of ^{14}C-urea, the retention time of urea was found to be 2.8-3 minutes (citrulline = 3.4 minutes).

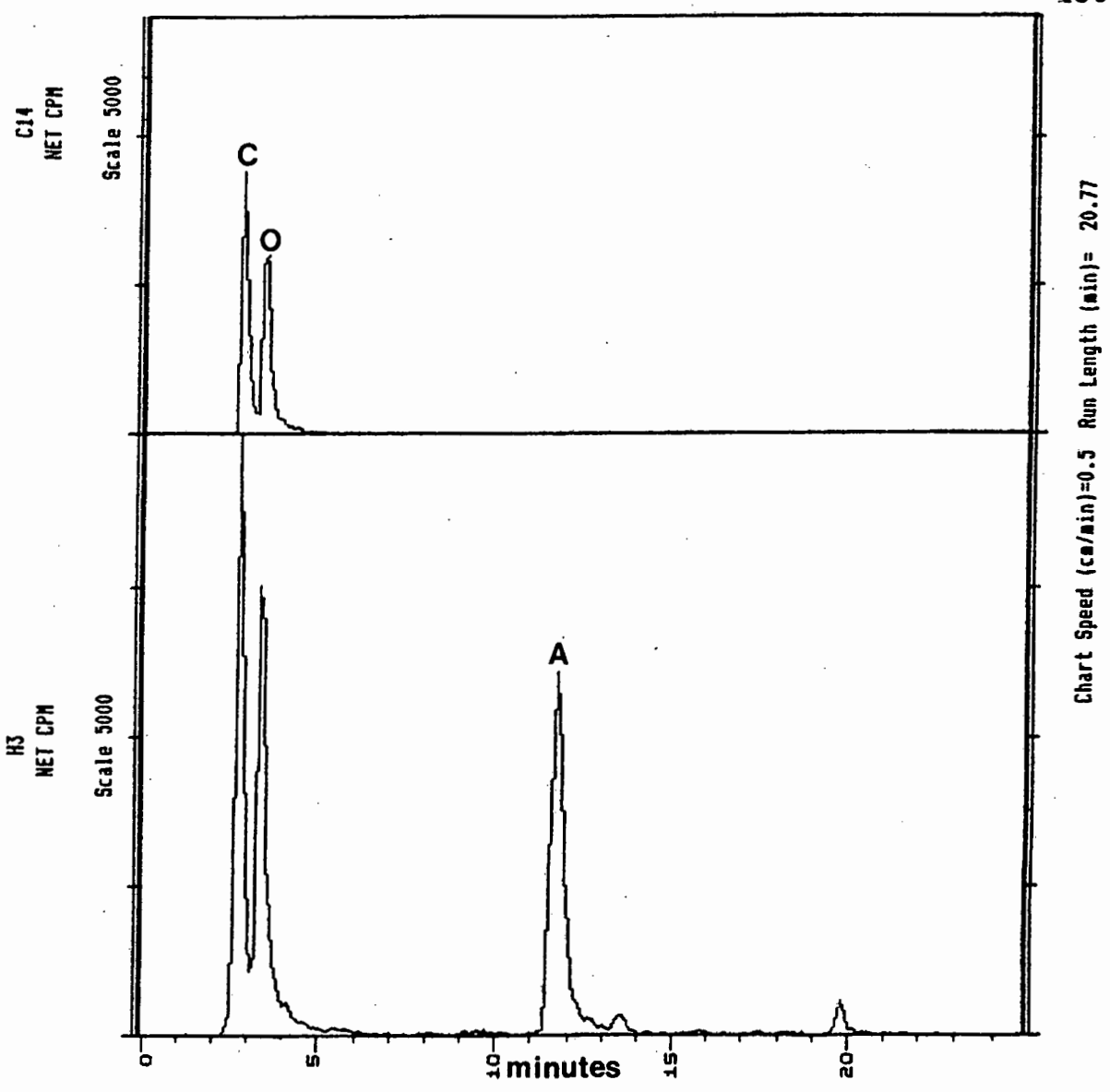

FIGURE 33: Reverse-phase/octane-sulphonate HPLC (buffer A = pH 4.5), separation of amino acid standards.

Citrulline (^{14}C), ornithine (^{14}C) and arginine (^{3}H) (pH 7) were separated on a C18-reverse-phase exchange column using a gradient system of Buffer A (0.1M Na acetate, 10mM octane-sulphonate, pH 4.5) and Buffer B (0.2M Na acetate: acetonitrile (10:3), 10mM octane-sulphonate) with an HPLC flow rate of 1ml/minute (see section 2.4.C.i for details of the gradient program). Amino acids were detected using a Flo-one-/Beta radiochromatography detector connected in series to the HPLC. C = citrulline (3 minutes), O = ornithine (3.5 minutes) and A = arginine (12 minutes).

THE EFFECTS OF pH ON THE ELUTION OF CITRULLINE AND ARGININOSUCCINATE

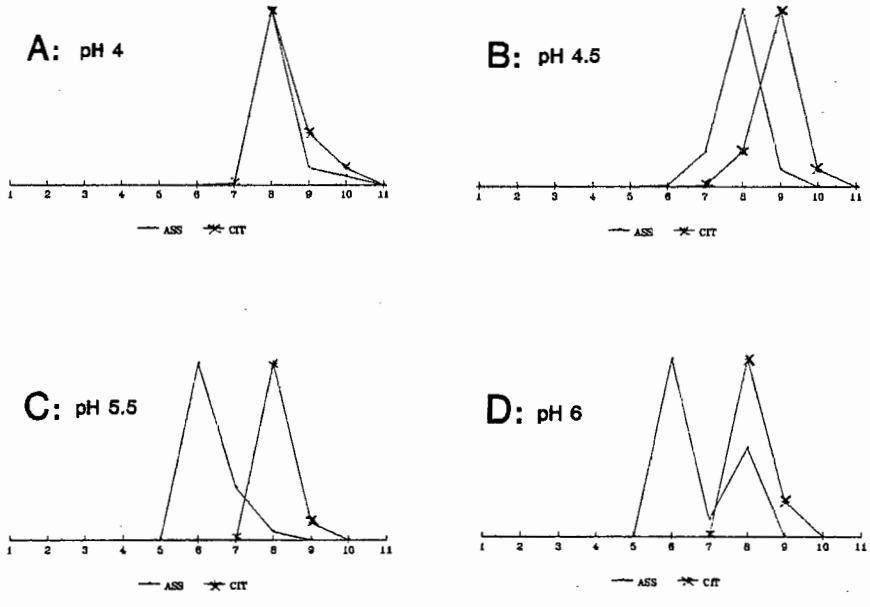

FIGURE 34: The effects of pH on the elution of citrulline and argininosuccinate.

Citrulline (CIT) and argininosuccinate (ASS) (pH 7) were separated on a C18-reverse-phase column (octane-sulphonate) using Buffer A of varying pH values (pH 4 - pH 6). HPLC fractions were collected at 30 second intervals and the positions of the amino acids detected by the addition of ninhydrin to the fractions. When buffer A is pH 6 two forms of argininosuccinate are separated (A and B forms).

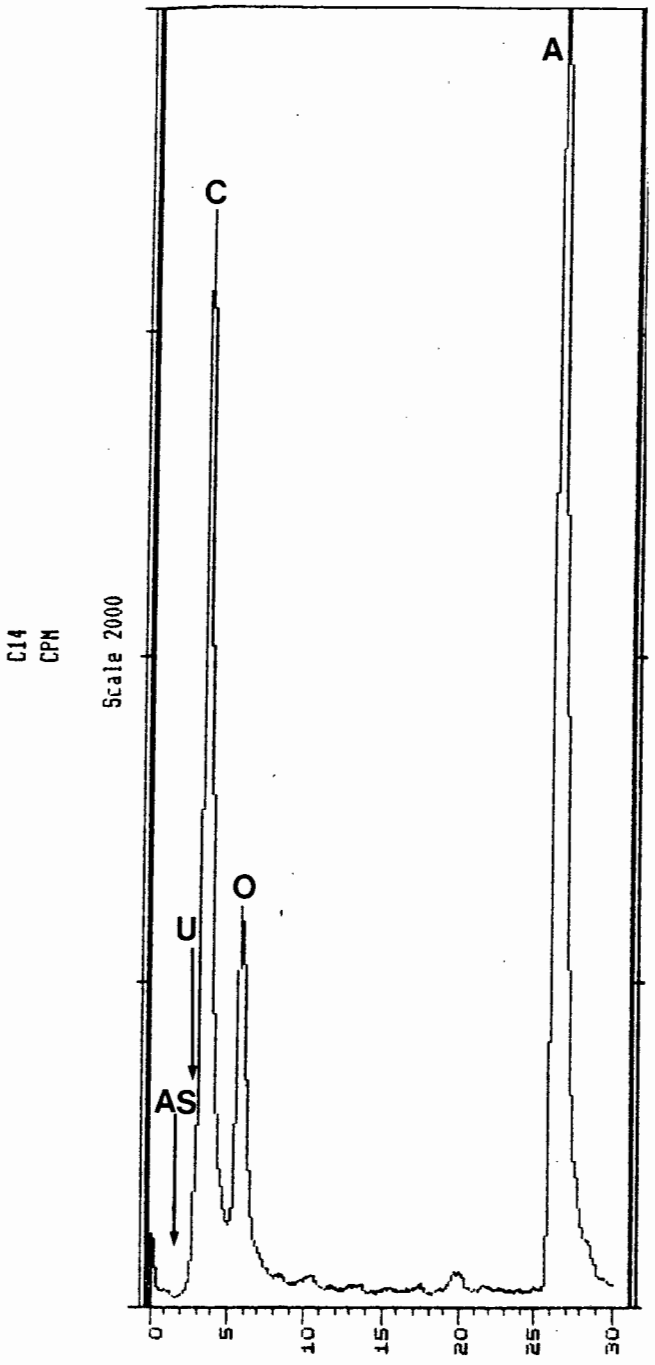

FIGURE 35: Reverse-phase/octane-sulphonate HPLC (buffer A = pH 5.5) standards profile.

A mixture of arginine, ornithine and citrulline (^{14}C) (pH 7) was separated on a C18-reverse-phase column using a gradient of Buffer A (0.1M Na acetate, 10mM octane-sulphonate pH 5.5) and Buffer B (0.2M Na acetate: acetonitrile (10:3), 10mM octane-sulphonate). Radioactive metabolites were detected using a Flo-one-/Beta radiochromatography detector. The relative positions of argininosuccinate (AS) and urea are also indicated. A = arginine (26.5 min), C = citrulline (3.4 min), O = ornithine (6 min), U = urea (3 min).

In order to utilise the HPLC information correctly, the quenching effects of the octane-sulphonate system were investigated. It was found that the efficiency of counting was constant throughout the run , regardless of the concentration gradient : ^{14}C in the 20-160 ^{14}C window on the radiochemical detector [97] = 24%, while in the open window 0-2048 efficiency of counting = 65%.

