A COMPARISON OF METABOLIC PATHWAY DYNAMICS IN MAN AND OTHER MAMMALS

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DEDICATED TO MY FAMILY

I declare that this thesis is my own work. It is being submitted for the Masters Diploma in Medical Technology, to the Cape Technikon, Cape Town. It has not been submitted before for any diploma or examination at any other Technikon or tertiary institution. The work was carried out at the Department of Chemical Pathology, Cape Town.

The opinions and conclusions drawn are not necessarily those of the Cape Technikon.

I. Baumgarten   29.10-93

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SUMMARY

The object of the present study was to determine whether there was a difference in the flux through two different metabolic pathways, the purine salvage pathway and the urea cycle, in skin fibroblasts from three species. A double label approach was adopted to measure the incorporation of the purine bases, hypoxanthine and adenine, and the amino acids, citrulline and arginine in acid precipitable material.

Before examining the between species variation it was necessary to examine different levels of possible variation such as the variation between individuals from each species, the variation between separate experiments and the replication error within experiments.

Eight individuals from each of three species, buffalo, human, and rhinoceros were examined with the labelled purine bases. Skin fibroblasts from four humans and eight rhinoceros individuals were also examined over varying passage numbers until the fibroblasts senesced, to determine the effect of ageing on the uptake of hypoxanthine and adenine.

The same four human fibroblast cultures were transformed with a transforming virus and examined to see the effects of transformation on the uptake of the purine bases, these transformed fibroblasts were compared with previously transformed rhinoceros fibroblasts. The uptake of

labelled citrulline and arginine was also examined in three individuals from each of the three species.

The major part of variation throughout the study was found to be at the between experiment level, despite stringently controlled conditions. This between experiment variation obscured any variation found within individuals from each species.

In spite of this major between – experiment variation, the results showed that there was significant variation between the three species in the uptake of hypoxanthine. Adenine uptake was similar in the buffalo and human, but was significantly different between both these species and the rhinoceros.

Citrulline uptake showed no variation between the three species, whereas arginine showed a significant variation between the rhinoceros and the other two species. Buffalo and human showed no significant variation in arginine uptake.

There was a significant increase in the uptake of hypoxanthine and adenine in transformed fibroblasts relative to untransformed fibroblasts.

As a consequence of the significant between-experiment variation demonstrated in this study, it is apparent that great care must be taken to standardize the conditions when using a double label approach, especially if the assay is to be used for the diagnosis of inborn errors of metabolism.

OPSOMMING

Die doel van hierdie studie was, om te bepaal of daar 'n verskil bestaan tussen die toestand van onvastheid van twee metaboliese paaie, nl. die purien-bergingspad en die ureum-siklus in vel fibroblaste van drie verskillende spesies. 'n Dubbel radioaktiewe merkingsmetode is gebruik om die inkorporasie van die purien basisse, hipoxantien en adenien en die aminosure, sitrullien en arginien in die suur presipiteerbare materiaal te meet.

Eerstens was dit nodig om die verskillende vlakke van moontlike variasie, soos variasie tussen individue van elke spesie, inter-eksperimentele variasie en die intra-eksperimentele replikasie variasie te bepaal, voordat die variasie tussen spesies ondersoek kon word.

Agt individue van die drie spesies, buffel, mens en renoster is met die radioaktiewe purien basisse ondersoek. Vel fibroblaste van vier mense en agt renoster individue is ook tydens verskeie passasies ondersoek, totdat die fibroblaste verouder het, om die uitwerking wat veroudering op die opname van hipoxantien en adenien het, vas te stel.

Dieselfde vier menslike fibroblast-kulture, is getransformeer met 'n transformeer-virus en daarna ondersoek om die uitwerking van transformasie op die opname van purien basisse te bepaal. Hierdie getransformeerde fibroblaste is met renoster fibroblaste vergelyk wat voorheen getransformeer is. Die opname van radio-aktiewe sitrullien

en arginien is ook in drie individue van elk van die drie spesies bestudeer.

Nieteenstaande streng gekontroleerde toestande, was die variasie op inter-eksperimentele vlak, die grootste. Enige variasie wat tussen individue van elke spesie gevind is, is deur hierdie inter-eksperimentele variasie verberg.

Ten spyte van hierdie groot inter-eksperimentele variasie, toon die resultate 'n beduidende variasie in die opname van hipoxantien, tussen die drie spesies. Alhoewel die opname van adenien in buffel en mens ooreengestem het, was daar 'n beduidende verskil in adenien opname tussen hierdie twee spesies en renoster. Daar was geen variasie in die opname van sitrullien in die drie spesies nie, maar wel 'n beduidende variasie in die opname van arginien by renoster en die ander twee spesies. Buffel en mens het geen beduidende verskil getoon in die opname van arginien nie.

Die opname van hipoxantien en adenien in getransformeerde fibroblaste was beduidend hoër as in nie-getransformeerde fibroblaste.

Die beduidende inter-eksperimentele variasie wat in hierdie studie gedemonstreer is, toon aan dat daar baie versigtig te werk gegaan moet word, om toestande te standardiseer wanneer die dubbele merkingsmetode gebruik word, veral wanneer hierdie toets vir die diagnose van aangebore metaboliese defekte gebruik word.

CONTENTS

ABBREVIATIONS

Ad	:	Adenine
AMP	:	Adenosine – 5'-monophosphate
APRT	:	Adenine phosphoribosyltransferase
Arg	:	Arginine
ASL	:	Adenylosuccinate lyase
ASL	:	Argininosuccinate lyase
ASS	:	Adenylosuccinate synthetase
ASS	:	Argininosuccinate synthetase
BME	:	Eagle's Basal Medium
Ci	:	Curie (2.2×10^{12} disintegrations per minute)
Cit	:	Citrulline
CSF	:	Cerebrospinal fluid
DMEM	:	Dulbecco's Modified Eagle's Medium
DPM	:	Disintegrations per minute
dTTP	:	deoxy Thymidine Tri-phosphate
EDTA	:	Ethylene diamine tetra-acetic acid
FCS	:	Foetal Calf Serum
HPRT	:	Hypoxanthine phosphoribosyltransferase
Hx	:	Hypoxanthine
IMD	:	Inherited metabolic disorders
IMP	:	Inosine – 5' – monophosphate
PPRP	:	5-phosphorylribose -1-pyrophosphate
PSN	:	Penicillin, Streptomycin and Neomycin
TCA	:	Trichloroacetic acid
TRIS	:	Tris (Hydroxymethyl) aminomethane

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1.0   INTRODUCTION

Different species of mammals display profound morphological diversity, and significant differences in physiology. Less well known is how different mammalian species are at the level of fundamental biochemical pathways, especially those catalyzed by "housekeeping" enzymes, these being those required for the normal functions of both undifferentiated and differentiated cells. This study seeks to determine whether this morphological diversity is paralleled by diversity at a biochemical level as measured by the flux through two short biochemical pathways: The first consists of four enzyme catalysis steps in the purine salvage pathways, and the second, two enzyme catalysis steps in the urea cycle.

In a pilot investigation, performed as a honours project (Sadler, 1990), the uptake of radio-labelled metabolites in cultured fibroblasts was compared in seven different mammalian orders, including one to three species within each order. A double label approach (Rosen *et al.*, 1977) was used and variation between species and between orders was found using several different isotope pairs. In order to define this variation more precisely, it was necessary to examine more individuals from each species. In order to assess the variation between species, it was necessary to define in addition the variation at a number of other levels, these are:

1. within experiment variation,

2. between experiment variation in the same cell line, and

3. between individual variation in the same species.

Cultured skin fibroblasts were chosen, as this cell type has been metabolically well characterized. The use of isotopically labelled substrates in cultured cells is a valuable means of measuring flux through metabolic pathways. The double label technique was described by Rosen *et al.*, (1977) as a method for screening the activity of metabolic pathways in fibroblast and peripheral blood cells. The method measures the radioactivity incorporated into the trichloroacetic acid (TCA) insoluble fraction of cells after incubation with two different radio-labelled substrates of the metabolic pathway under investigation. The isotopes used are typically ^{3}H and ^{14}C. As described by Rosen *et al.*, (1977) the ^{3}H labelled metabolite is usually not a substrate in the pathway to be examined using the ^{14}C labelled substrate and this serves as an internal control of metabolism in a cell population. A change in the ^{14}C : ^{3}H ratio would indicate a change in flux through the pathway as, for example, it might be caused by a defective enzyme. The method may suggest, but cannot identify an alteration in the activity of a specific enzyme, therefore additional specific enzyme assays would have to be performed. This technique has led to new insights into aspects of basic metabolism (Losman and Harley, 1978; Davidson and Harley,

1984; Steyn and Harley, 1984; Davidson *et al.*, 1984; Davidson *et al.*, 1985b and Steyn and Harley, 1985) which has, in turn led to new approaches in the diagnosis of inherited metabolic diseases and towards novel therapeutic measures (Harley and Berman, 1984; Davidson *et al.*, 1985a; Simmonds *et al.*, 1988 and Berman *et al.*, 1988).

The present study is a comparison between three species, representing the orders Artodactyla, Primata and Pernisodactyla, namely the buffalo (Syncerus caffer), the human (Homosapiens) and the rhinoceros (Diceros bicornis) and studies the uptake and incorporation of the purine bases hypoxanthine and adenine and the amino acids, citrulline and arginine. The double label approach used in this study differed from the standard approach in that the ^{3}H and ^{14}C labelled substrates feed into the same biochemical pathway and are separated by only a few catalytic steps.

Also examined were changes in the metabolic fluxes due to the ageing of fibroblasts in culture (Paz *et al.*, 1979) and changes due to viral transformation of the fibroblasts (van Doren and Gluzman, 1984).

1.2 HYPOXANTHINE AND ADENINE UPTAKE.

Hypoxanthine and adenine were chosen as one pair of labelled substrates since these are commonly used to investigate disorders of purine metabolism in man, (Rosen *et al.*, 1977; Paz *et al.*, 1979) and come together in the common product adenosine-5'-monophosphate (AMP) (Fig 1.) Hypoxanthine requires three steps catalysed by hypoxanthine phosphoribosyltransferase (HPRT), adenylosuccinate synthetase and adenylosuccinate lyase in its conversion to AMP, while adenine requires only one step catalysed by adenine phosphoribosyltransferase. In addition both substrates require transport into the cells by a facilitated diffusion mechanism.

Labelled ^{3}H adenine and ^{14}C hypoxanthine would therefore come together in a common product, AMP, and can then be quantified in acid precipitable material after eventual incorporation of the adenine nucleotide into nucleic acid. A decrease in the uptake of hypoxanthine would indicate a change in activity. Both HPRT and APRT are present in all mammalian tissue, the highest activity of HPRT being in the central nervous system. HPRT is an X chromosome-linked enzyme. The highest activity of APRT is in the liver and muscle. APRT is an autosomally linked enzyme. (Scriver CR *et al.*, 1989).

SALVAGE PATHWAYS OF HYPOXANTHINE AND ADENINE

PRODUCTS

FUMARATE ASPARTATE

AMP ← ADENYLO – SUCCINATE ← IMP
ASL ASS

APRT HPRT

PPRP

ADENINE HYPOXANTHINE

CELL MEMBRANE

ADENINE HYPOXANTHINE
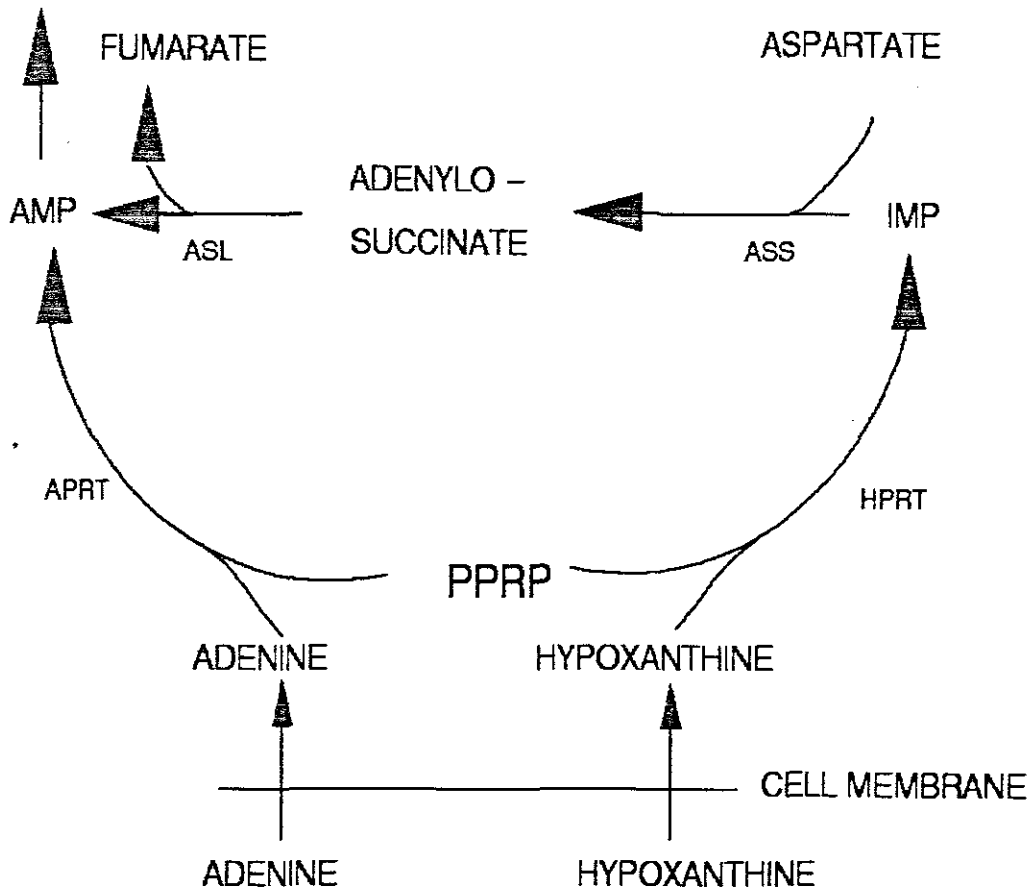
HPRT - HYPOXANTHINEPHOSPHORIBOSYLTRANSFERASE
ASS - ADENYLOSUCCINATE SYNTHETASE
ASL - ADENYLOSUCCINATE LYASE
APRT - ADENINEPHOSPHORIBOSYLTRANSFERASE

FIGURE 1

(courtesy of Prof. EH Harley)

In humans a variety of diseases are associated with deficiencies of HPRT or APRT. A partial deficiency of HPRT leads to purine over-production and gout. A virtually complete deficiency of HPRT leads to purine over-production and neurological disease, the Lesch-Nyhan syndrome. (Harkness *et al.*, 1990). APRT deficiency leads to the accumulation of adenine which *in vivo* is converted to 2,8 dihydroxyadenine by xanthine oxidase. This is relatively insoluble and causes urolithiasis, which can lead to renal failure (Christensen *et al.*, 1987).

Levels of activities of HPRT and related enzymes vary in a number of tissues in a variety of species (Harkness et al., 1990). For example there is no activity of HPRT or adenosine deaminase in horse erythrocytes, so that the erythrocyte concentration of phosphoribosylpyrophosphate, which together with hypoxanthine are substrates for HPRT, is as high in horses as in man with a deficiency in HPRT. However, HPRT activity is present in the blood lymphocytes and cells from the spleen and thymus (Peters and Veerkamp, 1979). Plasma levels of hypoxanthine and related substrates were measured in the horse and five other species. These species were chosen on the basis of varying erythrocyte HPRT levels. HPRT activity was also measured in a variety of tissues and compared to man and rabbit. Harkness *et al.*, (1990) used high performance liquid chromatography to measure the hypoxanthine, xanthine, uridine and cytidine in the plasma

and CSF. HPRT levels in equine tissues other than erythrocytes were similar to those in man and the rabbit. In the plasma and the CSF they found large species variation in hypoxanthine concentrations, these being about $50\mu M/L$ in the horse, rat, mouse and greyhound, $2\mu M/L$ in beagles and man, $10\mu M/L$ in rabbits and $20\mu M/L$ in sheep.