C.ii: Cation-exchange column.

Several problems were encountered with the separation of urea and citrulline from cell-supernatant fractions, with urea sometimes co-eluting with citrulline when using the reverse-phase HPLC system. In order to completely resolve these components and correctly identify citrulline peaks, a cation-exchange HPLC system was used in conjunction with the octane-sulphonate system. The cation-exchange system that was adopted was that used by the Beckman 6300 amino acid analyser . Several hours and a variety of buffers are required to separate the entire range of amino acids, so the method was shortened to allow for the definitive separation of urea and citrulline. The program was shortened to 35 minutes of Li-A, with Li-R removing the other amino acids from the column. Maintaining the cation-exchange column at 67°C was found to be necessary to achieve maximal urea / citrulline separation. Figure 36 shows the well-defined separation of these metabolites, with the retention time of urea at 6.9 minutes and citrulline at 29.6 minutes. Each peak represented 90-97% of the original radioactivity loaded onto the column.

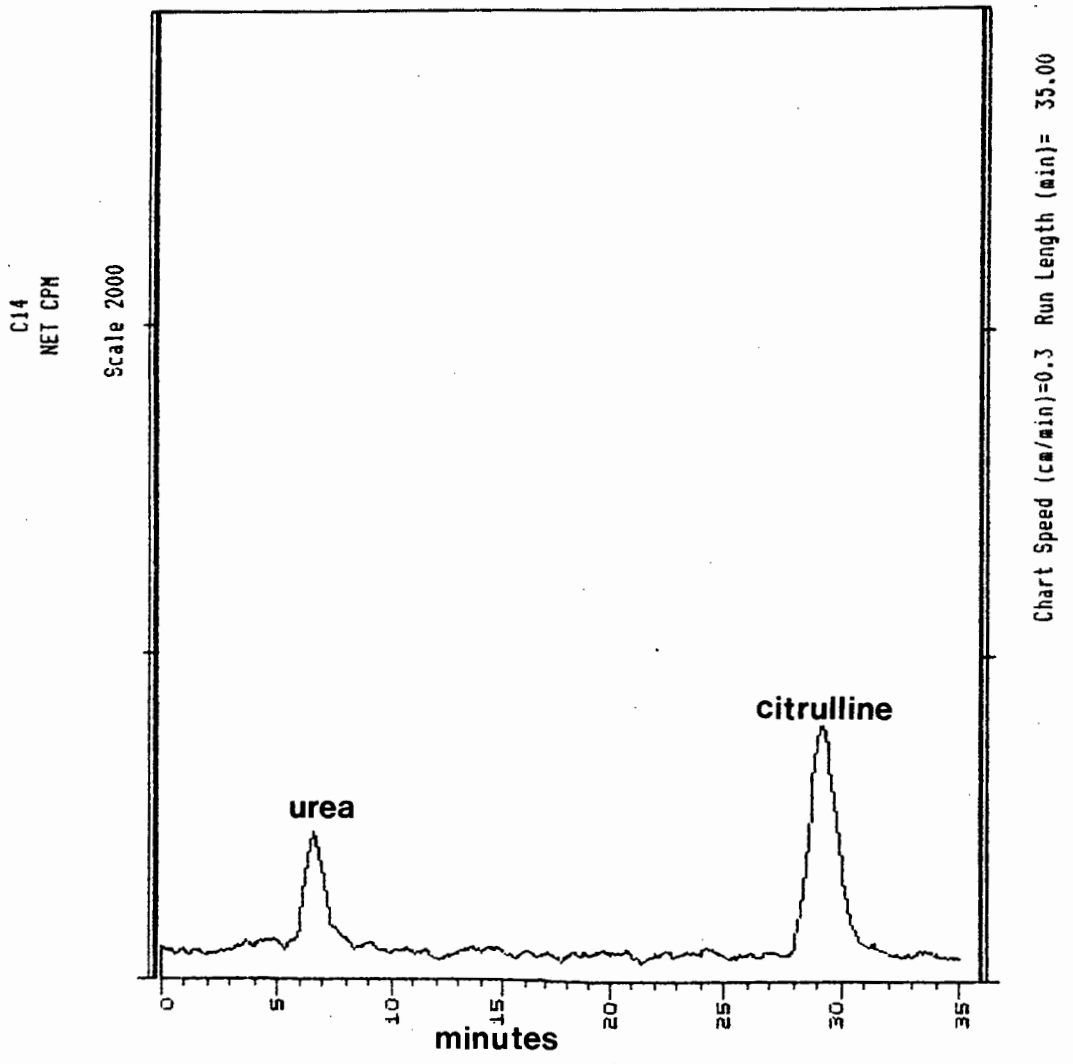

FIGURE 36: Cation-exchange separation of citrulline and urea.

A cation exchange column system (Li-A/Li-R) was used to separate citrulline from urea. The column was maintained at 67°C throughout the 35 minute run (HPLC flow = 0.3ml/minute). Radioactively labelled metabolites (pH 2.2) were detected by the Flo-one-/Beta radiochromatography detector connected in series to the HPLC. ^{14}C-citrulline = 29.6 minutes, ^{14}C-urea = 6.9 minutes.

The static quenching effects of Li-A were determined by calculating the efficiency of counting under the HPLC conditions. In the open window 0-2048 [97] ^{14}C counting efficiency was 66%.

The HPLC methods allow for accurate quantitative and qualitative analysis, with minimal hands-on manipulations allowing less room for experimental error.

2.6: DISCUSSION.

In order to study the NO˙ pathway in fibroblasts, an efficient separation system was needed to separate arginine, citrulline, ornithine, urea and argininosuccinate. Several methods are available to separate amino acids and three of these were analysed for their suitability to the separation of the components mentioned above. Thin-layer chromatography (silica plates), ion-exchange chromatography (Dowex-50) and two HPLC systems (reverse-phase octane-sulphonate and cation exchange) were optimised for the separation of the urea cycle metabolites.

The ion-exchange chromatography system separated arginine from citrulline, but the other amino acids were found to elute in overlapping positions making the analysis of separate amino acids difficult (figure 33A). The yields were high (97%) and the method quick and easily scaled up to allow for the analysis of many samples. However, the separation was not sufficient for the required purpose.

Thin-layer chromatography using silica plates provided well-defined separation of citrulline, ornithine and arginine (figure 32), although argininosuccinate migrated in two positions. The yields were relatively low at 74%, which may hinder the detection of small amounts of labelled metabolites. This method is also time consuming and requires the sample volume to be reduced to 5-10µl, which introduces a margin of error and adds to the low reproducibility of this method.

The most effective method with respect to separation efficiency, reproducibility and yield , was HPLC. Both systems gave clear, sharp standard peaks, with yields in the 90-97% range. Volumes of up to 100µl could be analysed, allowing for less loss during manipulations. HPLC also requires less hands-on time , which ensures greater reproducibility and accuracy. The disadvantage of both the methods used here, is that the programs are fairly long, requiring 37-53 minutes per sample run. The reverse-phase column gave clear separation of arginine , ornithine , citrulline and argininosuccinate (figure 35), while the cation-exchange system clearly separated urea from citrulline (figure 36). Although under preliminary test conditions the octane-sulphonate system sucessfully separated citrulline and urea, during cellular assay conditions it was found that the two HPLC systems needed to be used in conjunction with each other to definitively identify citrulline peaks.

From these results the HPLC methods were found to be more quantitative and reproducible than the other separation techniques, and were thus employed to investigate the nitric oxide pathway in fibroblasts. However, while awaiting the HPLC components, the thin-layer chromotography (silica plates) method was used for preliminary studies.

SECTION 2B: NITRIC OXIDE PRODUCTION IN FIBROBLASTS - PRELIMINARY TESTS AND THE NITRIC OXIDE ASSAY.

2.7: INTRODUCTION.

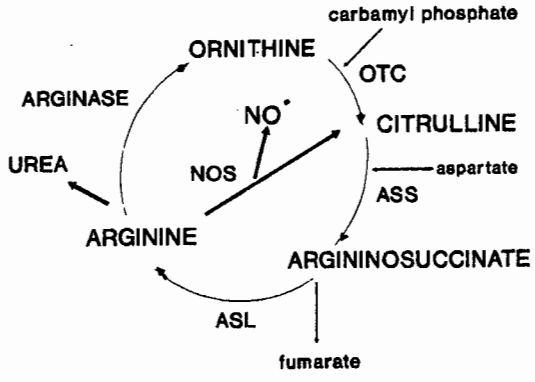

FIGURE 37: The Urea Cycle and the Nitric Oxide pathway.

NO· is produced when arginine is metabolised by NOS, to produce equimolar quantities of citrulline and NO· [63]. One assay system that is often used to detect iNOS activity is the ability of cells to form ^{14}C-citrulline from ^{14}C-arginine when stimulated by cytokines [54,59,60]. This method relies on the assumption that citrulline is not formed in these cells by any other pathway in the cell and that it is not readily metabolised.

The availability of F25, which is an ASS deficient fibroblast cell line, provided an interesting model to investigate NO· synthesis in fibroblasts. Citrulline is not readily metabolised by these cells (< 5% residual ASS activity) and should therefore accumulate in cytokine activated cells, if iNOS is operative in fibroblasts.

In designing the nitric oxide assay, it was important to be aware of the possibility of citrulline production via the ornithine transcarbamylase pathway (OTC). Arginase, ASS and ASL activities have been reported in fibroblasts [1,6], therefore the substrate for the OTC reaction, ornithine, will be formed by arginine metabolism in fibroblasts. Several reports [6,99] indicate that OTC activity is not present in fibroblasts. However, due to the importance of the absence of detectable OTC activity and the fact that we were working with mutant cells, OTC activity in the F25 and a control cell line F500 was investigated.

The majority of exogenous arginine is consumed by the cells to synthesize proteins, while another fraction is converted to urea by arginase activity. The contribution of these pathways to the utilisation of exogenous arginine was investigated, as well as ways of inhibiting these pathways (cycloheximide and valine treatment). This was performed to ensure that sufficient arginine remained in the medium to be utilised by iNOS and to limit the amount of radioactivity that needed to be used in the assay.

2.8: MATERIALS AND METHODS.

2.8.A: PRELIMINARY TESTS.

A.i: Ornithine transcarbamylase activity.

Confluent fibroblasts were trypsinised (section 1.3) and 300000 cells were plated into 3ml Falcon wells. The cells were incubated overnight in the presence of BME (5% FCS, PSN) and 5% CO_2 and then the medium replaced with 1ml medium Z containing 0.5µCi ^{14}C-U-arginine/well (Amersham 318mCi/mmol, universal label). After the required incubation times (0-10 hours), the medium was removed and the cells washed four times with ice cold saline to remove exogenous radioactivity. The cell supernatant fraction was collected by treatment with 0.5ml ice cold 5% TCA. The protein fraction was then collected by the further precipitation of cellular proteins with three washes of 5% TCA and the addition of 0.5ml 0.1M NaOH.

400µl of the protein fraction was dissolved in 6ml scintillation fluid (Packard Hionic-Flour) and counted in a liquid scintillation spectrometer (Beckman LS 6000IC). 80ul was used to determine the protein content of each sample (section 1.3). The cpm were normally expressed as cpm/cell number, however when protein levels differed significantly the cpm were expressed as cpm/µg protein. The dpm values were calculated where necessary by taking into account the background counts and the efficiency, which was 74% for ^{14}C in the open window, 0-1000.

Cell supernatant fractions were neutralised with 12 washes of an equal volume of water-saturated ether to pH 6. The samples were vortexed, centrifuged and the etherlayer removed at each wash. The volume of the samples was reduced from 500µl to 50-100µl by evaporation (Savant Speedvac SC110). The 1ml medium fraction was treated with an equal volume of 5% TCA to precipitate cellular proteins, ether neutralised and the volume reduced through evaporation. Cell supernatant and medium samples were analysed using the silica/ phenol/ propanol TLC system or the HPLC in combination with a (Flo-one/Beta series A100) radiochemical detector, using either the reverse-phase (sample pH = 7) or cation-exchange columns (sample pH = 2.2).

A.ii: Cycloheximide treatment.

F500 (control) fibroblasts (300000) were incubated in the presence of 0, 20, 50 or 100 µg/ml cycloheximide dissolved in 1ml medium Z. Inhibition of protein synthesis by cycloheximide was assayed as the cells' ability to incorporate 0.5µCi ^{14}C-U-arginine into acid precipitable material (protein), in the presence of increasing concentrations of cycloheximide over a 4 hour period. After the incubation period the medium, cell supernatant and protein fractions were collected and analysed as above.

A.iii: Arginase inhibition - Valine treatment.

F500 (control) fibroblasts (300000 cells) were incubated in 1ml medium Z (0.5µCi ^{14}C-U-arginine) with or without 50mM L-valine (Sigma) for 4 hours . Again the medium, cell supernatant and protein fractions were collected and analysed

as above. Protein levels were evaluated to assess differences in cell number due to the incubation conditions.

A.iv: Inhibition of ^{14}C-citrulline consumption by the addition of exogenous unlabelled citrulline.

0, 0.01, 0.1, 1 and 10mM concentrations of L-citrulline (Sigma) were added to the medium Z incubation medium (1ml/well). F500 (control) fibroblasts (300000 cells) were then incubated in these various solutions in the presence of 0.005µCi (0.1µM) ^{14}C-U-citrulline (58mCi/mmol, Amersham) for 4 hours. The metabolism of the trace amount of ^{14}C-citrulline by ASS in the presence of unlabelled citrulline was measured by the conversion of ^{14}C-citrulline into ^{14}C-arginine, which is then incorporated into protein. After the incubation period, the ^{14}C radioactivity content of the medium (200µl) and protein fractions (400µl) was analysed.

2.8.B: NITRIC OXIDE ASSAY - Finalised assay.

300000 F25 (ASS deficient) fibroblasts and a similar number of F500 (control) cells were plated into 3ml wells and allowed to grow to confluency in DMEM (10% FCS, PSN). Upon confluency the medium was removed and replaced with 1ml DMEM (10% FCS, PSN) containing 100U human, recombinant gamma interferon (ɣ-IFN) (Boehringer Mannheim) and 10µg lipopolysaccharide (LPS) (Sigma). The cells were incubated in the presence of these additives for 8 hours, at which time 2µCi ^{14}C-guanido-arginine (NEN products, 55.6mCi/mmol), 1mM citrulline (Sigma) and 50mM valine (sigma) were added and the cells incubated for a further 16 hours.

After the 24 hour incubation period the medium was removed, and the cells washed 4 times with ice-cold saline. The cell supernatant was collected by the addition of 0.5ml 5% TCA and finally the protein fraction collected by 3 washes of 5% TCA and the precipitate dissolved in 0.5ml 0.1M NaOH. The medium and cell-supernatant fractions were treated with 12 washes of water-saturated ether, freeze-dried to 50-100µl volumes and analysed on the cation-exchange HPLC system.

Amino acid analysis.

Medium from the nitric oxide assay was also analysed on the amino acid analyser to monitor the levels of arginine during the 24 hour assay. Medium fractions (400µl of 1ml) at time 0, 8 and 24 hours were collected and 20µl of an internal amino acid standard added (amino-ethyl cysteine, 2500µM). Cellular proteins were precipitated by the addition of 5-sulphosalicylic acid and the precipitate removed by centrifugation (5 minutes at 15000g). The resulting supernatant was filtered through a 0.2µm millipore filter and adjusted to pH 2.2 with 0.1M HCL or NaOH.

Samples were analysed on a Beckman system 6300 automated amino acid analyser. The amino acids were identified by the comparison of the trace to separations of standard amino acid mixtures, and their concentrations determined by their relative peak heights once corrected with the height of the internal standard using E.H.Harley's "Amino Acids" computer program.

2.9: RESULTS.

2.9.A: PRELIMINARY TESTS.

Originally all preliminary tests were performed in medium Z rather than DMEM. Medium Z is arginine-free, thus the uptake of ^{14}C-arginine and ^{14}C-citrulline by fibroblasts is optimal in this medium, allowing for a more sensitive assay. Exogenous arginine present in BME or DMEM lowers the specific activity of the added ^{14}C labelled component, making detection more difficult. Table 4 shows the extent of this dilution effect.

TABLE 4: THE EFFECTS OF ARGININE (0.3mM) ON THE UPTAKE OF ^{3}H-ARG AND ^{14}C-CIT IN F507 CONTROL FIBROBLASTS.

SAMPLE:	^{3}H-DPM:	^{14}C-DPM:	RATIO ^{14}C/^{3}H:
F507 IN BME	29944 33324	2234 2926	0.075 0.088
F507 IN MEDIUM Z	274750 274708	56928 54374	0.207 0.198

100000 control F507 fibroblasts were incubated in the presence of 1uCi ^{3}H-arginine and 0.5µCi ^{14}C-citrulline for 4 hours, in 1ml medium Z or DMEM. The acid precipitable material was collected and counted. Duplicate values are shown here.

A.i: Recovery yields.

All preliminary studies and the nitric oxide assay itself were based upon a basic assay to study the metabolism of ^{14}C-arginine by the fibroblasts. The assay involved the analysis of cellular components after specific incubations times in the presence of ^{14}C-arginine. It was essential that medium, cell supernatant and protein fractions were collected separately without interfraction contamination. The losses incurred during ether treatment and evaporation were also investigated to ensure that these were at a minimum.

A.i.a: Cellular fraction collection - Saline washing and TCA extraction.

After the required incubation period in each respective assay, the medium fraction was removed. To ensure that all the extracellular radioactity was removed from the fibroblast layer, the samples were washed with several washes of ice cold saline. 100µl of each 1ml saline wash were assessed for their radioactivity content. It was found that 4 washes were sufficient to reduce the levels of extracellular ^{14}C-arginine radioactivity to background levels (30-50 cpm). This washing prevents the contamination of the cell-supernatant fraction with medium components.

The precipitation of the protein fraction and the removal of cytoplasmic radioactivity by 3 washes of ice cold 5% TCA was previously described by Davidson et al [33]. This investigation was repeated and the carry-over of radioactivity to the protein fraction from a 0.5µCi solution

of ^{14}C-arginine at time 0, was found to be 0.2% after 3 TCA washes.

A.i.b: Water-saturated ether extraction.

The number of ether-extractions required to neutralise the TCA in the cell supernatant fraction was determined by measuring the pH of a 0.5ml solution of 5% TCA extract of 300000 F25 fibroblasts containing 0.5µCi ^{14}C-citrulline, after each ether wash. The radioactivity loss due to this process was measured by counting the radioactivity content of the ether layers after each wash. It was shown that 12 washes were sufficient to obtain a pH of 6 and that the radioactivity content of the 12 ether washes represented 1.5% of the inital radioactivity in the sample. This result indicates that no significant loss of citrulline was incurred during this neutralisation process.

A.i.c: Volume reduction: evaporation.

The loss of radioactivity incurred during the process of evaporation, to reduce the sample volume from 500µl to 50-100µl, was investigated by evaporating a 0.5ml TCA extract from F25 cells containing 0.25µCi of ^{14}C-citrulline. The loss at this stage was found to be 10% when compared to the duplicate sample.

Thus the overall loss of radioactivity incurred during the process of neutralisation and volume reduction of cellular extracts was found to be approximately 12%. This reduction should not seriously effect the detection of ^{14}C-citrulline, or effect the quantification of other arginine metabolites.

A.ii: Ornithine transcarbamylase activity.

A.ii.a: TLC.

The presence of detectable OTC activity in fibroblasts was initially assessed in the F25 and control F507 cell-lines (100000 cells) by measuring the amount of ^{3}H-citrulline formed by incubation with 1µCi ^{3}H-Arginine in 1ml medium Z over a 24 hour period (fresh medium at 12 hours). ^{3}H-arginine is generally labelled with tritium, which ensures that all arginine metabolites with be labelled, allowing for detection. Cell supernatant fractions were collected at 4, 8, 12 and 24 hour intervals, the volumes reduced by evaporation and then analysed on silica plates (phenol/propanol) [94], after being mixed with 10µl of a standard amino acid mix, for identification purposes.

The amount of radioactivity found to migrate with the citrulline standard (spot "C") is shown in figure 38. This figure shows a very slow accumulation of "C" over the 24 hour time period in both cell lines. If spot C represents citrulline, a much greater accumulation of citrulline would be expected in F25 (compared to the control), due to the argininosuccinate synthetase block.

The location of the tritiated arginine metabolites was determined by the positions of ninhydrin stained unlabelled standards. This was probably not accurate enough, as the radioactive spots may have been more diffuse than the unlabelled amino acids. This may well have led to loss of radioactivity, which could explain the unexpected result shown in figure 38.