1.2.1 AGEING OF FIBROBLAST CULTURES

The ageing of cells in culture has been studied for the past three decades. Hayflick and Moorhead, (1961) demonstrated that fibroblasts have a finite lifespan. Foetal fibroblasts have +/- 50 population doublings before cell division and growth ceases. Fibroblast doubling potential in adults is diminished and appears to be inversely proportional to the donor age, Hayflick, (1980). Hayflick, (1991) divides the life span of fibroblasts into three phases.

PHASE 1 : The primary cells derived from intact tissue and which have not undergone any subculture.

PHASE 11 : Cell strains which have:

a) a finite capacity to divide,

b) do not produce tumours when inoculated into experimental animals,

c) have the karyology of the tissue of origin, and

d) and are anchorage dependent.

PHASE 111 : The cells decline in growth potential and
have a large number of non-dividing or
slowly dividing cells. This stage is
also referred to as the senescent state.

Several studies have shown morphological and biochemical changes due to cellular ageing (Hayflick, 1980). Hayflick suggests that these changes occur in individual animals well before replicative failure of its somatic cell occurs. Therefore when studying the biochemical functions of fibroblasts in culture, ageing must be taken into account. Paz et al., (1979) described a slow but continual decrease (1.4%) in the HPRT/APRT ratio with each population doubling. They examined the initial rates of incorporation of labelled hypoxanthine into IMP and labelled adenine into AMP as well as the HPRT levels in cell free extracts. The HPRT declined more rapidly then APRT. Paz et al., (1979) put forward several interpretations to be considered as causes for this decline.

These are as follows:

1. HPRT is the product of a gene present in one functional copy on the active X-chromosome. The product of a single gene could become more vulnerable to statistical random alterations during the lifespan of the cultured cell than two autosomal APRT alleles. Therefore, enzymes coded

for on the X-chromosome are possibly more susceptible to deterioration in culture than autosomally coded enzymes.

2. Progressive increase in the number of altered proteins may result from certain cells reaching a state of terminal differentiation at each passage that the cells have accumulated. Hayflick, (1991) disputes the term differentiation in ageing cells and states that the replicating normal fibroblasts do acquire changes which result in losses in their physiological activity and these lead to cell death. These losses are more reasonably characterized as ageing than differentiation. Similarly the finite lifespan of cultured normal cells is more characteristic of the phenomenon of ageing than it is of differentiation.

In a study performed by Sugawara *et al.*, (1990) hamster cells which lacked the HPRT gene were fused with human cell strains which had translocations between X -chromosome and either chromosome 1 or chromosome 11, but were otherwise normal. The hybrid clones that escaped senescence indicate that a deletion of a critical portion of chromosome 1 had occurred. The hamster - human hybrid clones were selected in HAT - containing medium which means that the surviving cells must have retained the HPRT gene located on chromosome X.

In spite of a number of discrepancies and conflicting results, (Mullaart *et al.*, 1990), evidence has been obtained of the induction of DNA alterations with age at all levels eg. DNA chemical structure, DNA sequence organization and

gene expression. Such an alteration in gene expression may lead to qualitative and/or quantitative changes in protein which could well be the underlying cause of the age related dysfunctions of organs and cells. No proteins with altered primary structure have been found to appear during ageing, but there is a clear age related decrease in protein synthesis which may be responsible for the increased half lives of most proteins in old cells.

1.2.2 IMMORTALIZED (TRANSFORMED) FIBROBLASTS

Certain criteria have to be established before a cell strain can be referred to as immortalised or transformed.

These are according to Hayflick, (1990) :

1. the ability to produce tumours in laboratory animals,

2) alteration of the karyology as compared with the tissue of origin, and

3) anchorage independence .

In addition, immortalised cells grow in serum-depleted tissue culture medium unlike the mortal cell. They exhibit no contact inhibition and when confluent will continue to grow piled up on top of each other.

Human cells with a finite lifespan fused with immortal cells with a indefinite lifespan have been found to reacquire the property of senescence, (Sugawaka *et al.*, 1990), indicating that senescence is dominant over immortality. Sugawaka found that the hybrid cells that

escaped senescence had a deletion on the long arm of chromosome 1 and that the HPRT gene remained intact. Klein et al., (1991) using a nickle transformed chinese hamster cell line with a Xq chromosome deletion which had a normal chinese hamster X chromosome transferred to it, found that the normal hamster X chromosome induced 100% senescence in the colonies. The highly conserved nature of the X chromosome genes suggests that the human X chromosome may also contain a senescence - inducing gene. Loss or inactivation of this senescence gene by hypermethylation may be associated with the acquisition of immortality.

Negative growth control in transformed cells has been attributed to a tumour suppression gene and repression of the c-fos expression has been described in senescent human fibroblasts (Seshadri and Campisi, 1990).

In a study comparing the activity of HPRT in normal, developing and neoplastic tissue in rats (Wolheuter, 1975) found that in hepatomas and regenerating liver, viewed statistically, nearly all values lay outside the normal range, but in no consistent pattern relative to the growth rate. The lack of positive correlation with tumour growth rate sets the purine salvage enzyme, HPRT, apart from several enzymes of pyrimidine nucleotide synthesis. Thymidine kinase, carbamyl-aspartate transferase and dihydro-orotase all increase in activity with increasing growth rate in the Morris spectrum of hepatomas. Bravard et al., (1992) has reported in a study performed on rabbit

chondrocytes transformed with SV40, compared to untransformed rabbit chondrocytes, that several enzymes including HPRT are increased. In most cases, these variations were correlated with the number of chromosomes carrying the genes encoding for corresponding enzymes.

A study performed on gap junction communication (Rosen et al., 1988) using transformed normal, argininosuccinate synthetase deficient and argininosuccinate lyase deficient human fibroblasts showed that the transformed cell lines retained the same defects in the citrulline uptake as their parent lines and in addition transformation had no effect on citrulline metabolism in the normal fibroblast line.

1.3 CITRULLINE AND ARGININE UPTAKE.

Most cultured human cells (e.g. fibroblasts, amniotic fluid cells and lymphoblasts) do not contain ornithine transcarbamylase or carbamyl phosphate synthetase and therefore do not have a functional urea cycle (Fig 2, Pg. 14). They do possess the enzymes argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) which convert citrulline into arginine (Schimke, 1964; Tedesco and Mellman, 1967).

Cultured cells from patients with a deficiency of either enzyme are unable to convert citrulline into arginine at normal rates and this results in a decrease in the uptake of citrulline into protein (Kennaway and Curtis, 1981). Defects in ASS or ASL can be detected in cultured cells by

measuring the incorporation of radio-labelled citrulline into protein, assayed as acid precipitable material.

The advantage of this method over direct enzyme assays is the simplicity of the procedure and the small number of cells needed, factors especially useful in prenatal diagnosis. In humans, deficiencies of ASS and ASL are autosomal recessive disorders, resulting in citrullaemia or argininosuccinate aciduria respectively. A severe deficiency of ASS or ASL results in hyperammonaemia, neurological dysfunction, coma and death in the neonatal period.

Since these two enzymes are not constitutive in all tissues, they might therefore be expected to show a difference between species. For example Chinese Hamster cells, Don cells and V79, are deficient in ASS activity and have been used for metabolic cooperation studies (Hooper and Morgan, 1979; Gonzalez-Noreiga et al., 1980). Previous work shows that amniotic fluid cells have a much wider variation in citrulline incorporation activity then fibroblasts (Jacoby et al., 1981). They established that this was due to a wide variation in ASS activity, while ASL activity is much less variable. They also associated the high ASS activity with the fibroblast - like cell morphology and low activity with epitheloid morphology.

FIGURE 2
UREA CYCLE
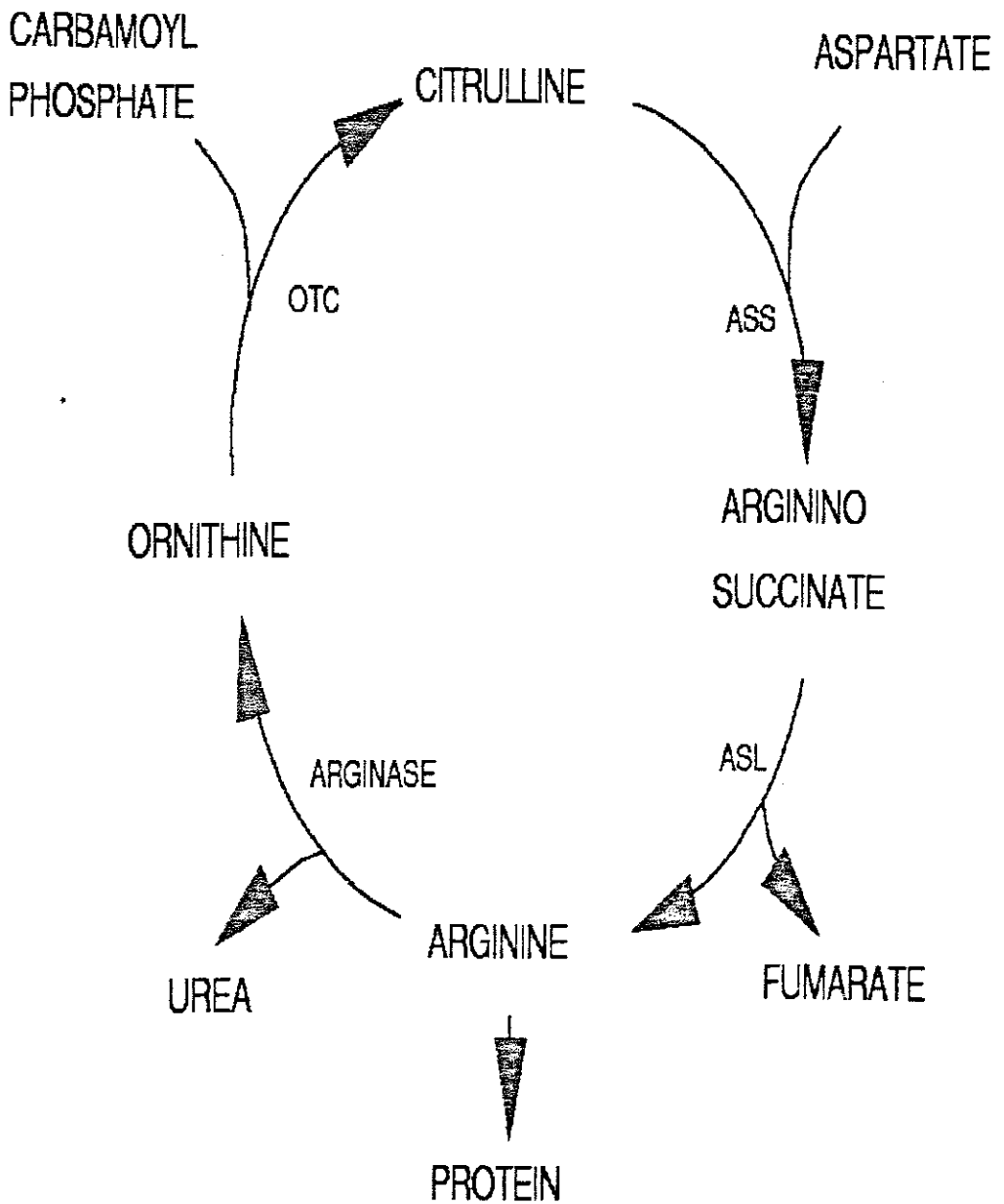
ASS - ARGININOSUCCINATE SYNTHETASE
ASL - ARGININOSUCCINATE LYASE
OTC - ORNITHINE TRANSCARBAMYLASE
Courtesy of Prof. E.H. Harley

2. MATERIALS AND METHODS

2.1 TISSUE CULTURE

2.1.1 PRIMARY CULTURES

The human fibroblasts were established from skin biopsies, 3 x 3 mm in size, taken from the forearm, of normal individuals. The skin biopsies were transported in a container with 3ml Dulbecco's Modified Eagle's medium (DMEM), (Gibco or Whitaker) supplemented with 10% Foetal calf serum, (Delta), this will be referred to as complete medium, to which penicillin (30mg/l), streptomycin (50mg/l), and neomycin (25mg/l) (PSN) was added. The animal fibroblasts were obtained from ear nicks taken from animals immobilized for the purpose of translocation. The dampened ear nicks were transported in plastic bags, shaved on arrival in the laboratory and scrubbed with Biotaine (Biocid limited, Woodstock) before being processed. The buffalo earnicks were received from the Thomas Baines nature reserve and the rhinoceros earnicks from various game parks in Natal including Umfolozi, Hluhlwe and Mkuzi.

The tissue was finely sliced into +/- 1mm x 1mm pieces covered with 0.5ml of DMEM supplemented with 10% foetal calf serum, (Complete medium) and PSN, in a 35mm x 10mm petri dish, (Corningware), the fragments of tissue were then dispensed into several 35mm x 10mm petri dishes and covered with a sterile coverslip. The coverslip was placed on top of the skin fragments to keep them in place, thus enabling the fibroblasts to attach to the surface of the tissue culture

dish. The medium under the coverslip, in contact with the fragments, becomes conditioned and is considered to be essential for fibroblast growth. 2.5ml of complete medium and PSN was added and the dishes were incubated in a Hotpack (model 351920, Hotpack Corp., Philadelphia.) humidified incubator in an atmosphere of 10% CO_2 at 37^OC. The tissue culture medium was changed twice weekly until a reasonable amount of cells had grown out of the explant. The coverslips were inverted, cell side up, and transferred into a new 35mm petri dish'. When both the original dishes and the dishes containing the coverslips were confluent, the cells were trypsinized (0.125% trypsin/0.5mM EDTA, (Adams, 1980) and seeded into a 50ml tissue culture flask (Greiner). The first trypsinization was designated as passage one. Subsequent subcultures were performed in the ratio of one flask into three flasks (1:3) and were recorded as the next passage number. The fibroblasts were frozen at an early passage number in complete medium plus 10% glycerol at 1^OC per minute.

The coding of the fibroblast cultures are given below:

Buffalo	Rhinoceros	Humans
A19 sub-adult	A113	F507 1 year old
A20 sub-adult	A114	F630 6 year old
A21 sub-adult	A118	F617 17 year old
A22 adult	A119	F600 22 year old
A23 adult	A120	F553 27 year old
A24 sub-adult	A122	F555 50 year old
A26 sub-adult	A123	F554 53 year old
A27 sub-adult	A124	F557 72 year old

2.1.2 MAINTENANCE OF FIBROBLASTS

Once the fibroblast cultures were established, antibiotic use was discontinued, since any operator manipulation errors likely to introduce mycoplasma would also introduce bacterial and fungal infections which are more readily observed. Antibiotics would therefore mask the introduction of mycoplasma due to unsatisfactory sterile technique. The fibroblasts were maintained with complete medium which was replaced twice weekly. Subculturing was performed when the fibroblasts were confluent and was done by decanting the growth medium, washing the flask out twice with trypsin/EDTA (Adams, 1980) to remove any FCS as this inhibits the action of trypsin. The cells were incubated at $37^{O}C$, containing 2ml of trypsin, with intermittent shaking until they were detached from the substratum. The cell

suspension was diluted with the required amount of complete medium and divided equally into three flasks.

Before freezing and during experimentation the fibroblasts were regularly screened for mycoplasma.

2.1.3 MYCOPLASMA SCREENING

Bisbenzamide fluorochrome stain, (Hoechst No.33258) was used to screen for mycoplasma using a modification of the method described by Chen, (1977). Fibroblasts were grown in 35mm X 10mm petri dishes on a coverslip in antibiotic free complete medium for four to five days, fixed with methanol: acetic acid (3:1), dried, and stained with the fluorescent stain, (0.5μg/ml Hoechst 33258 in Hanks Barium Salt Solution, (Highveld)) for 5 minutes and examined for mycoplasma using a fluorescent microscope (Nikon). It is essential to keep the cultures mycoplasma-free since these organisms may contribute to the overall metabolic fluxes observed. The fibroblasts used in this study were found to be free of mycoplasma contamination at all times.