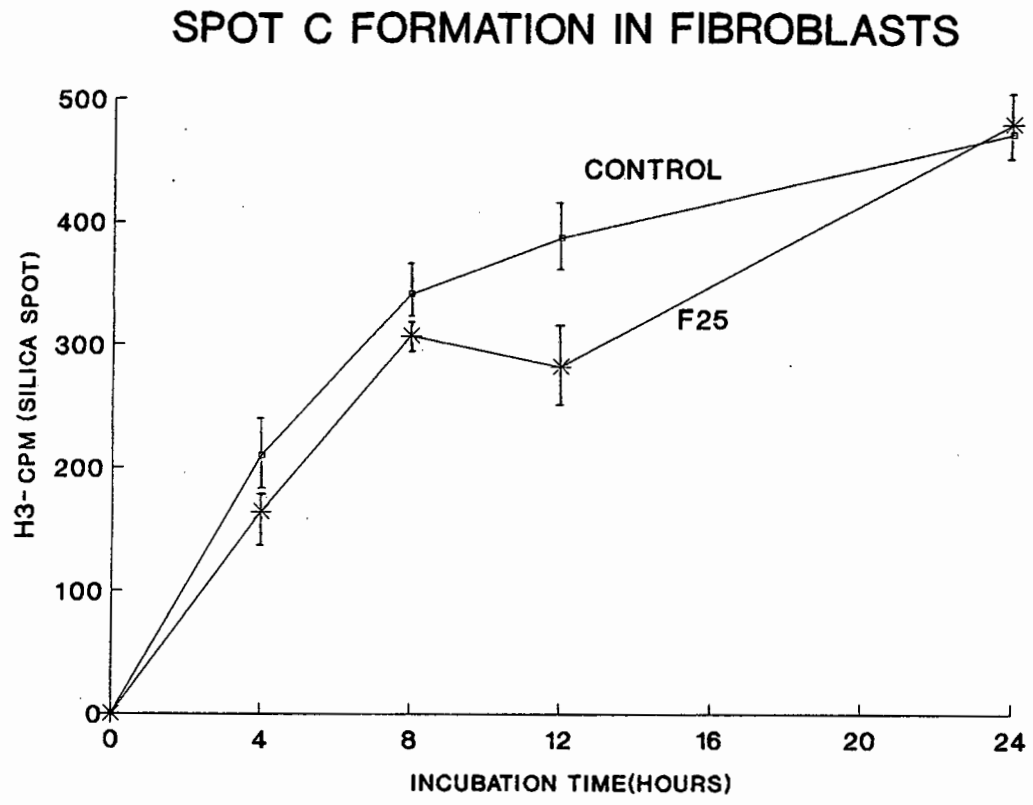

FIGURE 38: Ornithine transcarbamylase assay: Thin-layer chromotography analysis. Spot C formation in fibroblasts.

100000 F25 (ASS deficient) and F507 (control) fibroblasts were incubated in medium Z containing 1µCi ^{3}H-arginine over a 24 hour period. Cell supernatant fractions were collected at 4, 8, 12 and 24 hour intervals and the components separated on silica plates (phenol/propanol system). Unlabelled amino acid standards were used to identify the citrulline migratory position and the ^{3}H content of this spot (spot c) was determined at each time point. The error bars give an indication of the range of results in the duplicates.

To prevent this loss, the 4, 8 and 12 hour incubation time points were repeated using ^{14}C-U-arginine (another generally labelled arginine derivative) and the plates were autoradiographed to locate the labelled metabolites more accurately. Figure 39 represents an autoradiograph of a F25 8 hour time point and shows the formation of C-spot "C". The duplicate values of this experiment were poorly correlated however, so HPLC separation techniques were used to overcome the difficulties of quantitation using the silica plates.

A.ii.b: HPLC.

The OTC assay was repeated using ^{14}C-U-arginine (universal label) (0.5μCi) and the samples analysed on the HPLC. 300000 fibroblasts were used in the assay to increase the formation of the various metabolites, making them more easily detectable. A time course of 0-10 hours was performed and the medium (medium Z) as well as the cell supernatant fractions were collected at 2 hour intervals. The fractions were treated as in the methods and ^{14}C-arginine metabolites separated initially on a reverse-phase column (octane-sulphonate) [95,96].

To assess cell viability during the experiment, several controls were performed: 1) The medium was analysed to assess the levels of ^{14}C-U-arginine. 2) The incorporation of ^{14}C-arginine into ^{14}C-protein was monitored over the time period to ensure continuous cell growth. 3) Glucose levels in the medium were also analysed to ensure that enough glucose was present for cell utilisation.

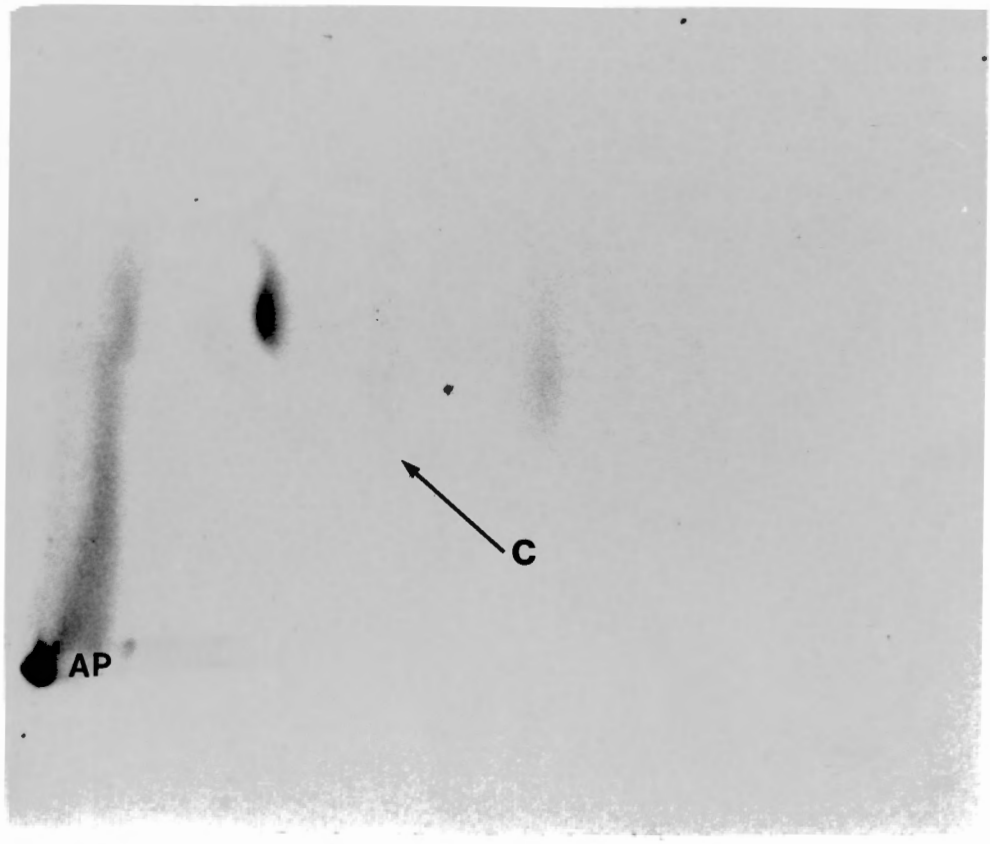

FIGURE 39: Ornithine transcarbamylase activity: Thin-layer chromatography analysis. Formation of ^{14}C-arginine metabolites.

100000 F25 (ASS deficient) fibroblasts were incubated for 0-12 hours in the presence of 0.5µCi ^{14}C-U-arginine (medium Z). Cell supernatant fractions were collected at 4-hourly intervals and separated on silica plates (propanol/phenol system). The silica plates were autoradiographed and ^{14}C-arginine metabolites identified according to the migration positions of standards. This autoradiograph represents an 8 hour sample, where C represents "spot C". AP = application point.

These controls were performed to ensure that the levels of arginine metabolites found in the medium and cell-supernatant fractions were due to normal cell functions, and not due to starvation or cell death. The results of these controls are shown in figure 40 and show that at 6 hours the medium needed to be replaced to replenish the ^{14}C arginine levels, allowing for constant ^{14}C-protein formation and an adequate glucose supply. The cells appeared normal under microscopic examination throughout the 10 hour experiment, showing no signs of large scale cell death.

Initially, samples were analysed on the reverse-phase / octane-sulphonate system and although the preliminary tests on this column (section 2.5.C) showed sufficient citrulline/urea separation, it was found that this was not always the case with the actual cellular samples. Therefore, samples that indicated the presence of ^{14}C-citrulline were analysed on the cation-exchange system (duplicate samples) to confirm the formation of ^{14}C-citrulline.

Figure 41 shows a typical amino acid profile of the cell-supernatant fraction from the F25 cell line analysed on the cation-exchange column, showing the formation of two distinct peaks. Peak X elutes in the predicted region for citrulline (29 minutes) and was initially assumed to represent this amino acid. Figure 42A shows the formation and accumulation of this labelled metabolite over the 10 hour incubation period.

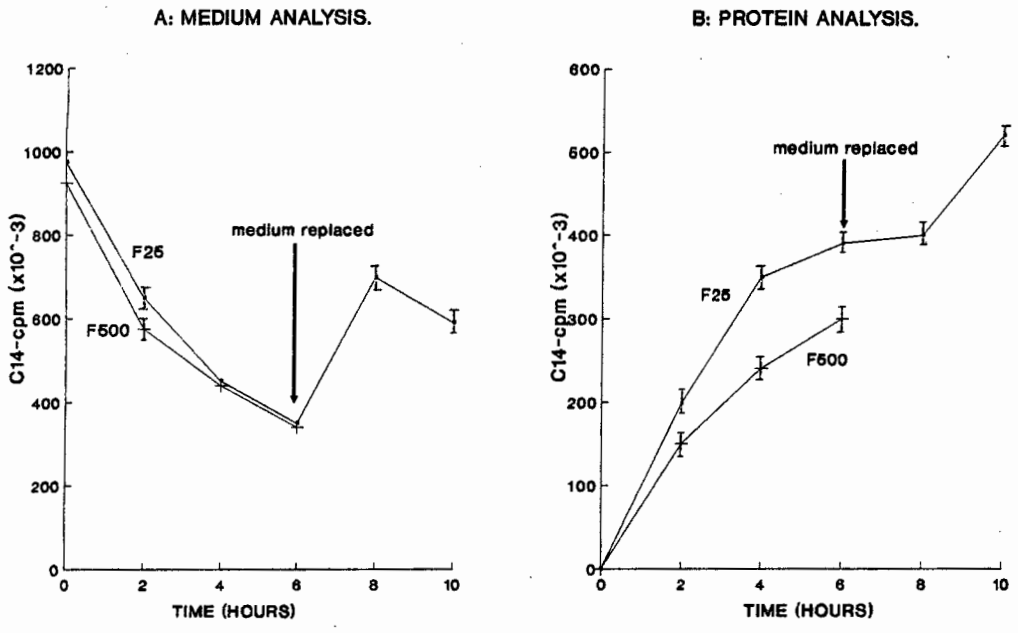

FIGURE 40: Ornithine transcarbamylase fibroblast assay: controls.

F25 (ASS deficient) and F500 fibroblasts (300000) were incubated in medium Z containing 0.5uCi ^{14}C-U-arginine over a 10 hour period (F500 = 6 hours). The medium (A) and the incorporation of ^{14}C-arginine into cellular protein (B) were monitored at 2-hourly intervals to monitor the presence of sufficient radioactivity in the medium and continuous cell proliferation during the incubation period. The medium was replaced at 6 hours to maintain glucose and radioactivity levels in the medium (>0.2μCi). The error bars represent the range of results in the triplicate wells.