2.1.4 SENESCENT (PHASE 111) FIBROBLASTS

Although the ages of the buffalo and rhinoceros were unknown, the human donors were selected to cover ages from 1 year old to 72 years old. The fibroblasts were maintained for four weeks after cell division ceased, to be certain that the cells had indeed senesced. They were examined on a regular basis using phase contrast microscopy, (Nikon), for

evidence of senescence including increased cell debris, enlargement of cells and absence of mitosis. If sufficient fibroblasts were available the hypoxanthine / adenine uptake was measured.

2.2 LABELLING CONDITIONS

The following isotopes were obtained from the Radiochemical Center Amersham, UK:

Isotope	Specific Activity
$(8-^3H)$ Adenine	21Ci/mmol
$(8-^{14}C)$ Hypoxanthine	25mCi/mmol
L-(carbamoyl - ^{14}C) Citrulline	58mCi/mmol
L-(2,3,4,5,-^3H) Arginine monohydrochloride	54Ci/mmol

The isotopes were added to the entire quantity of labelling medium for each experiment and not individually to each well, thereby avoiding pipetting errors.

Radioactivity was measured in a Beckman liquid scintillation spectrometer model LS 3801 using windows set at 0 - 425keV for ^3H and 425-675keV for ^{14}C respectively, with disintegrations per minute (dpm) calculated automatically from quench curves constructed using an external standard.

Double label assays were carried out on a weekly basis using serially passaged fibroblasts. Great care was taken to maintain consistency in the treatment of the cells before and during labelling. The following media were used for labelling :

Dulbecco's modified Eagle's medium with no foetal calf serum; Basal Eagle's medium with no foetal calf serum and Medium Z (NaCl 130mM, KCL 5mM, Hepes 15mM, D-glucose 8.3mM, phenol red 4mg/L, CaCl 1mM and MgCl 1mM pH 7.4 (Davidson *et al.*, 1984)).

2.2.1 PREPARATION OF TCA INSOLUBLE MATERIAL

After the labelling period the cells were washed three times with 5% trichloroacetic acid (TCA) to remove excess label and to precipitate protein while the cells were still attached to the tissue culture plates. After the final TCA wash the plates were drained and the cell residue was dissolved in 500μl 0.1N NaOH. Of this solution 90μl was used for protein determination (section 2.2.2), the remainder, 400ul, was mixed with 6ml of Hionic Fluor, (Packard) for quantitation of ^{3}H and ^{14}C scintillation spectrometry.

2.2.2 DETERMINATION OF CELL PROTEIN

Cell protein was determined using the Bradford method (Bradford, 1976) after washing the cells and precipitating with TCA. The TCA insoluble material was dissolved in 500μl of 0.1N NaOH. 90μl of this solution was added to 2ml distilled H_2O and 0.4ml Bio-rad protein assay dye reagent concentrate, (Bio-rad laboratories, Richmond, California) in a standard 10mm X 10mm path cuvette. The contents of the cuvette were immediately mixed and the absorbance at 595nm was read after 30-60 seconds in a Beckman spectrophotometer,

(Du-62) against a blank containing water instead of cell extract. Bovine serum albumin dilutions at concentrations of between 50 - 200μg/ml were used as standards.

2.3 THE ANALYSIS OF VARIANCE

The results were analysed using a nested analysis of variation implemented using the Statgraphics software package. This quantitates variation at a number of nested levels i.e. within individual experiments, between experiments', between individuals and between species.

For example: Four human fibroblast lines, seventeen experiments with three replicates would be analysed as follows.

Source of variation	df	Mean square	Expected mean square
Individual (A)	a-1	MSa 3301	$\sigma^2_e + n\sigma^2_b + nb\sigma^2_a$
Experiment (B)	a(b-1)	MSb 1559	$\sigma^2_e + n\sigma^2_b$
Error (rep)	ab(n-1)	MSe 35	σ^2_e

a = number of individuals = 4

b = number of experiments = 17

n = number of replicates = 3

MSa = mean square for individuals

MSb = mean square for experiments

MSe = mean square for replicates

Variance components

individual   experiment   rep [E(I)]
I   E

$Yijn = \mu + ai + aij + eijn$

$E(yyn) = \mu$

$E(ai) = 0$ $Var(ai) = \sigma^2_a$ = human variance

$E(aij) = 0$ $Var(aij) = \sigma^2_b$ = experiment variance

$Var(yijn) =$

$E(aijn) = 0$ $Var(aijn) = \sigma^2_e$ = replication variance

To test hypothesis: Ho $\sigma^2_a = 0$

= variation between humans is zero

If Ho is true then $\sigma^2_a = 0$ and $Exp(MSa) = \sigma^2_e + n\sigma^2_b$

This is the same as Exp(MSb)

To test Ho compare

$f = \dfrac{MSa}{MSb}$ or f a-1; a(b-1) = $f_{3,64}$ = 2.76

reject Ho if f > tabulated value

$f = \dfrac{3301}{1559} \sim 2 < 2.76$ Ho cannot be rejected at 5%

To estimate the variance component for humans

$Exp(MSa) - Exp(MSb) = nb\sigma^2_a$

Therefore use $\dfrac{MSa - MSb}{nb}$ to estimate σ^2_a $\dfrac{3301-1559}{3 \times 7} = 34.166$

To test hypothesis: Ho $\sigma^2_b = 0$

variation between experiments is zero

Use $f = \dfrac{MSb}{MSe} \sim f$ a(b-1), ab(n-1) = $f_{64,136}$ = 1.47

$= \dfrac{1559}{35} = 44.54 > 1.47$

Therefore reject hypothesis (Ho) and conclude that there is

a significant variation between experiments.

<u>To estimate the variation component between experiment :</u>

$Exp(\ MSb - MSe) = n\sigma^2_b$

so use $\quad \dfrac{MSb - MSe}{n}$ to estimate $\sigma_{2b} = \dfrac{1559 - 35}{3} = 508$

(Courtesy of Prof.J.M. Juritz, Dept. of Statistics, UCT)

2.4 MATERIALS AND METHODS FOR HYPOXANTHINE AND ADENINE UPTAKE

2.4.1 OPTIMAL CELL DENSITY AND WELL SIZE FOR THE UPTAKE OF LABELLED HYPOXANTHINE AND ADENINE.

To establish the optimal number of cells to be used, the cells were seeded out in varying amounts and into wells of varied capacity. These were six well plates, (Corningware 35mm wells), twelve well plates, (Corningware 25mm wells) and twenty four well plates, (Corningware 15mm wells). The cells were plated out in duplicate in varying amounts to give cell coverage corresponding to semi – confluent, just – confluent and over – confluent in the respective wells in complete medium and incubated overnight at $37^{o}C$ in an atmosphere of 10% CO_2. The fibroblasts were incubated overnight since this allows the cells ample time to attach to the bottom surface of the plates.

NUMBER OF CELLS SEEDED PER WELL

SIX WELL	TWELVE WELL	TWENTY FOUR WELL	
20×10^4	10×10^4	5×10^4	semi-confluent
40×10^4	20×10^4	10×10^4	just- confluent
60×10^4	40×10^4	20×10^4	over- confluent

After 24 hours DMEM with no foetal calf serum was prelabelled with ^{14}C hypoxanthine ($0.2\mu Ci/ml$) and ^3H adenine ($0.5\mu Ci/ml$) and added in the following amounts to the respective wells.

Six well plates	: 1.0 ml
Twelve well plates	: 0.75 ml
Twenty four well plates	: 0.5 ml

The plates were incubated at $37^{O}C$ at an atmosphere of 10% CO_2. After four hours the experiment was terminated with TCA as described in section 2.2.1.

Using the following amounts of 5% TCA for the different sized wells:

Six well plates   : 1.0ml

Twelve well plates   : 0.75ml

Twenty four well plates : 0.5ml

The cell precipitates were dissolved in following amounts of '0.1M NaOH and counted in 10mls Hionic Fluor, (Packard):

Six well dishes   — 1ml NaOH

Twelve well dishes   — 0.75ml NaOH

Twenty four well dishes — 0.5ml NaOH.

2.4.2 ESTABLISHMENT OF INCUBATION TIME AND MEDIUM TYPE TO BE USED FOR THE UPTAKE OF HYPOXANTHINE AND ADENINE

To establish the incubation time needed for the labelling of the cells and which medium for use to create optimal physiological conditions in which to perform the experiment, two media, Dulbecco's modified Eagle's medium (DMEM) and Eagle's basal medium (BME), with varying amounts of foetal calf serum, (0%, 1% and 5%) were compared by means of a time course.

For the time course using labelled DMEM the fibroblasts were trypsinized, seeded at 10 X 10^4 per well in complete medium and incubated overnight at 37^OC in an atmosphere of 10% CO_2. For the time course using BME, the fibroblasts were suspended in BME supplemented with 5% FCS, seeded at 10 X 10^4 cells per well and incubated overnight at 37^OC in an atmosphere of 5% CO_2. The media containing the varying amounts of FCS serum were prelabelled with ^{14}C hypoxanthine at $0.2\mu Ci/ml$ and ^3H adenine at $0.5\mu Ci/ml$. After 24 hours medium was replaced with the pre-labelled media and incubated in an atmosphere of 10% or 5% CO_2, respectively at 37^OC for 1/2 hour, 1 hour, 2 hours, 4 hours and 6 hours. The experiment was terminated as described in section 2.2.1; Pg 20.

2.4.3   EFFECT OF PASSAGE NUMBER ON THE UPTAKE OF LABELLED HYPOXANTHINE AND ADENINE

The effect of passage number was observed in four human and in eight rhinoceros skin fibroblast cultures. The fibroblasts were maintained for at least four weeks after signs of senescence became obvious, to ensure that there was no further significant growth. If there were sufficient fibroblasts available the hypoxanthine and adenine uptake experiments were performed.

2.4.4   EFFECT OF INCUBATION TIME PRIOR TO DUAL LABELLING WITH HYPOXANTHINE AND ADENINE.

Experiments were performed to determine whether performing the experiment at different times of the subculture would have an effect on the hypoxanthine and adenine uptake. These experiments measured the uptake of hypoxanthine and adenine starting at two hourly intervals after the initial overnight incubation. Three individual buffalo fibroblast lines were used at the same passage number and were plated out at the same time in complete medium. The uptake experiments were started at 21 hours, 23 hours, 25 hours and 27 hours respectively and terminated after the standard 4 hours of incubation in co-labelled medium. The medium was prelabelled for all four experiments and not individually for each experiment. The protein content of each well was also measured at the time of harvest.

Experiments were performed on four consecutive days in order to determine whether there was change in the uptake over longer periods of incubation time and the pre-treatment of the fibroblasts (see diagram below).

DIAGRAM OF EXPERIMENTAL PROTOCOL

MEDIUM
CHANGE
4 FLASKS

MEDIUM
CHANGE
3 FLASKS   EXP. 1   EXP. 2   EXP. 3   EXP. 4

SEED 1   SEED 2   SEED 3   SEED 4

FRI.   SAT.   SUN.   MON.   TUES.   WED.   THURS.   FRI.

TIME (DAYS)
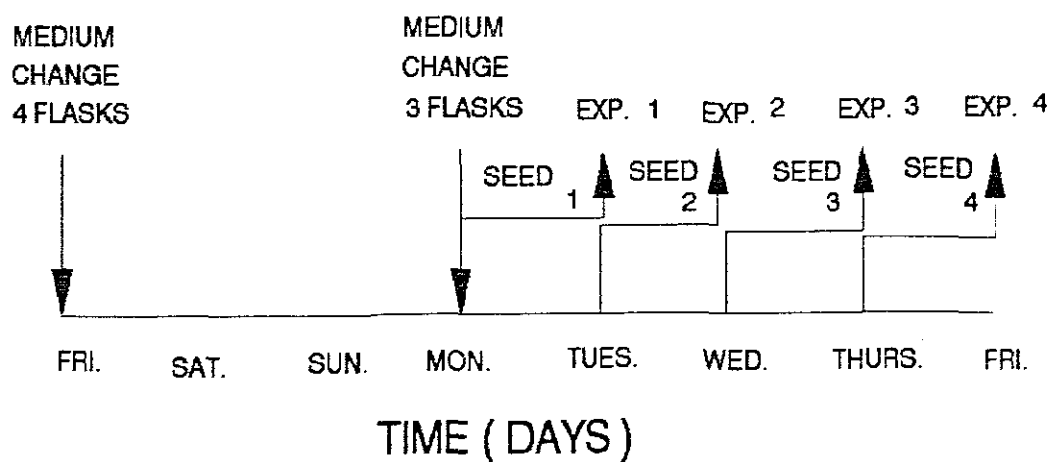
For example : Fibroblasts seeded into plates on Monday would have been without a routine medium change for two days, whereas the fibroblasts seeded on Tuesday would have had a routine medium change on Monday. On Wednesday, the fibroblasts would have had one day on the same medium and fibroblasts seeded on Thursday would have been without a medium change for two days. The same three individual buffalo, as above, were used, all three were at the same passage number but in four different flasks for the four days. The cells were plated out at the same time on a daily basis and the experiments were started 24 hours after incubation. The medium was pre-labelled for the entire experiment which was performed on four consecutive days.

2.4.5 HYPOXANTHINE PHOSPHORIBOSYLTRANSFERASE ENZYME ACTIVITY IN TRANSFORMED AND UNTRANSFORMED FIBROBLASTS

2.4.5.1 PREPARATION OF CELL LYSATE.

Two 250ml flasks, (Nunc) of confluent fibroblasts were trypsinized and washed twice in 0.9% NaCl. The cell pellet was suspended in 100ul of 50mM Tris-HCL, pH 7.8 and 150mM NaCl, freeze thawed six times in an acetone dry ice bath, centrifuged for 30 minutes at 10,000 rpms, (Sigma 2mk centrifuge) (Galloon and Harley, 1988) and passed through a modified Penefsky column (Grubmeyer and Penefsky, 1981) packed with 0.5ml of Sephadex G50 coarse beads in a 1ml

syringe. The cell lysate was loaded and the column centrifuged at 1000rpm for one minute. Protein determinations were performed and the cell lysate diluted on the basis of this to give +/- $100\mu g$ protein per $20\mu l$.

2.4.5.2 ENZYME ASSAY

The assay measures the amount of labelled IMP formed from ^{14}C hypoxanthine and PPRP as described by Wohlheuter, (1975) and Steyn and Harley, (1984). All the assays were performed at 37^0C and each assay had a final volume of $100\mu l$ containing 50mM Tris-HCL, pH 7.8, 14mM MgCl, 4mM PPRP. ^{14}C Hypoxanthine and dTTP were used at the appropriate concentrations. Assay tubes were brought to 37^0C for one minute before the reaction was started by adding $20\mu l$ of the cell lysate. Four aliquots of $20\mu l$, taken at four minute intervals, were applied to DE-81 anion exchange paper pre-ruled in squares of 25mm x 25mm and pretreated in 50mM EDTA, pH 8.0. Eight aliquots were applied to one strip of anion exchange paper. This was dried and washed in a five liter beaker with running water for one hour. The anion exchange paper was dipped into 99.9% ethanol to strengthen the paper. Each square was cut out and put into 10ml of Hionic fluor, (Packard) and left for 24 hours before measuring the radioactivity in a Beckman scintillation counter. The data was analysed by linear regression, using the method of least squares (Bevington, 1969) and the reaction was regarded as linear when a correlation of greater then 0.99 was obtained.

All assays were performed in duplicate and one unit of enzyme activity was defined as pmol/min/µg protein.

2.4.6   ESTABLISHMENT OF IMMORTALIZED (TRANSFORMED) FIBROBLASTS

Four of the rhinoceros and the four of human fibroblast cultures were immortalized by infection with recombinant adenovirus SV40 virions carrying a defective origin for replication, (This was a gift from Dr.Y.Gluzman, Cold Spring Harbor). The fibroblasts were exposed to the virus, at a concentration of 1µl in 10ml of complete medium for 2-3 days, after which the virus-containing medium was discarded and the fibroblasts maintained in the usual manner. The cultures were examined on a regular basis using phase contrast microscopy for changes in morphology and in growth patterns. Fibroblasts previously immortalized in this laboratory were shown to exhibit the typical changes associated with immortalization (Rosen et al., 1988). For example growth of colonies in soft agar can be observed after ten days, staining of monolayers using haematoxylin and eosin or methylene blue to examine the morphology of the cells. Untransformed fibroblasts are unable to grow in soft agar since they are anchorage dependent (Hayflick, 1991). The transformed fibroblasts also had a decreased requirement for exogenous serum derived growth factors and failed to show density dependent inhibition of growth. Virus transformed fibroblasts multiply much faster, and were

therefore subcultured at a ratio of 1 flask into 6 flasks weekly.

The human transformed fibroblasts were plated at the same concentrations as the human untransformed fibroblasts and were assayed from the first passage after infection with the transforming virus to monitor the changes in the uptake of hypoxanthine and adenine as the transformed fibroblasts took over the culture (Fig 10, pg.61).

Hypoxanthine and adenine uptake reached maximum levels in all four human transformed fibroblasts after passage 5, and measurements were made between passage 5 to 8. The fibroblasts showed increased numbers of multinuclear cells, the growth rate had increased and the cells were growing in colonies on top of each other instead of, as in the case of untransformed fibroblasts, becoming quiescent once reaching confluence. The rhinoceros fibroblasts were previously transformed and had been frozen at the 5th passage. These were retrieved from the liquid nitrogen and were also examined from the 5th passage to the 8th passage.

Designations of transformed fibroblasts are :

Rhinoceros			Humans		
BR33	transformed	TBR33	F507	transformed	TF507
BR34	"	TBR34	F553	"	TF553
BR35	"	TBR35	F557	"	TF557
BR36	"	TBR36			TF200

2.5 DEVELOPMENT OF A STANDARD METHOD FOR THE UPTAKE OF CITRULLINE AND ARGININE

2.5.1 OPTIMAL CELL DENSITY AND WELL SIZE FOR THE UPTAKE OF LABELLED CITRULLINE AND ARGININE

A similar procedure to that used for purine uptake was performed to optimize the amino acid uptake. To establish the optimal number of fibroblasts to be used the cells were seeded in varying amounts and into wells of varying capacity: 24 well (15mm), 12 well (25mm) and 6 well (35mm) plates, (Corningware).

For the purpose of the comparison of the three different sized wells, fibroblasts were suspended in BME and 5% FCS, counted, (Coulter counter), and seeded into the various sized tissue culture dishes in numbers that give rise to semi-confluent, just - confluent and over - confluent coverage. The fibroblasts were incubated overnight at 37^OC in a CO_2 atmosphere of 5%.

Number of cells seeded per well.

	Semi-confluent	confluent	over-confluent
6 well	20×10^4	40×10^4	60×10^4
12 well	10×10^4	20×10^4	40×10^4
24 well	5×10^4	10×10^4	20×10^4

^{14}C citrulline and ^{3}H arginine were added to BME with no FCS to give specific activities of 8μCi/ml and 1μCi/ml respectively. The labelled medium was added to the respective wells in the following amounts.

Six well dishes   - 1ml

Twelve well dishes   - 0.75ml

Twenty four well dishes - 0.5ml

The dishes were incubated at 37°C in a 5% CO_2 atmosphere for four hours. The experiment was terminated with TCA as described in section 2.2.1; Pg 20 using the following amounts:

Six well dishes   - 1ml

Twelve well dishes   - 0.75ml

Twenty four well dishes - 0.5ml

The following amounts of 0.1M NaOH were added to each well to dissolve the cell precipitate:

Six well dishes   - 1ml

Twelve well dishes   - 0.75ml

Twenty four well dishes - 0.5ml

The NaOH mixture was counted in 10ml of Hionic Fluor, (Packard). Eagle's Basal medium (BME) supplemented with 5% FCS was used since it contains the least amount of arginine needed to maintain cells in a quasi-physiological condition (Davidson *et al.*, 1984).

2.5.2   ESTABLISHMENT OF INCUBATION TIME AND MEDIUM TYPE TO BE USED ON THE UPTAKE OF LABELLED CITRULLINE AND ARGININE

To establish a suitable medium in which to do the uptake experiment a time course was performed comparing BME and Medium Z (Davidson *et al.*, 1984), a phosphate buffer solution with glucose, using varying amounts of FCS, 0%, 1%

and 5%. The cells were suspended in BME containing 5% foetal calf serum, plated into the 24 well plates and incubated in a 5% CO_2 atmosphere at 37°C overnight. The BME and Medium Z with varying amounts of FCS was prelabelled with ^{14}C citrulline (8μCi/ml) and 3H arginine (1μCi/ml). The fibroblasts were incubated at 37°C in an atmosphere of 5% CO_2 for the BME and without CO_2 for Medium Z for periods of 1/2 hour, 2 hours, 4 hours and 6 hours respectively.

Radioactivity in cell protein was precipitated and measured as in the purine uptake experiments. Fewer individuals and fewer experiments were performed due to the high cost of the labelled citrulline.

2.5.3   EFFECT OF INCUBATION TIME PRIOR TO DOUBLE LABEL OF CITRULLINE AND ARGININE.

To examine the effects of varying the time interval between seeding the cells and starting the experiments, the fibroblasts of three buffalo individuals, of the same passage number were seeded in triplicate into four separate sets of tissue culture dishes and incubated for 21 hours, 23 hours, 25 hours and 27 hours respectively before starting the experiments. The medium was prelabelled for the entire experiment and the fibroblasts were labelled for the standard four hour period. A sample of the dissolved cell precipitate from each well was measured for protein content.

Experiments were then performed on four consecutive days in order to determine whether there was a change in the

uptake due to longer periods of incubation time and whether
the pre-treatment of the fibroblasts had any effect.

DIAGRAM OF EXPERIMENTAL PROTOCOL

MEDIUM
CHANGE
4 FLASKS

MEDIUM
CHANGE
3 FLASKS   EXP. 1   EXP. 2   EXP. 3   EXP. 4

SEED   SEED   SEED   SEED
1   2   3   4

FRI.   SAT.   SUN.   MON.   TUES.   WED.   THURS.   FRI.

TIME (DAYS)
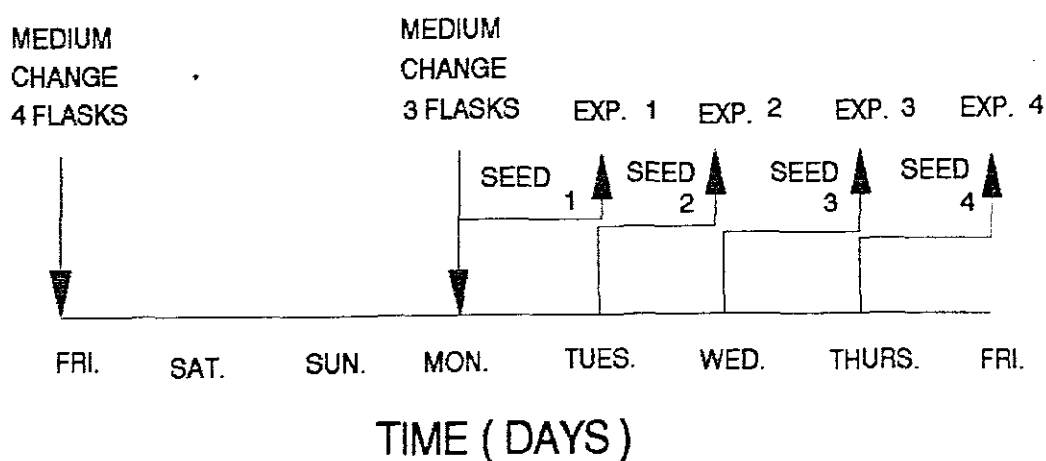
For example: Four different confluent flasks for each
individual were used and these flasks were routinely

maintained for the four day duration of the experiment. Fibroblasts seeded into plates on Monday would have been without a routine medium change for two days, whereas the fibroblasts seeded on Tuesday would have had a medium change on Monday. Fibroblasts seeded on Wednesday would have been without a medium change for one day and those seeded on Thursday were without a medium change for two days.

The same individuals were used as in the previous experiment, and the three individuals were at the same passage number. The fibroblasts were counted, seeded into the tissue culture dishes on consecutive days and incubated for twenty four hours before starting the experiments. The same batch of labelled medium was used for the entire experiment, which was performed on four consecutive days in triplicated wells.

3.0 RESULTS

3.1 EFFECT OF CELL DENSITY ON THE UPTAKE OF LABELLED HYPOXANTHINE AND ADENINE.

In order to examine the effect of the degree of confluence of a fibroblast culture on the uptake of hypoxanthine and adenine varying number of cells were seeded into wells of varying capacity. The results (Fig 3) show that more dispersed cells incorporated more of both substrates then the crowded monolayer. Adenine uptake was more sensitive to cell number then hypoxanthine, this being illustrated in the figure showing the ratio of isotope uptake plotted against cell number (Fig 3 C). Figure 3 D and E shows the uptake of hypoxanthine and adenine decreasing sharply with confluence with the results expressed as dpm/cell, the size of the well does not effect the uptake of either labelled substrate (Fig 3 F and G).

For subsequent experiments twenty four well plates seeded with 10×10^4 cells per well were chosen for the following reasons :

1. eight individuals could be assayed in triplicate in one plate thus making the comparison more accurate, and

2. less isotope was used.

FIGURE 3
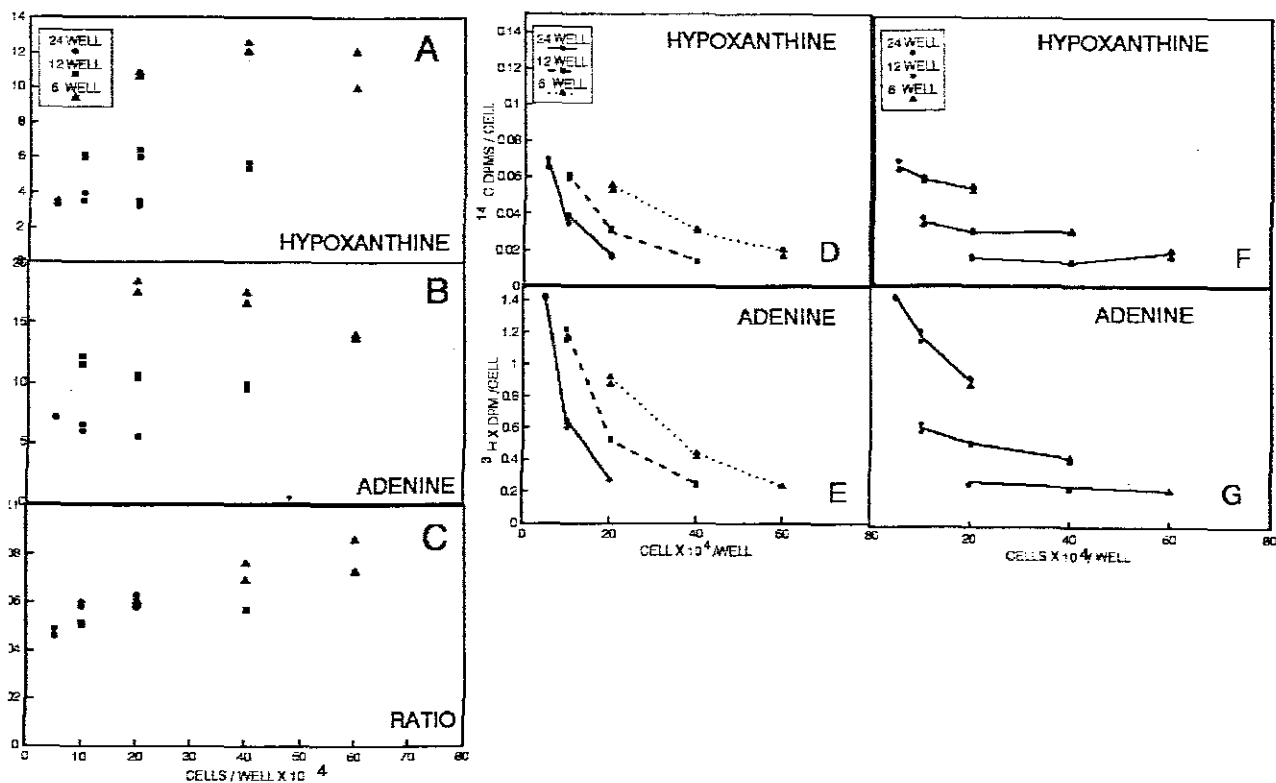
Figure 3 A,B and C shows the effect of well size and cell number on hypoxanthine and adenine uptake expressed as dpm / cells x 10^4 The fibroblasts were seeded in varying concentrations and in different sized wells.

Figure 3 D and E shows the effect of cell density on the uptake of hypoxanthine and adenine in the same sized well expressed as dpm/cell

Figure 3 F and G shows the degree of cell confluence in three different sized wells on the uptake of hypoxanthine and adenine, expressed as dpm/cell.

3.1.2   EFFECT OF INCUBATION TIME AND MEDIUM TYPE ON THE

UPTAKE OF LABELLED HYPOXANTHINE AND ADENINE.

The time course experiment, using buffalo fibroblasts, showed, as was observed by Paz *et al.*, (1979), that uptake, especially of hypoxanthine, is not strictly linear with time. Hypoxanthine showed a lag period of about half an hour before the isotope was incorporated at a linear rate into TCA perceptible material (Fig 4). Foetal calf serum at proportions greater then 1% resulted in a decrease in the ratio of hypoxanthine to adenine incorporated, although it was not clear whether this was previously due to a decrease in hypoxanthine uptake and increasing adenine uptake, or a combination of the two.

FIGURE 4
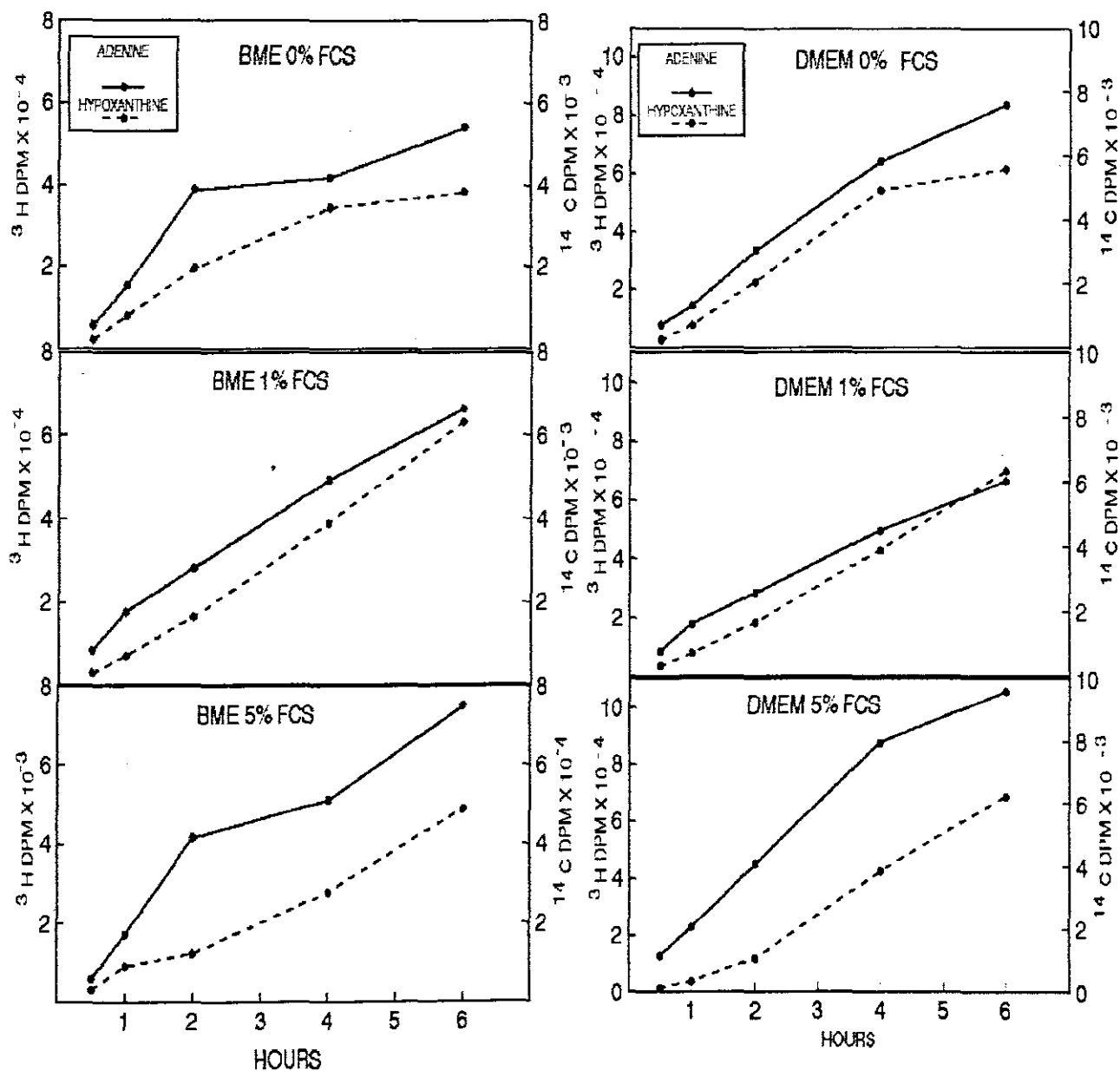
Figure 4 compares the effect of two media, DMEM and BME, with varying amounts of FCS over a period of 6 hours in order to determine the optimal physiological conditions and time needed for the double label experiment.