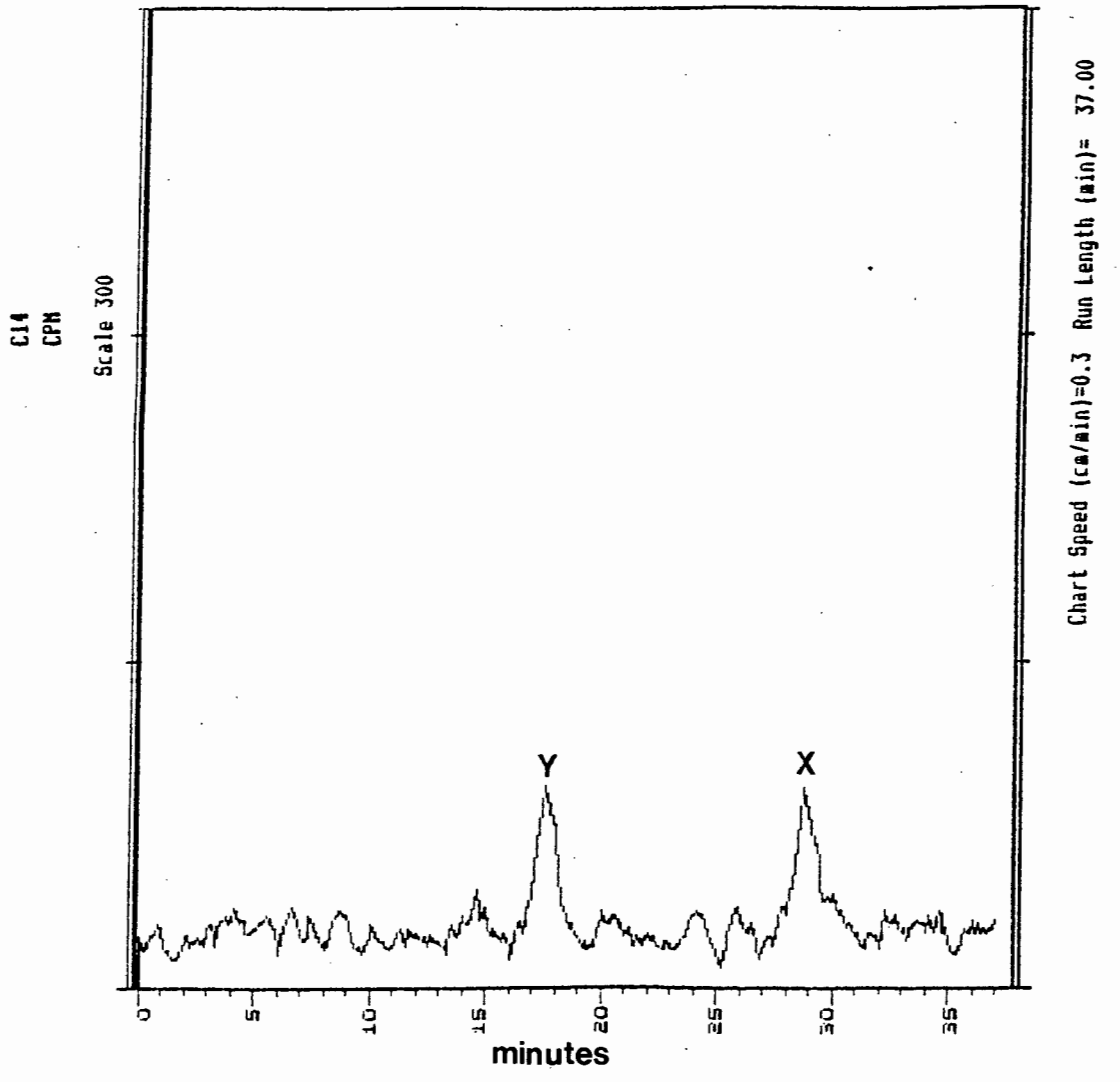

FIGURE 41: Ornithine transcarbamylase assay: HPLC analysis of F25/F500 cellular supernatant fractions.

This figure represents the typical separation profile of ^{14}C-arginine metabolites found in the cellular supernatant fraction of F25/F500 cells, analysed on a cation-exchange column (Li-A/Li-R) during the OTC 10 hour assay. 300000 F25 (ASS deficient) fibroblasts were incubated in the presence of 0.5µCi ^{14}C-U-arginine over a 10 hour period in medium Z and samples collected at 2-hourly intervals. Two major peaks were identified in this fraction : peak X and peak Y.

F25 cells should accumulate ^{14}C-citrulline due to the ASS blockage, however figure 42A seems to indicate "citrulline" depletion at 6 hours and then a gradual accumulation again, when the medium was relenished with ^{14}C-arginine. Due to the unexpected nature of the metabolism of peak X, the OTC experiment was repeated for a control cell line, F500, but only over a 6 hour period to establish the trend of the curve. If the species represented by peak X was in fact citrulline, very low levels of this peak would be expected in the control due to its continual metabolism by ASS and ASL activity. However, it was found that peak X formation followed the same curve as that shown for F25, with depletion of metabolite X at 6 hours. Interestingly, 3 times more of this metabolite was produced in the control cell line, which further suggested that peak X did not represent citrulline.

Proline and glycine elute very closely to citrulline on a typical amino acid profile from the amino acid analyser, so the possibility of these amino acids being represented by peak X was investigated. On the cation-exchange program used on the HPLC system, it was demonstrated that proline eluted very closely to citrulline, and may be a likely candidate for peak X in the OTC experiment. Glycine eluted somewhat earlier at 27 minutes. To confirm this result, ^{14}C-proline and ^{14}C-citrulline standards were used to spike the OTC cell-supernatant samples, and the the identity of peak X was confirmed as proline. Figure 42B also shows the continous accumulation of urea and proline in the medium extracts over the 10 hour incubation period.

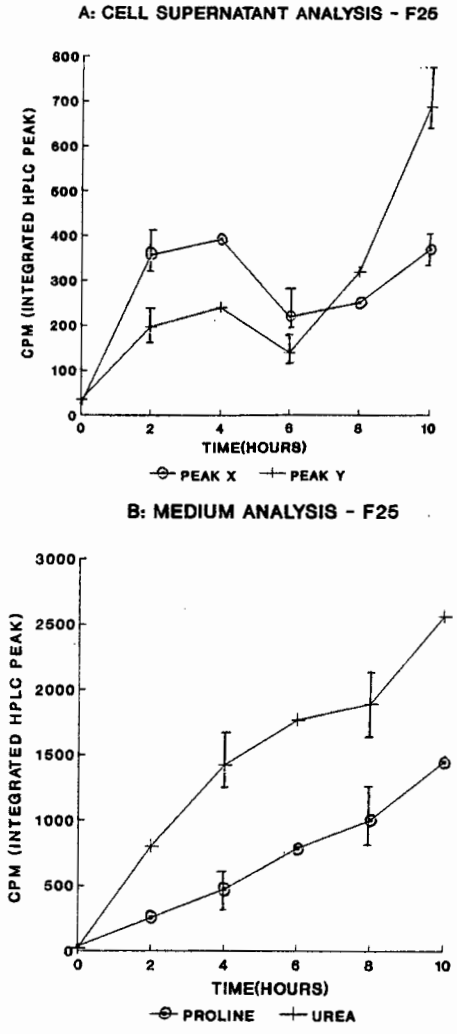

FIGURE 42: Ornithine transcarbamylase assay: Accumulation of ^{14}C-arginine metabolites in the medium and cellular supernatant fractions.

300000 F25 (ASS deficient) fibroblasts were incubated in 1ml medium Z containing 0.5µCi ^{14}C-U-arginine for 0-10 hours (medium replaced at 6 hours). Cellular supernatant and medium fractions were collected at 2-hourly intervals and analysed on a cation-exchange HPLC column (Li-A/Li-R system). **A)** The accumulation of ^{14}C-cpm in peak X and peak Y in the cellular supernatant fractions. **B)** The accumulation of ^{14}C-cpm in the identified proline and urea integrated peaks in the medium. The error bars represent the range of results seen in the duplicate wells.

The shape of the proline curve (figure 42 peak X) may reflect the cells utilisation of proline, where a small pool is maintained in the cell and any excess accumulates in the medium. When ^{14}C-proline formation was slowed due to the fall in exogenous arginine levels at 6 hours, this intracellular pool was diminished, explaining the dip in the curve.

Citrulline and proline were also separated on silica TLC plates and were found to elute in the same position, explaining the earlier TLC results (section 2.9.A.ii.a), which suggested the formation of $^{3}H/\ ^{14}C$ citrulline (spot C) from labelled arginine.

These combined results show that detectable levels of labelled citrulline could not be demonstrated from the metabolism of labelled arginine over a 10 hour incubation period. This indicates that OTC activity is not detectable in human cultured fibroblasts, which is in agreement with previous reports [6,99].

The proline that was formed from the metabolism of arginine in the above OTC experiment, was formed by the ornithine-keto aminotransferase (OKT) pathway which is found in fibroblasts [6]. Another product of this pathway is glutamate (see figure 43) and this was investigated as a possibility for the metabolite represented by peak Y (17.5 minutes) in figure 41. The accumulation of metabolite Y in the F25 cell-supernatant fraction over the 10 hour period, is shown in figure 42A.

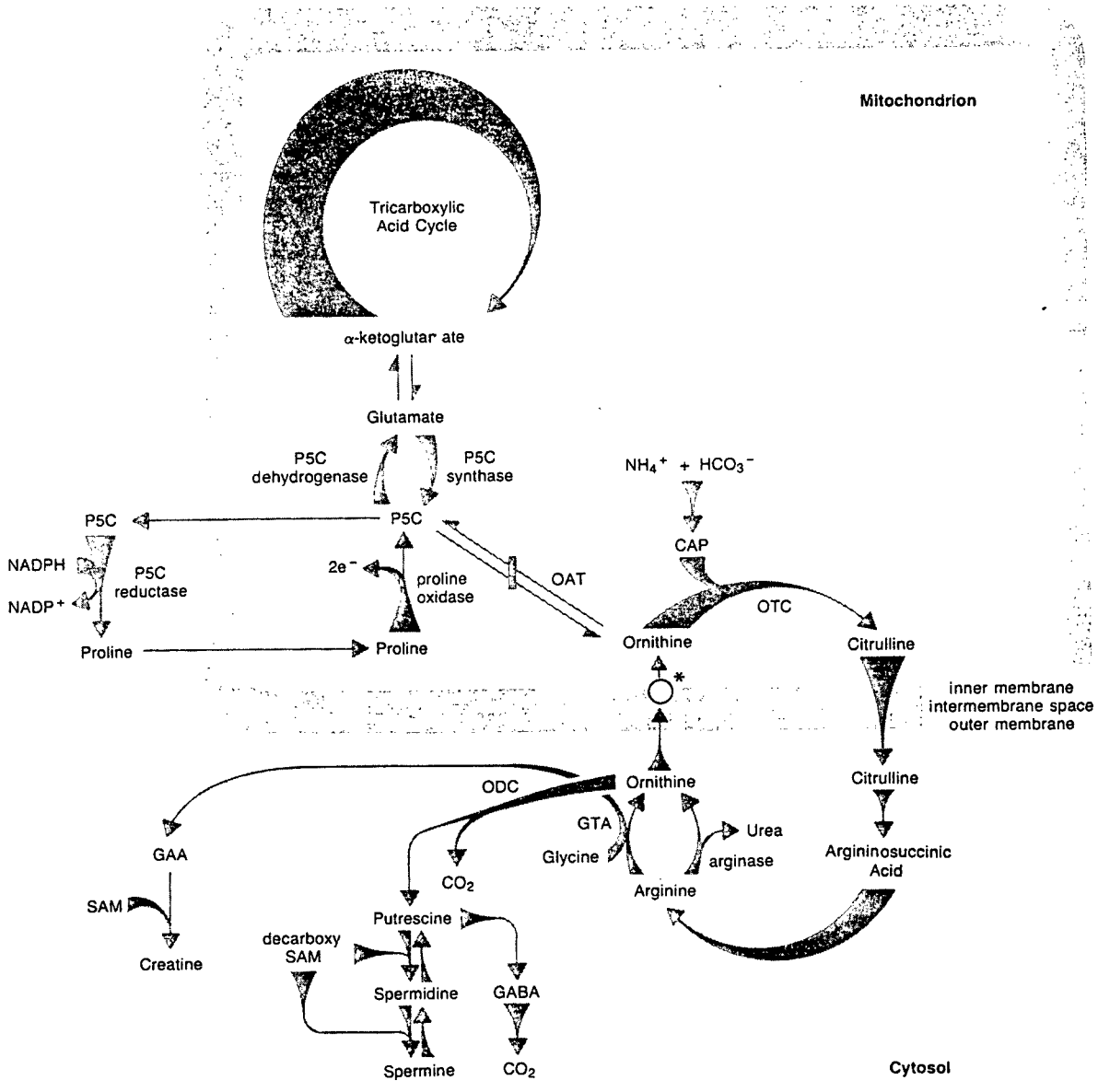

FIGURE 43: Intracellular amino acid metabolism [1].

Diagram of amino acid metabolism, demonstrating the link between the urea cycle and the ornithine keto-amino transferase (OKT, OAT) pathway. CAP = carbamyl phosphate synthetase, OTC = ornithine transcarbamylase.

HPLC and TLC analysis of glutamate's migration position relative to other standards, provisionally confirmed its identification as the metabolite in peak Y.

From the measurement of arginine metabolites during this investigation, it is possible to get an approximate estimation of the arginine flux with respect to the urea cycle and the flow of arginine towards the synthesis of protein. Calculations were made according to the amount of ^{14}C-protein and ^{14}C-urea formed after 4 hours (figures 40 and 42B) during the OTC experiment. The cpm results were converted to dpm values to ensure accuracy.

Firstly, after the 4 hour incubation period, the amount of ^{14}C-arginine in the medium had been reduced by 53%, with 45% of the available ^{14}C-arginine having been incorporated into acid precipitable material. Secondly, from the amount of ^{14}C-urea that was formed over the 4 hour period, it was calculated that only 0.8% (14nM) of the available ^{14}C-arginine (1.6μM) was metabolised by arginase activity. This result shows a preferential flux of arginine molecules towards protein production rather than urea formation by a ratio of 56:1.

These results do not accurately represent the absolute amounts of the various arginine metabolism pathways, but instead give an indication of the scale of the utilisation of arginine for protein synthesis after a 4 hour incubation period.

A.iii: Cycloheximide treatment.

Due to the large consumption of ^{14}C-U-arginine by protein synthesis, the possibility of blocking this pathway was investigated as a means of promoting the metabolism of arginine by other pathways.

The effects of a cycloheximide concentration range of 0-100µg/ml on F500 fibroblasts were investigated, to find the minimal concentration necessary to halt protein synthesis in these cells, and maximise flux to the other metabolites. ^{14}C-arginine was added at the same time as cycloheximide to monitor the levels of protein synthesis and metabolism of arginine. The acid precipitable fraction was collected to monitor protein synthesis, medium collected to monitor glucose levels and cell-supernatant fractions analysed on both the reverse-phase and cation-exchange HPLC systems. The cells were also visualised throughout the incubation period and most of the wells showed no signs of ill effects, although some cell death was noted in the wells containing 100µg/ml cycloheximide.

Figure 44A shows the inhibitory effects of cycloheximide on protein synthesis, with 20µg/ml allowing only 3.8% of the original protein synthesis and 50-100µg/ml between 1 and 2%. This shows the effective blockage of this pathway by cycloheximide. Figure 44B shows the resulting increase in the formation of arginine metabolites (urea, proline, glutamate) as a result of protein synthesis inhibition.

EFFECTS OF CYCLOHEXIMIDE

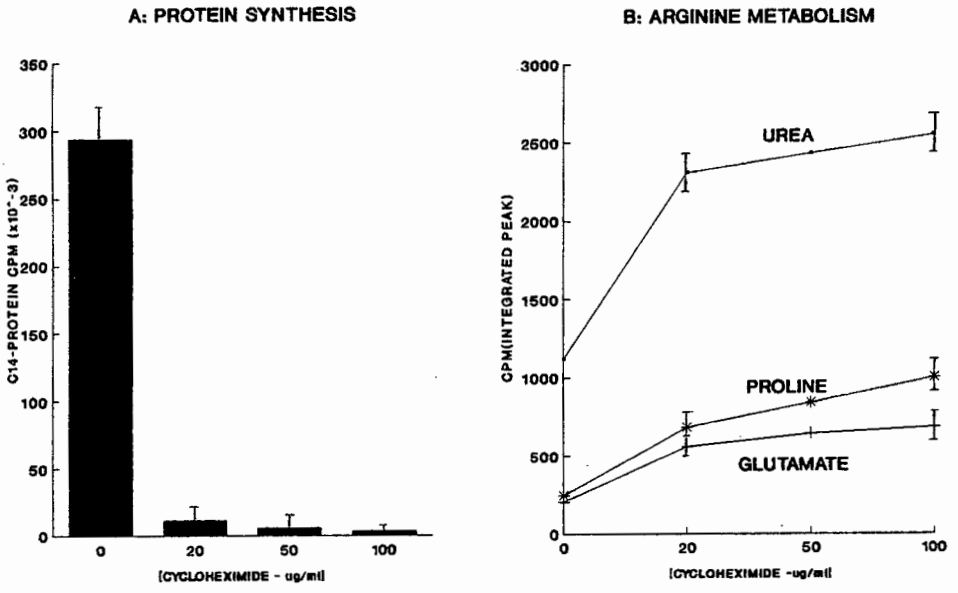

FIGURE 44: The effects of cycloheximide on protein synthesis and arginine metabolism in control fibroblasts.

300000 F500 (control) fibroblasts were incubated in medium Z containing 0-100µg/ml cycloheximide. 0.5µCi ^{14}C-U-arginine was included in the medium to analyse arginine metabolism in the presence of the cycloheximide. (A) represents the amount of ^{14}C-protein formed in the presence of the various concentrations of cycloheximide. (B) Through the HPLC analysis (reverse-phase and cation-exchange) of medium and cellular supernatant fractions, the formation of ^{14}C-urea, ^{14}C-proline and ^{14}C-glutamate was monitored. The error bars indicate the range of the duplicate results.

The glucose analysis of the medium after the incubation period, revealed that cells incubated with 100µg/ml cycloheximide utilised glucose at a slightly faster rate.

From these results, 50µg/ml cycloheximide was chosen as the level necessary to inhibit protein synthesis maximally and stimulate the increase in metabolism of arginine by other pathways, without detrimentally effecting cell viability.

A.iv: Arginase inhibition - Valine treatment.

Some researchers [88,100] have used arginase and ASS inhibitors to maximise the formation of citrulline by arginine via the nitric oxide pathway. The major enzyme of the urea cycle in fibroblasts that may cause a diversion of ^{14}C-arginine is arginase. Knowles et al [100] reported that in liver cells, this enzyme could be effectively inhibited in the presence of 50mM L-valine, while others [95,101] report that ornithine also inhibits arginase activity (43%-67%).

The effects of valine on the arginase activity in fibroblasts (F500) were investigated. 50mM valine and ^{14}C-U-arginine were incubated together with the fibroblasts and then the protein, medium and cell supernatant fractions evaluated.

Firstly, it was shown that the presence of valine caused a 33% reduction in protein synthesis. Secondly, arginase activity results in the formation of ornithine, which as can be seen in figure 45 leads to the formation of proline and glutamate by OKT. Analysis of the medium and cell-supernatant

fractions revealed the following results:

TABLE 5: EFFECTS OF VALINE ON ARGINASE ACTIVITY.

METABOLITE:	+ VALINE (cpm):	- VALINE (cpm):
UREA	3387	6511
ORNITHINE	2607	4500
PROLINE	120	682
GLUTAMATE	140	685

F500 (control) fibroblasts were incubated in the presence of 0.5µCi ^{14}C-U-arginine and 50 mM L-valine for 4 hours. The medium and cell supernatant fractions were collected and analysed on the cation-exchange and reverse-phase HPLC systems. The amounts of ^{14}C-cpm in each of the ornithine, urea, proline and glutamate integrated HPLC peaks were recorded (averages of triplicate results).

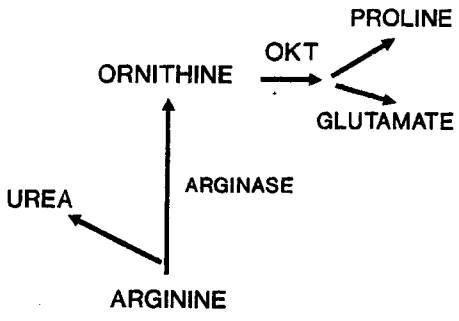

FIGURE 45: The arginase and OKT reactions.

These results show the effect of valine on arginase activity, with a 50% decrease in both urea and ornithine production. The larger decrease in proline and glutamate may also reflect the inhibitory effects of valine on enzymes other than arginase. Although the inhibition is not 100%, 50mM L-valine does partially inhibit arginase activity in addition to

slowing protein synthesis, both of which will help promote the metabolism of arginine to citrulline by iNOS if this pathway is operative in fibroblasts.

A.v: Inhibition of ^{14}C-citrulline metabolism by addition of unlabelled citrulline.