The lag in the hypoxanthine uptake was present in human fibroblasts as well as in buffalo fibroblasts (Fig.5). Based on the results of the time course it was decided to use DMEM without FCS. DMEM has the additional advantage that it contains no hypoxanthine, and is also the medium on which the fibroblasts are maintained routinely. FCS was omitted as it does contain varying amounts of hypoxanthine which could influence the uptake of labelled hypoxanthine. The incubation time was set at 4 hours since uptake of hypoxanthine and adenine was effectively linear over this time. $0.2\mu Ci$ ^{14}C hypoxanthine and $0.5\mu Ci$ ^{3}H adenine was used for all the purine base uptake experiments.
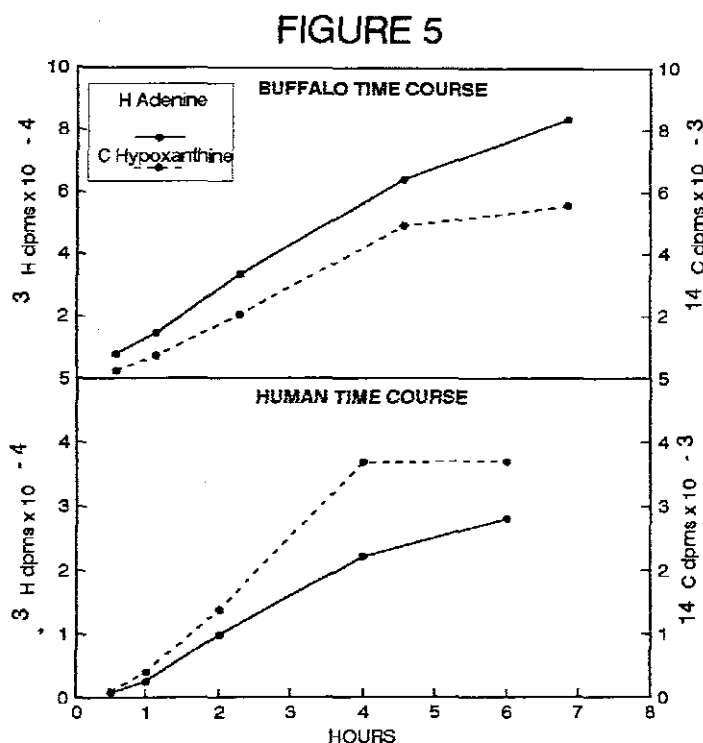
FIGURE 5

Figure 5. A time course performed in DME with no FCS, prelabelled with hypoxanthine and adenine over a 6 hour period using buffalo and human skin fibroblasts.

3.1.3 A MEASUREMENT COMPARISON; LABELLED UPTAKE IN TERMS OF CELL NUMBER, CELL PROTEIN AND $^{14}C/^{3}H$ RATIO.

Measurement of uptake of isotope was measured in one of three ways:

1.) relative to cell number, this being the number of cells originally seeded in the dish the day prior to the experiment,

2.) relative to cell protein, measured in a sample of the cell extract, and

3.) the ratio of uptake of the one label relative to the other.

In all cases measurements were performed in triplicate and with each experiment performed throughout this entire study a 50μl aliquot of the labelled medium was counted. The coefficient variation for ^{14}C hypoxanthine was 2.35% and for ^{3}H adenine 5.5%.

TABLE 1

THE COEFICIENT OF VARIATION IN REPLICATES WITH (%) THE UPTAKE OF HYPOXANTHINE AND ADENINE

	HYPOXANTHINE	ADENINE	HYPOXANTHINE	ADENINE	RATIO
Relative to	cell number	cell number	protein content	protein content	Hx / Ad
HUMAN	11.5	4.6	12.3	7.6	7.6
BUFFALO	13.36	5.9	11.73	7.5	8.79

The results of 36 separate uptake experiments on human and buffalo fibroblast cultures were analysed for within experiment variations by measurement of the coefficient of variation (Table 1). Results were measured over twenty eight passages in four human fibroblast cultures and over ten passages in eight rhinoceros fibroblast cultures. The results showed that the least error between replicates within a experiment is accrued when measurement is made relative to cell number. Correcting for protein or correction by using the ratio of one isotope value divided by the other did not significantly improve the results.

3.1.4   **EFFECT OF PASSAGE NUMBER ON THE UPTAKE OF LABELLED HYPOXANTHINE AND ADENINE**

A slight but significant decrease in adenine uptake was observed in both the human and rhinoceros cultures. This was approximately linear with passage numbers, (Fig 6). A more pronounced decrease to approximately half the initial uptake was seen in the hypoxanthine uptake in the human fibroblasts, which despite the wide scatter of results was significant at the 1% level. The effect was more pronounced for the rhinoceroses. The ratio of hypoxanthine to adenine reflects this difference in the degree of decline between the uptake of the two isotopically labelled substrates, even in the human fibroblast cultures, the slope of the regression is still significantly different from zero, despite the wide scatter.

FIGURE 6
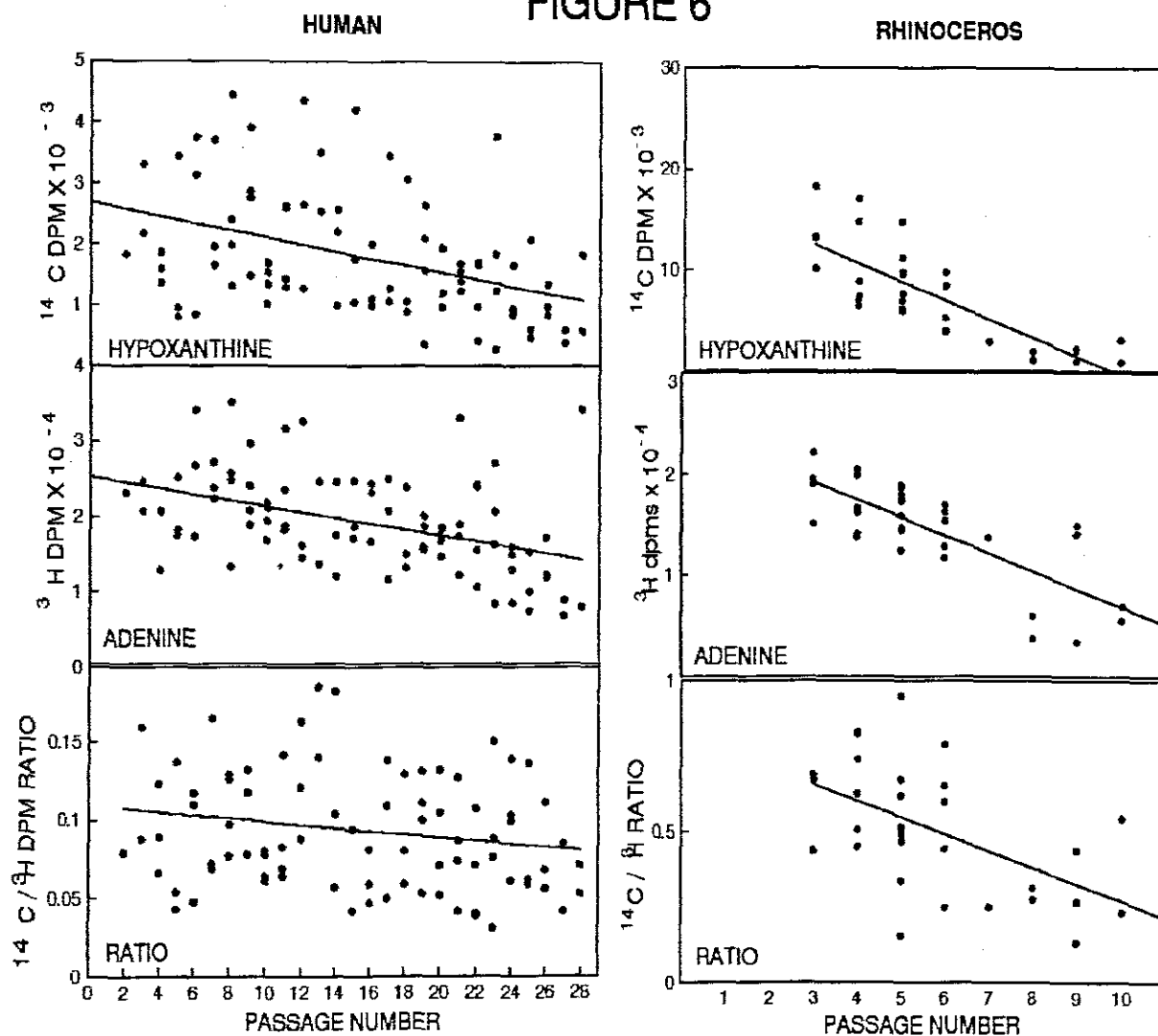
Figure 6 shows the general trend in the uptake of hypoxanthine and adenine with the age of the fibroblasts. Uptake was measured as a function of passage number, combining the results from four individual human fibroblast cultures and eight individual rhinoceros fibroblast cultures.

In Figure 7 the results were plotted for each separate individual, demonstrating considerable variation in the degree of decline in isotope uptake with passage number. These results indicate that a comparison between the uptake of these substrates between species or individuals should ideally be done at similar passage numbers. The age of the individuals at the time of biopsy was known for humans. However, no significant difference in the pattern of uptake relative to the physical age of the individual at the time of biopsy was demonstrable, nor was the decline in hypoxanthine or adenine uptake that was seen with the total results demonstrable separately in all cases.

FIGURE 7
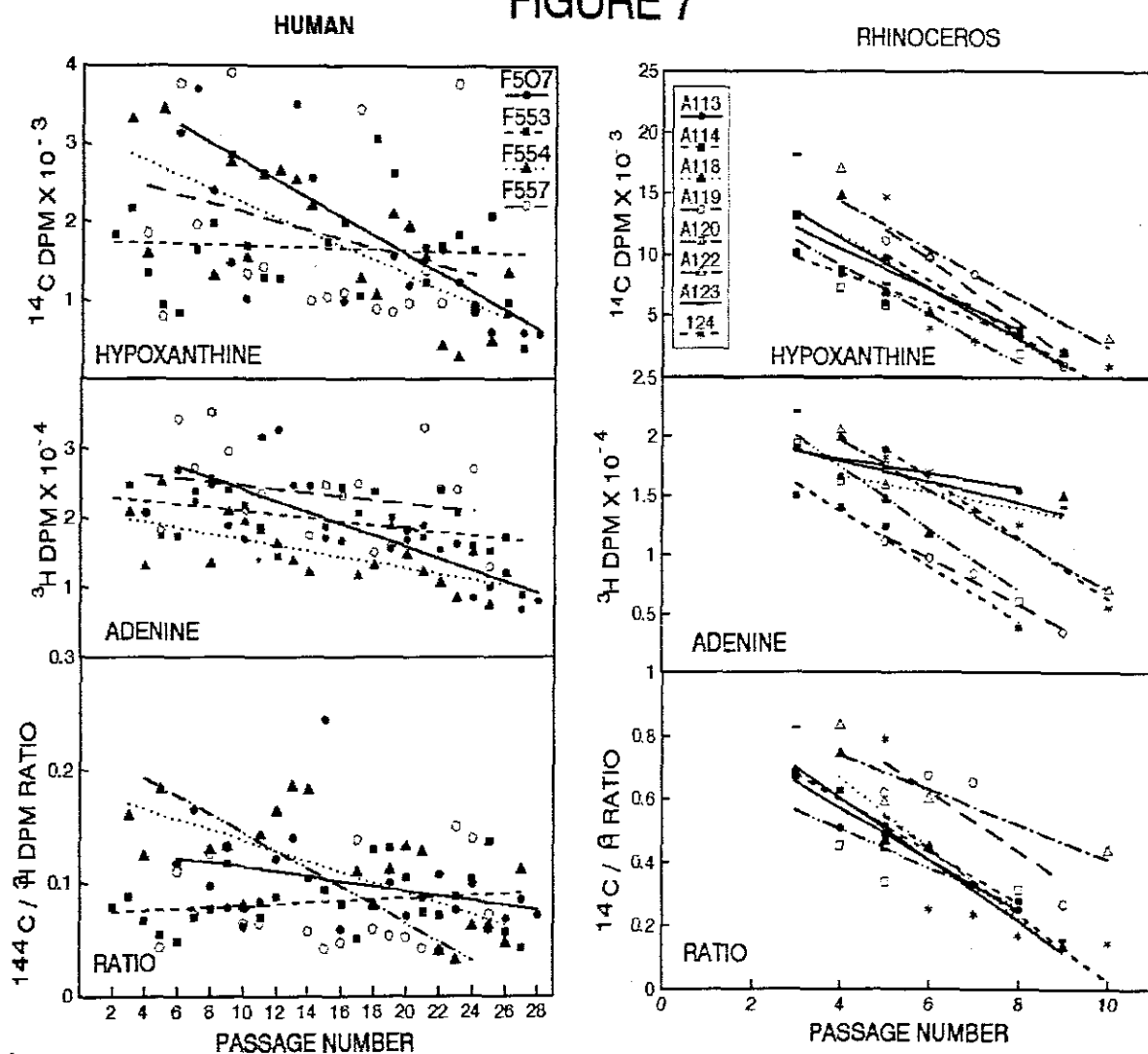
Figure 7 shows the effect on hypoxanthine and adenine uptake with passage number in four donors of different ages at the time of biopsy.

Human lines: F507 - 1 year old, F553 - 27 year old F554 - 50 year old and F557 - 69 year old. The age of the rhinoceroses were unknown.

TABLE 2
ANALYSIS OF VARIATION COMBINING THE RESULTS OF VARYING NUMBER OF PASSAGES

	4 CELL LINES 17 passages	4 CELL LINES 10 passages	4 CELL LINES 4 passages	8 CELL LINES 4 passages
HYPOXANTHINE				
individual	.00	13.76	15.92	49.65***
experiments	93.32***	79.19***	77.10***	43.80***
replications	6.68	7.05	6.98	6.56
ADENINE				
individual	21.10***	18.72*	48.68*	33.20*
experiments	74.58***	76.29***	44.86***	65.56***
replications	4.32	4.99	6.46	4.24
RATIO				
individual	23.55***	40.19***	33.49	61.90***
experiments	50.61***	51.90***	52.62***	29.40***
replications	25.84	7.90	13.89	9.00

*** significant at 0.5%
* significant at 5%

The effect of combining the results at different passage numbers on the amount of between individual fibroblast cultures, and between - experiment, variation, is shown in Table 2.

This shows that when comparing hypoxanthine uptake in four individual fibroblast cultures, the percentage of the variation attributable to between-experiment variation decreases from 93.32 % over the first 17 passages, to 43.80% when the results are combined from just the first four passages. At the same time the between-individual variation is increased to a significant value. Increasing the number of individual fibroblast cultures from four to eight individuals enhanced the between-individual variation. A

similar , but lesser, effect was demonstrable in the adenine uptake results. In the following comparisons individuals of each species at the same passage numbers were used.