Although the purpose of this study was to utilise ASS deficient fibroblasts (F25) to investigate the potential NO$^{\bullet}$ pathway, some difficulty was incurred in the detection of citrulline during the NO$^{\bullet}$ assay (see 2.8.B). One of the possibilities to explain this result was that because only small levels of citrulline may be produced via the NO$^{\bullet}$pathway (10μM over 24 hours [77]), any residual ASS activity may metabolise this ^{14}C-citrulline. Therefore to minimise ^{14}C-citrulline consumption in these fibroblasts, the dilution effects of exogenous L-citrulline on the metabolism of a trace amount of ^{14}C-citrulline by ASS was investigated. The tests were performed on F500 (control) cells to maximise ^{14}C-citrulline uptake levels for detection purposes. The effects of 0.01, 0.1, 1 and 10mM L-citrulline on the utilisation of 0.005μCi ^{14}C-citrulline (0.1μM) to form ^{14}C-protein was investigated. Figure 46 shows the ^{14}C-cpm incorporated into acid precipitable material after the incubation period and shows the efficient dilution effects of the added citrulline.

1mM added exogenous L-citrulline was found to be adequate to minimise the metabolism of trace amounts (0.1μM amounts) of ^{14}C-citrulline.

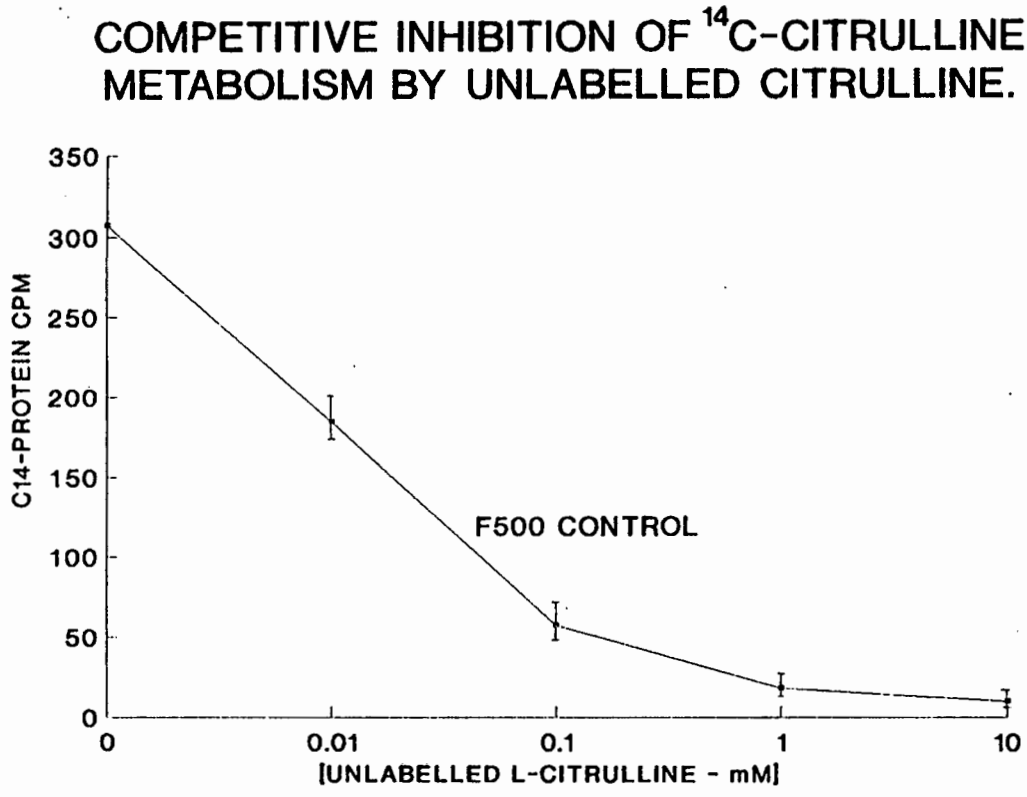

FIGURE 46: Competitive inhibition of ^{14}C-citrulline metabolism by ASS with unlabelled L-citrulline.

The effects of unlabelled L-citrulline on the metabolism of ^{14}C-citrulline (0.1μM) were investigated in F500 (control) fibroblasts. 300000 cells were incubated with 0.005μCi ^{14}C-citrulline (medium Z) in the presence of 0, 0.01, 0.1, 1 and 10mM L-citrulline for 4 hours. ASS activity was assayed by measuring the incorporation of ^{14}C-cpm into acid precipitable cellular material (^{14}C-citrulline->^{14}C-arginine->^{14}C-protein). The error bars represent the range of results from the triplicate wells.

2.9.B: NITRIC OXIDE ASSAY.

Using the information from the preliminary studies, several attempts were made to induce the nitric oxide pathway in fibroblasts.

B.i: Experiment 1.

When the project was initiated little was understood about the mechanism by which interferon could stimulate activity of iNOS. It was not known whether cytokine induction was pre or post-translational or that exogenous arginine levels of between 0.2mM and 0.3mM were required for maximal iNOS activity [7]. So due to the preliminary findings of the large consumption of label by the protein synthesis pathway, the inital study utilised the blockage of this pathway by 50µg/ml cycloheximide to try and promote $NO^{\bullet}$ formation, and maintain a high level of ^{14}C-arginine in the cytoplasm.

The actual experiment involved incubation of the F25 fibroblasts with 100U γ-IFN, in the presence or absence of 10µg/ml LPS for 18 hours in DMEM, to activate iNOS. The length of the incubation period and amounts of cytokines needed for iNOS activation were decided upon by reference to other studies [7,54,58,77]. The medium was then removed and replaced with medium Z (to enhance ^{14}C-arginine uptake) containing 0.5µCi ^{14}C-U-arginine (universal label) and 50µg/ml cycloheximide. The incubation was then continued for a further 4 hours and the cell-supernatant fractions analysed (HPLC) for the formation of ^{14}C-citrulline. No citrulline was detected under these incubation conditions.

In hindsight this result is not surprising, as several key elements of the experimental design were found to be inappropriate in the light of recent publications. Firstly, interferon induces iNOS at the transcriptional level, which then requires protein synthesis to allow the formation of the enzyme [60,71]. Secondly, although iNOS has been isolated and then assayed (similar to the method used here), the in vitro assays were performed over short periods of time (10-30 minutes) [73,88,100], and at present no indication is given as to the half life of this enzyme. It is possible that over the 4 hour period, in the absence of further protein synthesis and interferon stimulation, any iNOS that was produced during the 18 hour γ-IFN stimulation period was rapidly degraded and did not therefore allow the production of detectable levels of citrulline. Finally, for maximal activity of iNOS, exogenous arginine at levels of above 0.25mM were shown to be required [7]. Under the assay conditions mentioned above, only 1.5µM arginine was added in the form of ^{14}C-U-arginine, which would not have been enough to allow for maximal enzyme activation.

In light of the above, several points were noted that could have contributed to the absence of detectable citrulline under the above experimental conditions:

1) Continual cytokine stimulation is required to allow the production of sufficient levels of iNOS.

2) Although IFN and LPS may stimulate transcription over the 18 hour period, the inclusion of cycloheximide in the incubation medium would prevent the synthesis of iNOS protein.

3) Sufficient iNOS may be produced in the 18 hour period, however under the incubation conditions , sufficient extracellular arginine was not available to allow for maximal enzyme activity.

4) The citrulline may be produced in very small quantities and it is possible that even the 5% residual ASS activity in F25 may result in the metabolism of this ^{14}C-citrulline pool.

B.ii: Experiment 2.

In an attempt to circumvent these problems, the assay was remodelled to take into account the above considerations. Firstly, the OTC experiment (section 2.9.A.ii.b) showed that large amounts of proline and glutamate can be formed from ^{14}C-arginine metabolism during the incubation, and that a large peak of proline may readily interfere with the detection of a small citrulline peak, due to the very close retention times of these two amino acids (cation-exchange column). So as to avoid this problem the NO˙ experiment was performed with ^{14}C-guanido-arginine. The ^{14}C-label of this arginine preparation is positioned on the carbon that forms part of the guanido group in arginine, which is cleaved by arginase to form ^{14}C-urea. Thus the only labelled metabolite that can be formed from the reactions of arginase, OKT and

OTC, is urea. The only other possible labelled metabolite that may arise from the metabolism of ^{14}C-guanido arginine is citrulline, if it is produced by the NO$^{\cdot}$ pathway. This enables the sensitivity of the assay to be enhanced as it prevents thee overlap of potentially major labelled species such as prolne (OKT pathway).

Secondly, it was proposed that the incubation period of 4 hours in the presence of ^{14}C-arginine may not have been long enough to allow the accumulation of detectable levels of ^{14}C-citrulline. So to overcome this problem, as well as the need for extracellular arginine levels in the > 0.2mM range [7] and the requirement of the presence of IFN for continual iNOS production, the experiment was performed as follows: F25 and F500 (control) cells were incubated for 8 hours in DMEM (0.5mM arginine) containing 100U γ-IFN and 10mg LPS. This step should allow for the inital activation of iNOS. 2μCi of ^{14}C-guanido arginine was then added to the medium, as the iNOS substrate, and the incubation continued for a further 16 hours. Cell supernatant and medium fractions were then collected. Such large amounts of ^{14}C-arginine were necessary to allow for the lagre consumption of arginine for protein synthesis, especially over the 16 hour incubation period.

Due to the long incubation time of 16 hours in the presence of the isotope, it was important to try and limit the utilisation of ^{14}C-arginine by other pathways (arginase). Other researchers have used urea cycle inhibitors to promote NO$^{\cdot}$ formation [88,100]. The effects of valine were

investigated in the preliminary studies and revealed that 50mM L-valine was sufficient to reduce arginase activity by 50% to 60% (section 2.9.A.iv). The added valine was also shown to reduce protein synthesis by 33%. Unlabelled citrulline (1mM) was found to be effective in minimising the metabolism of ^{14}C-citrulline (section 2.9.A.v), thus promoting ^{14}C-citrulline accumulation. So in an attempt to maximise NO$^{\bullet}$ production and ^{14}C-citrulline formation, 50mM valine and 1mM L-citrulline were added at the same time as the ^{14}C-guanido-arginine in the above experimental profile. A schematic summary of this NO$^{\bullet}$ experiment is shown in figure 47.

Controls:

To ensure that the system was operating optimally and that all the essential components were present, several controls were performed. Firstly, the extracellular arginine levels in the medium were measured at time 0, 8 hours and 24 hours to ensure that arginine levels remained above the critical 0.25mM level. Amino acid analysis showed this to be the case. Secondly, the inhibition of arginase activity was measured by comparing cells treated with and without 50mM L-valine in the presence of the cytokines and ^{14}C-guanido-arginine. It was shown that the presence of 50mM valine over the 16 hour incubation period effectively reduced the amount of ^{14}C-urea produced by 66%.