3.1.5   WITHIN SPECIES VARIATION USING LABELLED HYPOXANTHINE AND ADENINE

Before comparing between-species variation, it was necessary to analyze in detail the amount of within-species variation found in the double label isotope uptake experiments. ·

Eight individual fibroblast cultures from human, buffalo and rhinoceros were therefore studied in four separate experiments at similar passage numbers, and the results of ^{14}C hypoxanthine and ^{3}H adenine uptake experiments were plotted for each cell line in Fig.8. It is immediately apparent that there is a high degree of scatter in all the results.

FIGURE 8
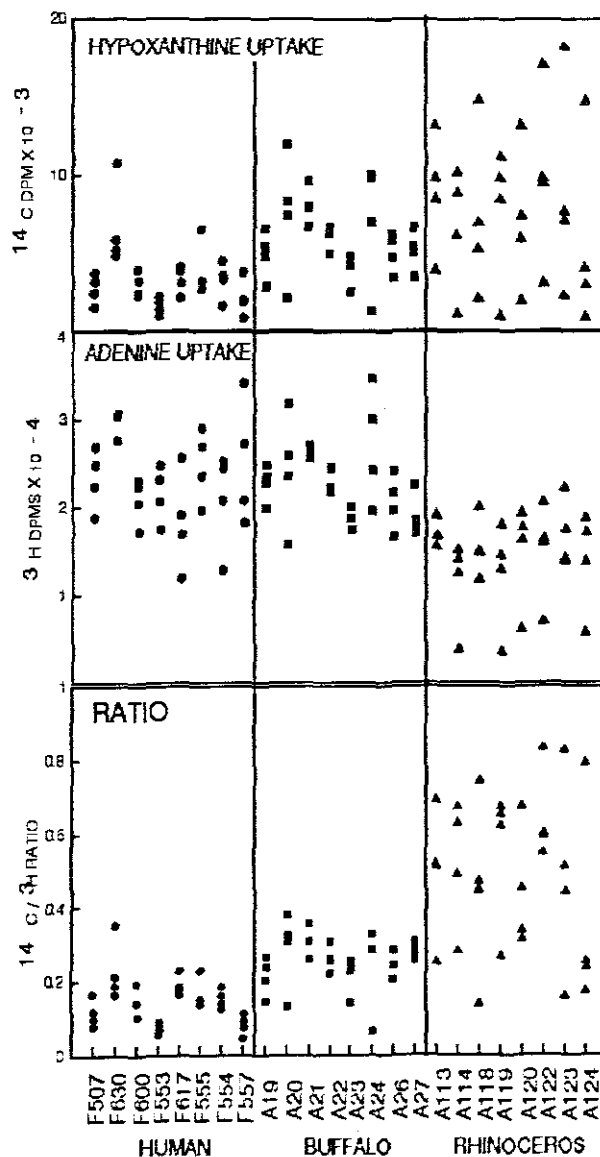
Figure 8 shows the scatter in the uptake of ^{14}C hypoxanthine and ^{3}H adenine for the eight different individuals of each species and for four separate experiments. Points are the mean of triplicate results from each experiment.

TABLE 3
ANALYSIS OF VARIANCE OF HYPOXANTHINE AND
ADENINE UPTAKE WITHIN SPECIES

	number of	HUMAN %variation between	BUFFALO %variation between	RHINOCEROS %variation between
HYPOXANTHINE				
individuals	8	49.65***	10.94	.00
experiments	4	43.80***	80.49***	97.35***
replicates	3	6.56	8.57	2.65
ADENINE				
individuals	8	33.20*	28.93*	4.03
experiments	4	62.56***	60.27***	90.90***
replicates	3	4.24	10.80	5.07
RATIO Hx/Ad				
individuals	8	61.60***	.00	.00
experiments	4	29.40***	87.76***	93.16***
replicates	3	9.00	12.24	6.84

*** significant at 0.5%

* significant at 5%

The analysis of variance is documented in Table 3. Only a small component of the variation was attributable to triplication error. There is little significant variation within individual buffalo or rhinoceros fibroblast lines, but the variation becomes more significant within individual human fibroblast lines. A major component of the variation is between individual experiments, which is significant at the 0.5% level for all three species and for both substrates.

3.1.6   BETWEEN SPECIES VARIATION USING LABELLED

HYPOXANTHINE AND ADENINE

TABLE 4
ANALYSIS OF VARIANCE OF HYPOXANTHINE AND ADENINE UPTAKE BETWEEN SPECIES

		HYPOXANTHINE	ADENINE	RATIO
	Number of	% variation between	% variation between	% variation between
SPECIES	3	31.34***	44.56***	64.42***
INDIVIDUAL	8	.00	12.06*	.00
EXPERIMENT	4	65.71***	39.31***	32.82***
REPLICATES	3	2.95	4.06	2.71

*** significant at 0.5%
*   significant at 5%

When the analyses of variance is extended to include the between-species variation, (Table 4), the between species variation becomes the predominate component in the total variation for adenine, and a major component for hypoxanthine. This, together, with Fig 8 (Pg. 52) shows that the rhinoceros has a significantly greater uptake of hypoxanthine relative to humans or buffalo and a slightly lower uptake of adenine.

TABLE 5
HYPOXANTHINE AND ADENINE UPTAKE BETWEEN SPECIES
ANALYSIS OF VARIANCE USING A PAIRWISE COMPARISON

	number of	HUMAN/BUFFALO % variation between	HUMAN/RHINOCEROS % variation between	BUFFALO/RHINOCEROS % variation between
HYPOXANTHINE				
species	2	38.19***	42.80***	9.44***
individual	8	15.47*	.00	.00
experiment	4	41.86***	55.35***	87.02***
replicates	3	4.48	1.86	3.54
ADENINE				
species	2	.00	54.51***	57.43***
individual	8	15.99	6.78	7.42
experiment	4	62.23***	35.05***	31.71***
replicates	3	21.78	3.65	3.43
RATIO				
species	2	55.99*	72.00***	52.56***
individual	8	10.48*	.00	.00
experiment	4	29.28***	26.03***	43.99***
replicates	3	4.24	1.97	3.46

*** significant at 0.5%
* significant at 5%

Analyses of variance between pairs of species, Table 5, shows that there was a significant variation in the hypoxanthine uptake between each of the pairs. There was no significant difference in the adenine uptake between human and buffalo, although the rhinoceros had significantly lower uptake relative to either human or buffalo. When the ^{14}C hypoxanthine :^{3}H adenine uptake ratios were compared, the buffalo and human were only significantly different at the 5% level, although both showed a highly significant difference with respect to the rhinoceros.

3.1.7　EFFECTS OF CONDITIONS PRIOR TO LABELLING ON THE UPTAKE OF LABELLED HYPOXANTHINE AND ADENINE

Since replicate results were very similar, reflected in their small contribution to the total variance, and since the between-experiment variation constituted the major component of the overall variation, it appeared that variation within a fibroblast culture varied in some unknown way over relatively long time periods. Uptake was therefore measured between experiments conducted at two hourly intervals, or at daily intervals (Fig 9 and Table 6), to see if long time scale effects could be observed.

FIGURE 9
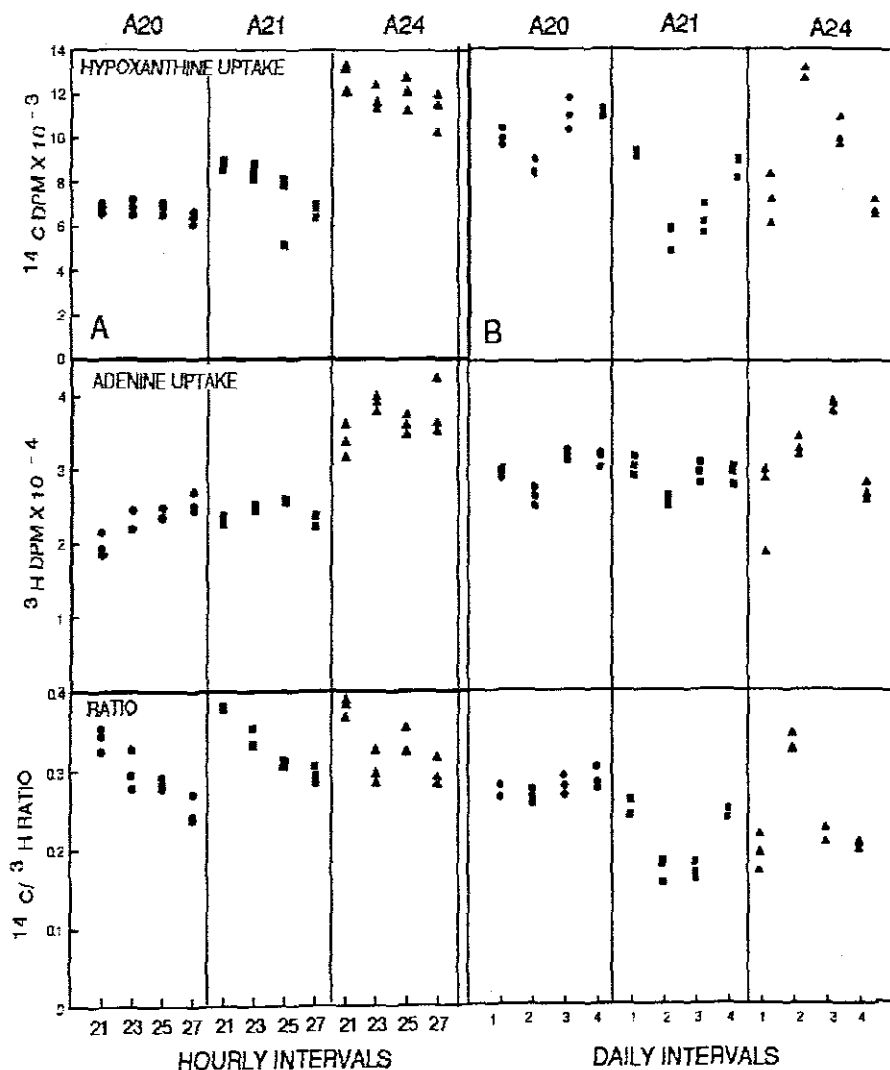
Figure 9 A shows the effect of hypoxanthine and adenine uptake of extending pre-incubation times beyond the standard 24 hours after seeding the fibroblasts into tissue cultures plates.

Figure 9 B shows the effect over a period of 4 days starting each experiment at the same time during each day, using fibroblast cultures from different flasks at varying times since the last medium change. The same three buffalo

fibroblast cultures at the same passage number were used for these experiments and were confluent at the time of seeding.

TABLE 6

	NUMBER OF	HYPOXANTHINE % variation	ADENINE % variation	RATIO % variation
TWO HOURLY INTERVALS				
individuals	3	92.26 ***	90.04 ***	4.06
experiment	4	4.79 ***	5.78 ***	82.03 ***
replicates	3	2.96	4.18	13.91
DAILY INTERVALS				
individuals	3	13.15	.00	24.51
experiment	4	80.13 ***	90.79 ***	70.08 ***
replicates	3	6.73	9.21	5.41

*** significant at 0.5%

In the shorter term study, suggestions of a progressive trend in either increasing or decreasing uptake of substrates was seen in some individuals, accompanied by a significant value for the between-experiment component of the variation. However, the overall value of this component of the variation was, under these conditions, very much less than the between-individual component of the variation which was over 90% for each substrate. On the other hand the between-individual component of the variation was very low,

and not significant, when the ratios of hypoxanthine to adenine uptake were compared. When the experiments were conducted at longer intervals the between-experiment component of the variation now predominated (over 80%) for the hypoxanthine and adenine uptake as well as in the ratio.

This result showed that long term physiological changes take place in cultured fibroblasts, with the interesting result that if a comparison between three fibroblast cultures were to take place on one occasion only, however much replicated, a significant difference in uptake of hypoxanthine or adenine would be the expected outcome. However, when results from many experiments are combined, this apparent between individual variation is seen to be a consequence of marked variation in uptake even in the same cell line, when experiments are performed on different occasions.

3.1.8 EFFECTS OF TRANSFORMATION ON THE UPTAKE OF HYPOXANTHINE AND ADENINE

Fibroblasts exposed to an adenovirus/SV40 recombinant virus showed morphological changes typical of transformed cells (Van Doren and Gluzman, 1984). Most cells were irregular in shape, there was a marked increase in the nuclear cytoplasm ratio and nuclei were hyperchromatic with prominent nucleoli. The transformed cells showed a disorganised growth pattern with cells growing on top of each other in islands unlike untransformed fibroblasts which

grew in ordered parallel monolayers. The uptake in all four lines increased significantly up to the 5th passage and stayed high until about the 8th passage after which the uptake of both substrates decreased, almost reaching the original levels as at the beginning of transformation, as the cells senesced.

Cessation of growth and subsequent death of the transformed fibroblasts occurred with a fibroblast culture established from a 1 year old human individual senescing at 57 doublings and a fibroblast culture established from a 72 year old human individual at 44 doublings. Interestingly, despite the enhanced growth rate and isotope incorporation in the human transformed cell lines, their lifespan from the time of transformation was only moderately greater than that of corresponding untransformed fibroblasts. Each of the four transformed fibroblast lines eventually senesced at about 44-57 population doublings as compared with 40-46 population doublings for the corresponding four untransformed fibroblast cultures.

FIGURE 10
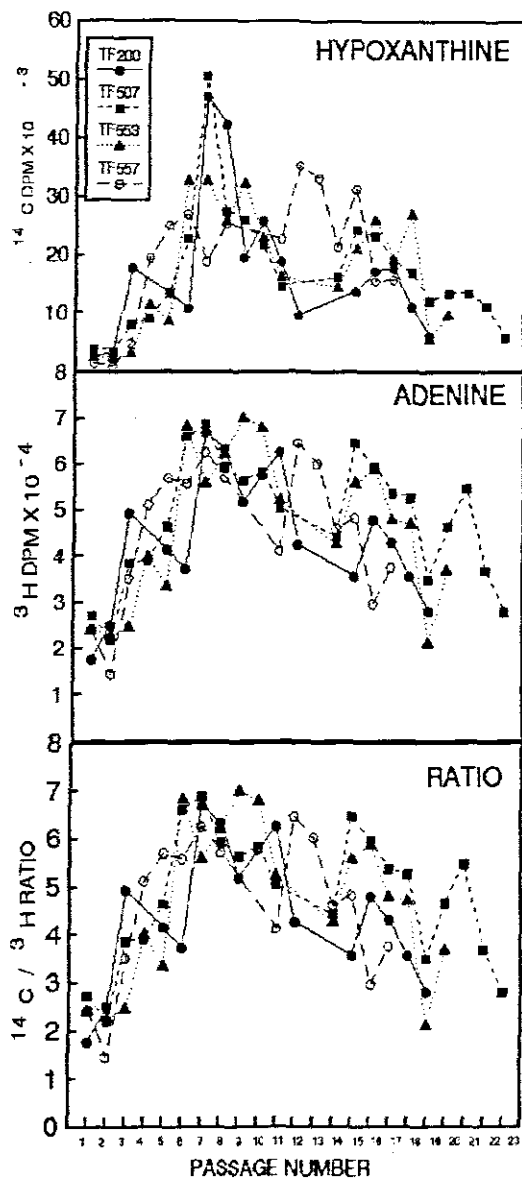
Figure 10 shows the change in hypoxanthine and adenine uptake from the time of infection with the transforming virus to senescence of four human transformed fibroblast cultures.

TABLE 7

ANALYSIS OF VARIANCE OF HYPOXANTHINE AND ADENINE UPTAKE

BETWEEN INDIVIDUAL HUMAN TRANSFORMED FIBROBLASTS

	NUMBER	HYPOXANTHINE % variation between	ADENINE % variation between	RATIO % variation between
INDIVIDUALS	4	.00	22.27	.00
EXPERIMENTS	5	89.32 ***	63.79 ***	90.53 ***
REPLICATES	3	10.68	13.93	9.47

*** significant at 0.5%

Table 7 illustrates the variation within individuals for transformed fibroblasts and shows that there was no significant variation between the transformed fibroblasts, and that the major significant variation was between experiment for both the substrates at 0.5% level.