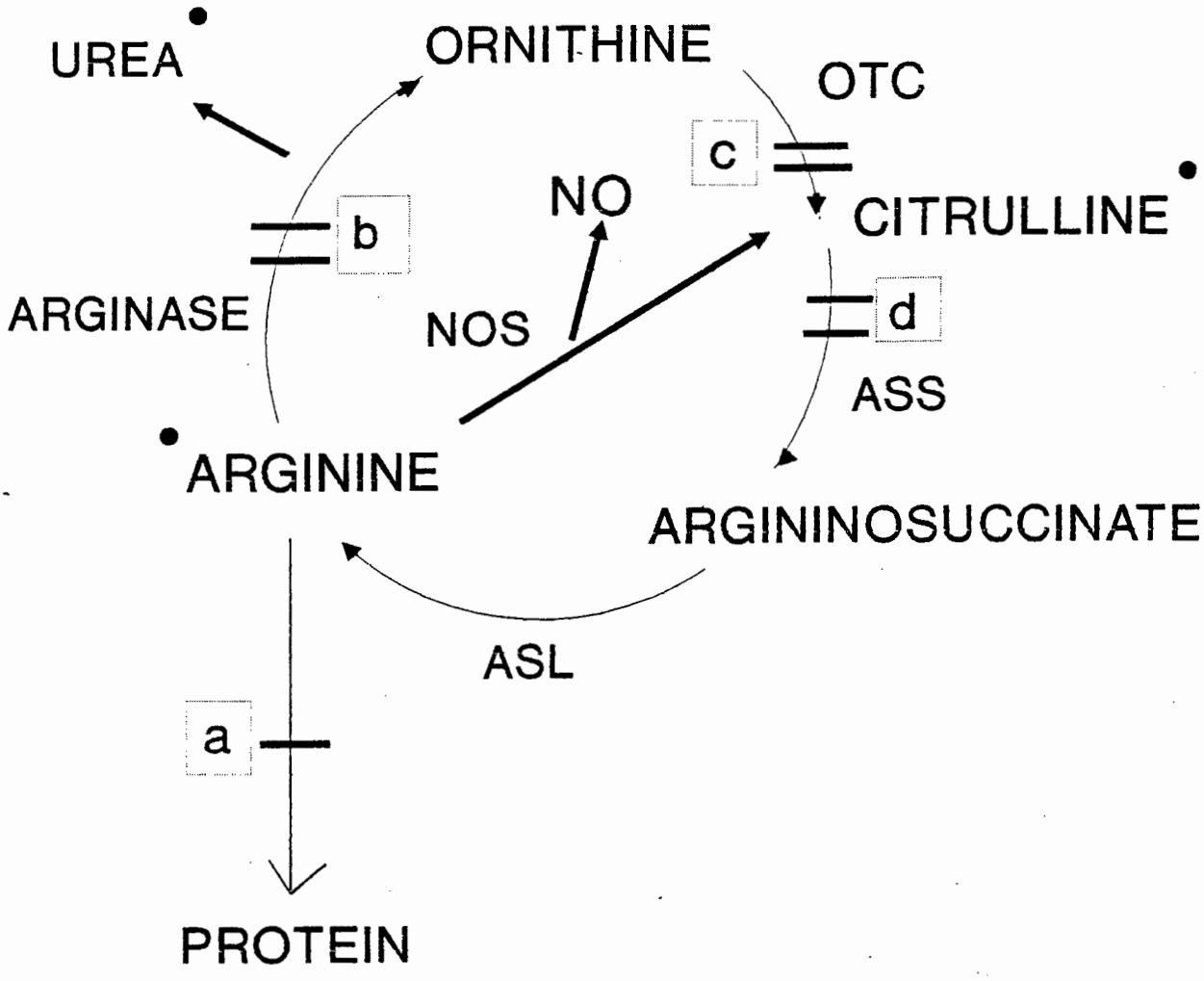

FIGURE 47: A schematic representation of the nitric oxide assay (experiment 2) in human fibroblasts.

A simplified diagram of the main enzymes and metabolites that were considered during the design of the nitric oxide assay and the steps that were taken to promote the formation of NO by blocking these pathways. (a) = 50mM valine reduces protein synthesis by 33%. (b) = Arginase activity is reduced 50-70% by 50mM valine. (c) = OTC activity was shown to be absent in fibroblasts. (d) = ASS activity is reduced in F25 (1-5%) and 1mM citrulline prevents the metabolism of trace amounts of ^{14}C-citrulline by ASS. ● = Possible ^{14}C-metabolites when incubating with ^{14}C-guanido arginine.

With regards to ^{14}C-citrulline metabolism, cytokine stimulated control fibroblasts (DMEM, 0.5mM arginine) were incubated in the presence of 50mM L-valine with or without 1mM L-citrulline as well as 0.05μCi ^{14}C-citrulline, and the amount of ^{14}C-protein generated after the 16 hour incubation period measured.

It was found that no significant ^{14}C-protein was formed under these circumstances, indicating the effectiveness of the added 1mM citrulline in diluting the ^{14}C-citrulline and minimising its metabolism by ASS. Amino acid analysis showed that both citrulline and valine were found in substantial amounts in the medium after the 16 hour incubation period.

Finally, the experiment was performed in the presence and absence of the cytokines as a comparison and the experiment was performed in triplicate to ensure accuracy. γ-IFN and LPS were always used in conjunction with one another as this has been found to be the optimal stimulation in a number of systems [77,82].

The cell-supernatant and medium fractions of both the F25 and F500 samples were analysed on the reverse-phase and cation-exchange columns after the 24 hour incubation period of the experiment. No ^{14}C-citrulline was detected after using these revised incubation conditions.

2.10: DISCUSSION.

The aim of this section was to use the F25 (ASS deficient) fibroblast cell line to demonstrate whether induction of the $NO^{\cdot}$ pathway (iNOS) by cytokines could be achieved in cultured human fibroblasts. Several $NO^{\cdot}$ assay systems in other cell lines [54,59,60,88,100] have used the fact that equimolar amounts of citrulline and $NO^{\cdot}$ are produced by the metabolism of L-arginine by NOS [63]. In the F25 fibroblast cell line, this citrulline should accumulate due to the absence of significant ASS activity, allowing for the detection of accumulating citrulline in response to cytokine stimulation.

$NO^{\cdot}$ has been shown to operate in both signal transduction and in host immune defense mechanisms (macrophage cytotoxicity) [60,63,71]. Several similarities exist between macrophages and fibroblasts that suggests that fibroblasts may play a role in the general immune response. These include the synthesis of BH_4 [77,80,81,82], which has been directly linked to the production of $NO^{\cdot}$ in murine fibroblasts after cytokine stimulation [77], as well as the increased synthesis of IDO in response to this stimulus [82]. It is therefore possible that fibroblasts may participate in the general immune response through the production of $NO^{\cdot}$ by iNOS activity.

The basic $NO^{\cdot}$ assay system that was adopted in this study was as follows: Activation of the ASS deficient fibroblasts with γ-interferon and lipopolysaccharide, to

induce the expression of iNOS. The iNOS enzyme activity was then assayed by measuring the accumulation of ^{14}C-citrulline from ^{14}C-arginine in cell-supernatant and extracellular medium fractions. ^{14}C-citrulline and other arginine metabolites were separated and quantified using two HPLC systems (reverse-phase / octane-sulphonate and cation-exchange).

Preliminary studies were performed to optimise the NO$^{\cdot}$ assay and ^{14}C-citrulline detection system. Firstly, OTC activity was tested in fibroblasts, with the results confirming previous reports [6,99] of the absence of OTC activity in fibroblasts. This result implied that arginine could only give rise to citrulline if it was metabolised by NOS, via the NO$^{\cdot}$ pathway. Secondly, ways of inhibiting other arginine metabolic pathways were investigated, in an attampt to maximise the flow of arginine to citrulline via iNOS activity. These tests included the inhibition of protein synthesis by cycloheximide (50μg/ml) and the inhibition of arginase activity by valine (50mM). It was later found that complete protein inhibition was not appropriate for the NO$^{\cdot}$ assay so cycloheximide was not used, although valine was incorporated to reduce arginase activity (50-70%). Finally, although ASS activity in the F25 (ASS deficient) was only 1-5% of the control, it was proposed that this activity may allow for the metabolism of small amounts of accumulating ^{14}C-citrulline over the long incubation periods. The addition of 1mM

unlabelled citrulline was found to be effective in diluting small amounts of ^{14}C-citrulline (0.1μM), to prevent its metabolism by ASS. Valine (50mM) and citrulline (1mM) have previously been used in the analysis of the NO$^{\cdot}$ pathway in liver [88,100].

Several attempts were made to detect NO$^{\cdot}$ production in fibroblasts, with the most comprehensive assay incorporating a total of 24 hours of exposure to γ-interferon and LPS to induce iNOS expression, with iNOS activity being measured for 16 hours during this cytokine exposure. The iNOS activity was assayed by measuring the formation of ^{14}C-citrulline from ^{14}C-guanido-arginine in the presence of 50mM valine and 1mM citrulline, over the 16 hour period. However, in all these experiments ^{14}C-citrulline production was not detected.

There are many parameters that need to be established to definitively answer whether human fibroblasts are able to express iNOS activity. Firstly, in some cell lines iNOS activity can be induced and detected after several hours stimulation with γ-interferon (6-24 hous) [54,58,72]. However, human fibroblasts have shown very slow responses to cytokines, requiring 96 hours to show some effects of interferon stimulation (increased IDO synthesis) [81]. It is possible therefore that the 24 hour stimulation used in this study, is not long enough in studies with human fibroblasts. Secondly, it was not established if the cytokine combination was actually having any effect on the fibroblasts. It is essential to establish a control to ensure that these

additives are activating the cell. For example, by monitoring an increase in IDO or BH_4 synthesis [77,81,82].

Finally, the indirect assay of $NO^{\cdot}$ production by the formation of ^{14}C-citrulline from ^{14}C-arginine may not be the best assay system to use if only small amounts of $NO^{\cdot}$ are produced. Long assay times (>24 hours) require very large amounts of radioactivity with most of the label being incorporated into protein. The detection of $NO^{\cdot}$ by the formation of nitrates and nitrites is more suitable to extended incubations and may be more suitable for this system. The number of cells used in this study may also not be sufficient if very small amounts of iNOS are induced, with the radiochemical detector system only being able to define peaks containing more than 300dpm (2.4nM of ^{14}C-guanido-citrulline) without ambiguity.

The implications of $NO^{\cdot}$ production in fibroblasts are fascinating, with the potential for diagnostic testing in the area of $NO^{\cdot}$ disorders. The involvement of fibroblasts in the general immune response by their production of $NO^{\cdot}$ is also a new idea, that may uncover a different perspective to generalised immunology. However, it appears that identification of iNOS activity in fibroblasts requires more extensive investigation than was possible during the course of this study.

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