TABLE 8

ANALYSIS OF VARIANCE OF HYPOXANTHINE AND ADENINE UPTAKE BETWEEN
INDIVIDUAL HUMAN TRANSFORMED AND UNTRANSFORMED FIBROBLASTS

	NUMBER of	HYPOXANTHINE % variation between	ADENINE % variation between	RATIO % variation between
TF/F	4	90.16 ***	94.32 ***	90.61 ***
INDIVIDUALS	4	.00	.99	.00
EXPERIMENTS	5	8.35 ***	3.85 ***	8.57 ***
REPLICATES	3	1.50	.83	.82

*** significant at 0.5%

Table 8 illustrates the variation between untransformed
fibroblasts and transformed fibroblasts showing that over
90% of the variation is attributable to the variation
between transformed and untransformed fibroblasts. The
degree of this difference is such that the between
individual fibroblast culture component of the variation is
no longer a significant contribution to the overall
variations.

FIGURE 11

F - FIBROBLASTS
TF - TRANSFORMED FIBROBLASTS
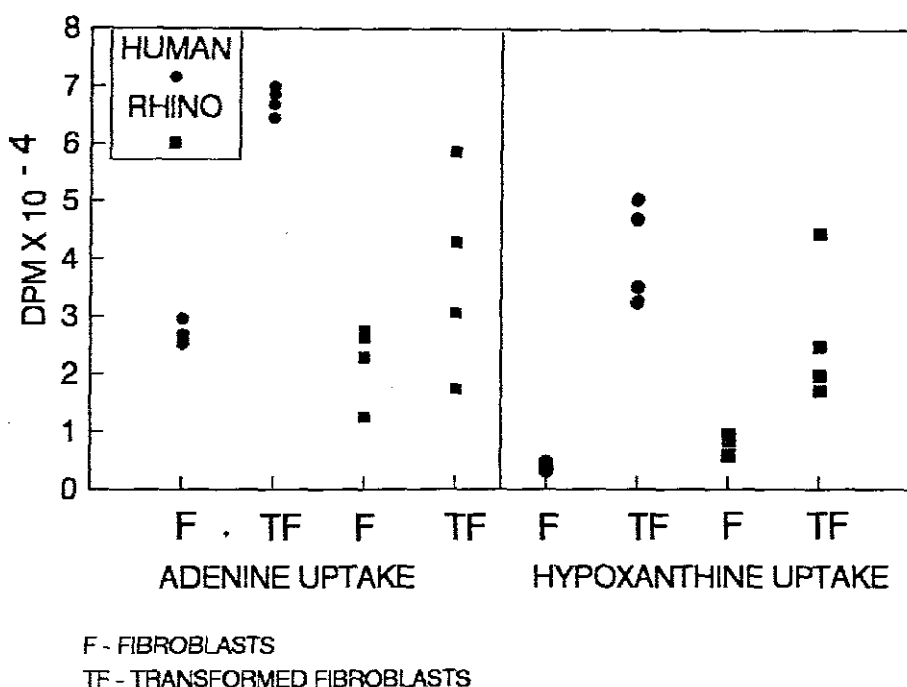
Figure 11 compares the effects of transformation on uptake of hypoxanthine and adenine in human and rhinoceros fibroblasts.

In every case uptake was enhanced by transformation but hypoxanthine uptake was increased proportionally more than adenine, all increases being significant in an analysis of variance at 0.5%. To determine whether the increase in hypoxanthine uptake was a consequence of the increased HPRT activity in the cell, the activity of the enzyme was measured in both transformed and untransformed fibroblasts and compared to hypoxanthine uptake into acid precipitable material.

FIGURE 12
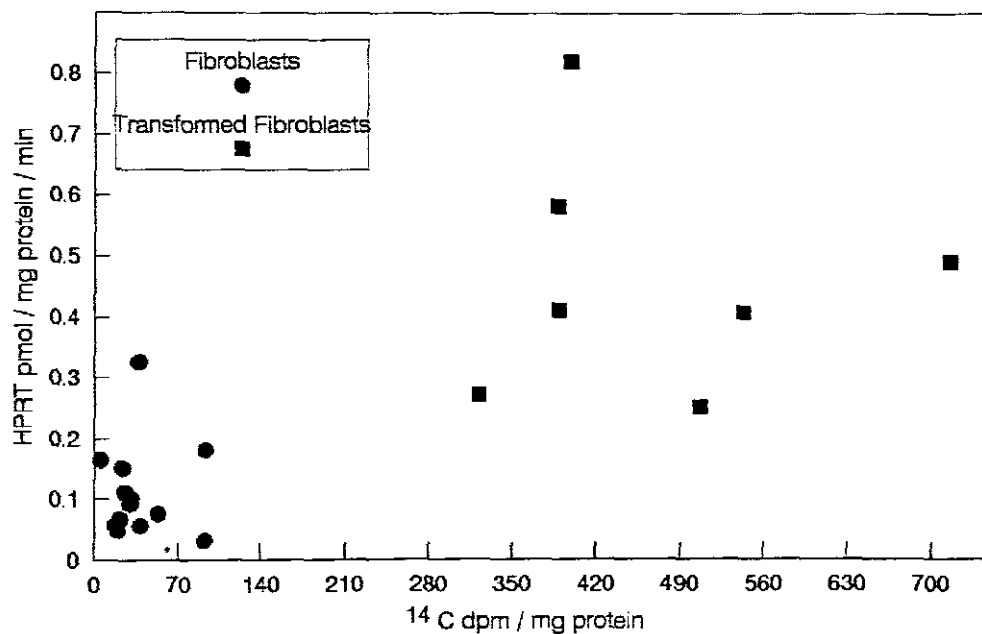
Figure 12 correlates HPRT activity in transformed and untransformed human fibroblasts, with hypoxanthine uptake into acid precipitable material.

The results (Figure 12) showed that although the HPRT activity was clearly increased in transformed cells, the increase could only partly account for the comparable increase in hypoxanthine uptake, and within either the transformed group or the untransformed group, there was no linear correlation between HPRT activity and hypoxanthine uptake. Other factors were therefore contributing to the overall uptake into TCA precipitable material.

TABLE 9

ANALYSIS OF VARIANCE OF HYPOXANTHINE AND ADENINE UPTAKE BETWEEN HUMAN AND RHINOCEROS TRANSFORMED FIBROBLASTS

	NUMBER	HYPOXANTHINE % variation between	ADENINE % variation between	RATIO % variation between
SPECIES TF	2	39.83	71.56 **	44.02 *
INDIVIDUALS	4	18.41	9.07	13.41
EXPERIMENTS	2	36.04 ***	15.29 ***	36.87 ***
REPLICATES	3	5.72	4.08	5.70

*** significant at 0.5%
** significant at 1%
* significant at 5%

Table 9 illustrates the variation between human and rhinoceros transformed fibroblasts.

When comparing human and rhinoceros transformed fibroblasts, in contrast to untransformed fibroblasts, no significant variation in the hypoxanthine uptake was found and the variation in the adenine uptake was only significant at the 1% level. Again, the predominate variation was between experiments documented in Table 9 and Fig 11. The differences in substrate uptake between species therefore became unapparent when the fibroblast cultures were transformed.

3.2   **THE EFFECT OF CELL DENSITY ON THE UPTAKE OF CITRULLINE AND ARGININE.**

In order to examine the effect of the degree of confluence of a fibroblast culture on the uptake of citrulline and arginine a varying number of cells were seeded into wells of varying capacity. For the total dpm counts, both the citrulline and arginine uptake increased in a manner which was less than directly proportional to cell number with arginine being slightly more sensitive to cell number (Fig 13 A, B and C). When the figures were corrected to dpm/cell, Fig 13 D and 13 E, the citrulline decreased slightly with cell density in the same wells whereas the arginine uptake decreased sharply with an increase in cell density. This accounts for the increase in the ratio seen in Fig 13 C.

The effects of the same cell density in different sized wells (Fig 13 F and G) was fairly constant showing that well size had no effect on the uptake. It was therefore decided to use the twenty four well tissue culture dishes at a cell density of 10×10^4 cells/well since:

1) less label and fewer cells were needed, and

1) three individuals from three species performed in triplicate could be examined on one plate.

FIGURE 13
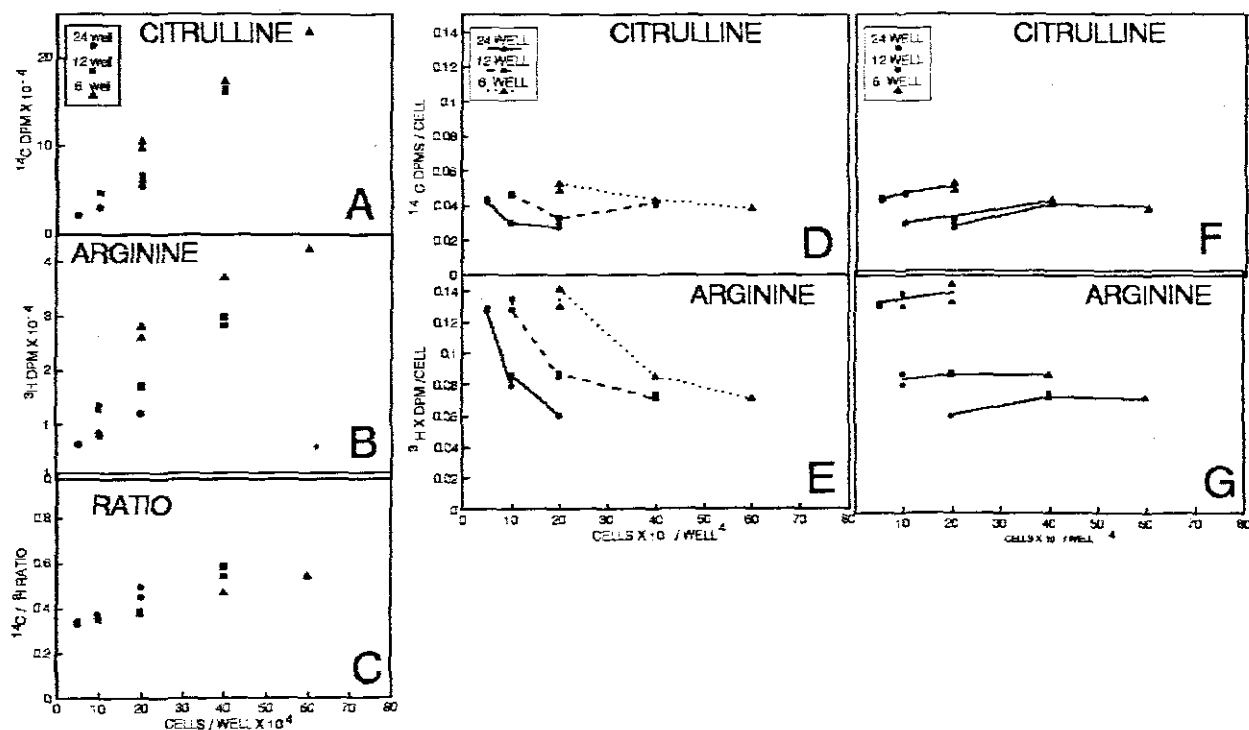
Figure 13 A, B and C shows the effect of well size and cell number on labelled uptake of citrulline and arginine, expressed dpm / cells x 10^4

Figure 13 D and E shows the effect of cell density on the uptake of citrulline and arginine in the same sized well expressed as dpm/cell.

Figure 13 F and G shows the effect of cell confluence in three different sized wells on the uptake of citrulline and arginine expressed as dpm/cell.

3.2.1 EFFECT OF INCUBATION TIME AND MEDIUM TYPE ON THE UPTAKE OF CITRULLINE AND ARGININE.

Incubating in labelled medium for varying times (Fig 14) showed that the uptake of both citrulline and arginine was linear over a period of four hours in BME without FCS. The citrulline uptake showed a short lag period which was more obvious with the BME and 5% FCS. In Medium Z uptake was only linear up to four hours in the presence of 5% FCS.

BME without FCS was selected since it is more physiological than Medium Z and contains only 21mg/l of arginine, which is the minimum required amount for normal cell growth. This keeps the intracelluar arginine pool low and maximises the intracellular specific activity of labelled arginine (Davidson et al., 1985b). Another reason for avoiding additions of FCS was that it contains varying amounts of arginine which could interfere with the results. A four hour labelling period was chosen as standard since the citrulline and arginine uptake was approximately linear over this time period.

FIGURE 14
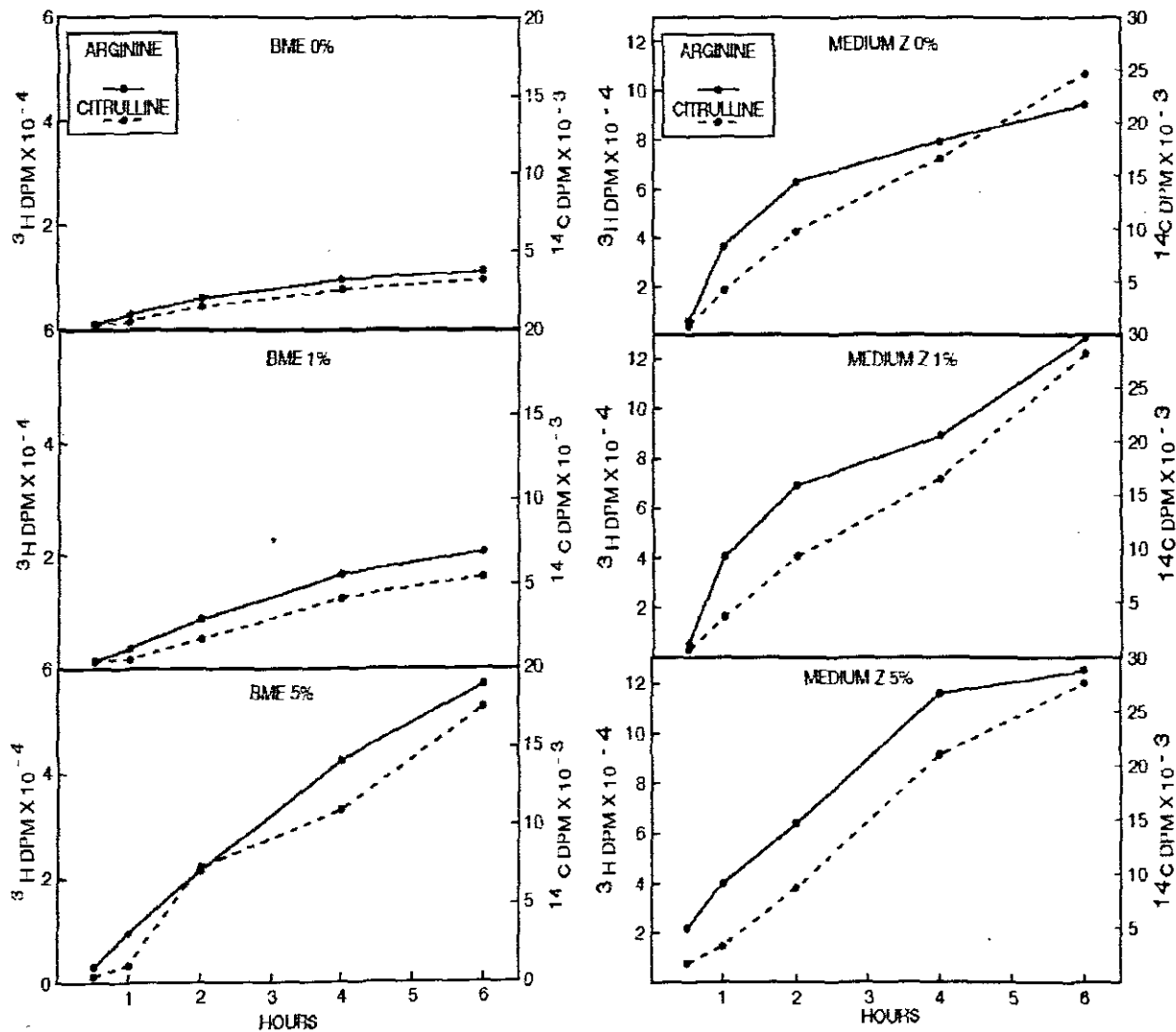
Figure 14 compares the effects of two media BME and Medium Z with varying amounts of FCS over a period of 6 hours in order to establish optimal physiological conditions and time needed in which to perform the citrulline and arginine uptake experiments.

3.2.2   A COMPARISON OF MEASUREMENT; LABELLED IN TERMS OF
          CELL NUMBER, CELL PROTEIN AND $^{14}C/^{3}H$ RATIO.

Isotope uptake was measured in acid precipitable
material, and expressed in one of three ways:

1.   relative to cell number, i.e. the number of cells
       originally seeded into the dish the day prior to
       the experiment,

2.   relative to cell protein, measured in a sample of
       the cell extract, and

3.   as the ratio of uptake of one label relative to
       the other.

In all cases the measurements were performed in
triplicate.

TABLE 10
COEFICIENT OF VARIANCE IN REPLICATES WITH THE UPTAKE OF CITRULLINE AND ARGININE   (%)

	CITRULLINE	ARGININE	CITRULLINE	ARGININE	RATIO
	cell number	cell number	protein content	protein content	cit/arg
HUMAN	6.5	6.9	14.3	14.9	8.9
BUFFALO	5.5	3.4	8.9	9.8	6.9

Table 10. The results of 14 separate uptake experiments on human and buffalo fibroblast cultures were analysed for within experiment variations by measurement of the coefficient of variation.

As in the purine uptake (Table 1, Pg 45) the results, Table 10 shows that the least error was accrued when the measurement was made relative to cell number. Correcting for protein did not improve the results. Correction by using the ratio of one isotope value relative to the other was better than the protein correction, but still not an improvement on the use of cell number. The coefficient of variation for the two species, buffalo and human, were comparable. Values were

accumulated over 14 experiments using three individuals from two species. With each experiment a 50ul aliquot of the labelled medium checked to ensure that the label added in the medium was constant between experiments. The coefficient of variation, for the labelled medium used throughout the entire study, was 1.2% for ^{14}C citrulline and for ^{3}H arginine 9.6%.

Fewer individuals were used in these experiments as compared to the hypoxanthine and adenine uptake experiments due to the expense of the ^{14}C citrulline therefore the effect of ageing of cultures was not examined. Since the age of fibroblast cultures had a significant effect on uptake of purines, in the previous section (3.1.4), fibroblasts in the current set of experiments were used at comparable passage numbers.

3.2.3   **WITHIN SPECIES VARIATION USING LABELLED CITRULLINE**
           AND ARGININE

Before comparing the between species variation, it was necessary to analyse in detail the amount of within-species variation found in uptake of citrulline and arginine. Fibroblast cultures from three different individuals from each of the three species, human, buffalo and rhinoceros, were labelled in three separate experiments, and the results are shown in Fig.15. The analysis of variance is documented in Table 11 and Table 12.

FIGURE 15
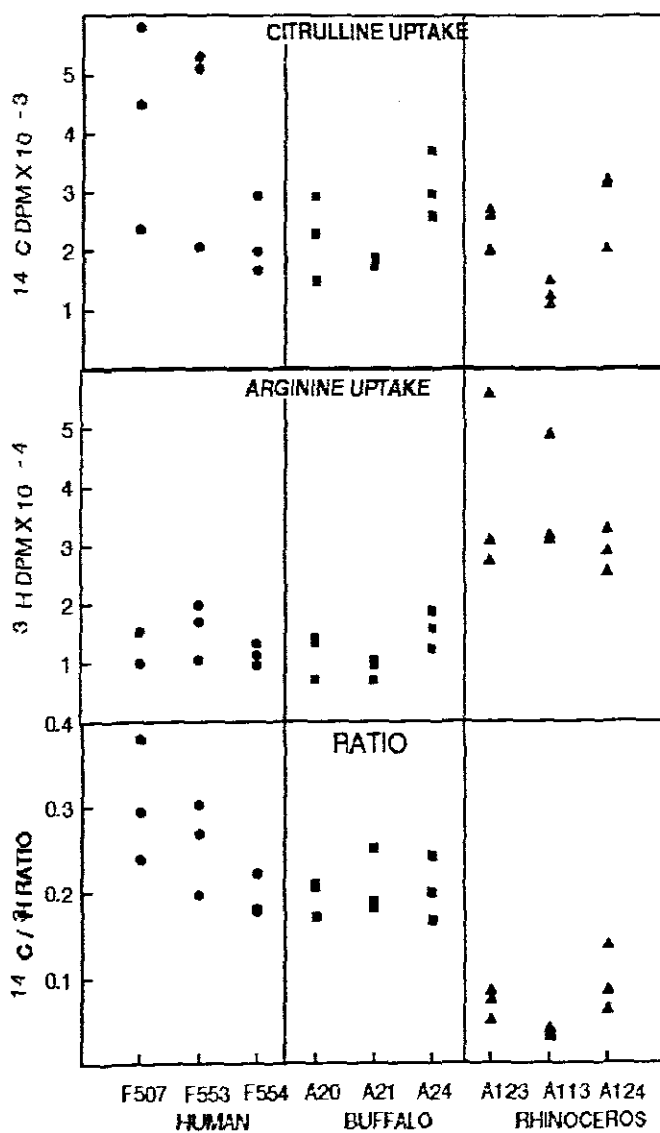
Fig 15 shows the scatter in the uptake between three different individuals of each species for three separate experiments performed on different days. Points are the mean of triplicate results.

TABLE 11
ANALYSIS OF VARIANCE OF CITRULLINE AND ARGININE
UPTAKE WITHIN SPECIES

		HUMAN	BUFFALO	RHINOCEROS
	number of	% variation between	% variation between	% variation between
CITRULLINE				
individuals	3	19.98	53.14	64.60*
experiments	3	77.83***	42.74***	34.33***
triplication	3	2.20	4.11	1.06
ARGININE				
individuals	3	5.92	45.83	0.00
experiments	3	88.76***	53.25***	97.55***
triplication	3	5.32	0.92	2.45
RATIO Cit/Arg				
individuals	3	41.25	0.00	53.37
experiments	3	51.12***	84.29***	45.99***
triplication	3	7.63	15.71	0.64

*** significant at 0.5%
* significant at 5%

Table 11 shows the variation between individuals from each of the three species.

This showed that there was no significant variation between the three individual fibroblast lines for either citrulline or arginine uptake, except for the rhinoceros fibroblast cultures, where it was significant only at the 5% level. The most significant component of variation was between individual experiments, which is significant at the 0.5% level for all three species. Only a small component of the variation was attributable to triplication error.

3.2.4 BETWEEN SPECIES VARIATION USING LABELLED CITRULLINE AND ARGININE.

There was no significant variation between the three species in the citrulline uptake. There was, however, a significant variation in the arginine uptake and the ratio, significant at the 0.5% level, which together with Fig 15 showed that the rhinoceros had a significantly greater increase in the uptake of arginine relative to the human and buffalo. This high uptake of arginine relative to citrulline in the rhinoceros, results in much lower ratio values then in the other two species.

TABLE 12
ANALYSIS OF VARIANCE OF CITRULLINE AND ARGININE
UPTAKE BETWEEN SPECIES

	number of	CITRULLINE % variation between	ARGININE % variation between	RATIO CIT/ARG % variation between
SPECIES	3	12.4	76.43***	77.83***
INDIVIDUAL	3	30.65*	0.00	7.44*
EXPERIMENT	3	54.97***	22.94***	12.92***
TRIPLICATION	3	1.93	0.63	1.81

*** signifivant at 0.5%
*   significant at 5%

Table 12 shows the analysis of variance when all three
species are compared.

TABLE 13
CITRULLINE AND ARGININE UPTAKE BETWEEN SPECIES
ANALYSIS OF VARIANCE USING A PAIRWISE COMPARISON

	number of	HUMAN/BUFFALO % variation between	HUMAN/RHINOCEROS % variation between	BUFFALO/RHINOCEROS % variation between
CITRULLINE				
species	2	17.53	19.47	.00
individual	3	21.49	25.81	60.15 **
experiment	3	58.87 ***	53.20 ***	37.71 ***
replicates	3	2.11	1.52	2.14
ARGININE				
species	2	.00	75.92 ***	79.21 ***
individual	3	29.48	.00	.00
experiment	3	67.71 ***	23.31 ***	20.29 ***
replicates	3	2.81	.77	.50
RATIO				
species	2	18.28	83.42 ***	89.11 ***
individual	3	22.42	7.42 *	1.66
experiment	3	43.23 ***	8.30 ***	8.20 ***
replicates	3	16.06	1.04	1.02

*** significant at 0.5%
** significant at 1%

The analysis of variance between pairs of species is shown in Table.13.

There was no significant variation between the buffalo and human in either citrulline or arginine uptake. There was a variation, significant at the 0.5% level, between both the buffalo and the human relative to the rhinoceros in the uptake of arginine and in the ratio. There was no significant between-species variation for the citrulline uptake.

3.2.5   EFFECTS OF CONDITIONS PRIOR TO LABELLING ON THE

LABELLING THE UPTAKE OF CITRULLINE AND ARGININE.

Since the between-experiment variation constituted the major component of the overall variation, uptake was measured at two hourly intervals on the same day or at daily intervals (Fig 16 and Table 14), as in the purine uptake experiment (Fig 9, Pg. 57 and Table 6, Pg.58). Some suggestion of a progressive trend in either increasing or decreasing uptake of substrates was seen over the shorter time period. In the experiments performed at two hourly intervals the between-individual variation was predominant and was accompanied by a significant experimental variation. When the experiments were conducted at longer intervals (Fig 16 B) the between-individual component of the variation was low and only significant in the ratio of citrulline : arginine, whereas the between experiment components of variation again provided the major and most significant component of the variation as in the purine uptake experiments.

FIGURE 16
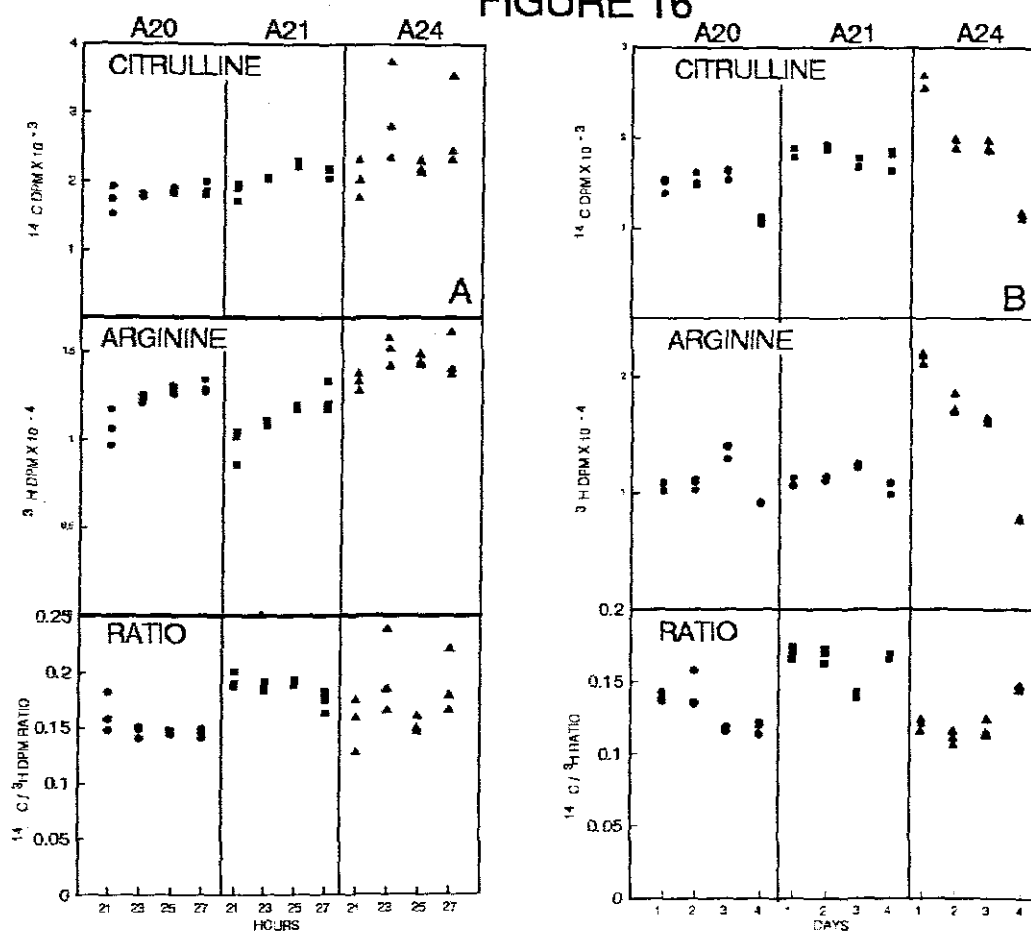
Figure 16 A shows the effect of citrulline and arginine uptake extending pre-incubation times beyond the standard 24 hours. The uptake experiments were started at two hourly intervals after the initial 24 hours.

Figure 16 B shows the long term effect over a period of four days, using four different flasks from each individual, which during the course of the experiment were maintained routinely. The same three buffalo fibroblast cultures were used for both the experiments and were confluent at the time of seeding.

TABLE 14

ANALYSIS OF VARIANCE OF CITRULLINE AND ARGININE UPTAKE USING DIFFERENT PRE-LABELLING INCUBATION INTERVALS

	NUMBER OF	CITRULLINE % variation	ARGININE % variation	% variation
TWO HOURLY INTERVALS				
individuals	3	40.00 *	63.13 **	39.48 *
experiment	4	19.29 *	23.85 ***	18.94 *
replicates	3	40.71	13.02	41.58
DAILY INTERVALS				
individuals	3	16.71	23.43	62.59 ***
experiment	4	80.74 ***	75.42 ***	32.72 ***
replicates	3	2.55	1.16	4.69

*** significant at 0.5%
** significant at 1%
* significant at 5%

Table 14 documents the variation on citrulline and arginine uptake using different pre-labelling incubation intervals.

It was noticed that in this particular set of experiments one individual buffalo fibroblast culture (right side of the panels in Fig 16 A and B) proved more difficult to disperse on trypsinizing, with cells tending to remain in clumps. This could effect the accuracy of seeding equivalent number of cells and could account for the poor triplication and wider between-experiment scatter in the results.

4. DISCUSSION

The analysis of variance enabled the amount of variations attributable to four nested levels of causation to be dissected out, these levels being between replicates, between experiments, between individual fibroblast cultures and between species. The variation within the replicate results of any individual fibroblast culture was only a small component of the total variation for uptake of the purine bases and the amino acids. It was instructive that this was not reduced by correcting for protein content of the cell lysate. The error of the protein measurement would appear to exceed the sum of the pipetting error when cells were seeded into the triplicate wells, plus the error in the steps involved in transferring the acid precipitable material from the wells to the counting phial.

Cell density had a marked effect on uptake of both the purine bases. This was probably a function of the area of the cell exposed to the medium and available for transport, but the greater sensitivity to cell density of adenine uptake relative to hypoxanthine cannot readily be accounted for by this mechanism alone. Uptake of both the amino acids increased equally with increase in cell number, however cell density did not have as great an effect on the uptake of citrulline as on the uptake of arginine, as illustrated by Fig 13 B, Pg.69. The arginine uptake decreased sharply with the increase of cell density. As a consequence of these effects every uptake experiment was performed under

identical conditions of cell density and time after cells were subdivided into triplicate wells. However, in spite of this, the between-experiment component was the major and most significant factor in the variation on within-species uptake for both the purine bases and the amino acids.

Jacoby *et al.*, (1981) described a difference in the activities of the urea cycle enzymes in amniotic fluid cells between cells with a fibroblast-like morphology and a epithelial cell-like morphology. The fibroblasts in the present study all had the same morphology.

The magnitude of this between-experiment variation was such that in the purine uptake, only one of the three species (human), showed a significant between-individual variation at the 1% level. In the amino acid uptake experiments only, the rhinoceros showed a significant between individual variation. This was with citrulline uptake at the 5% level.

The experiments observing how variation in purine and amino acid uptake related to the timing of the experiment (Fig 9 A, Fig 9 B, Pg. 57; Fig 16 A and 16 B, Pg. 81) suggests that unknown physiological or environmental factors can affect uptake of these substrates significantly over time periods in excess of a few hours after the seeding of the cells into the tissue culture plates and before the double label experiments were performed. The fibroblasts would probably have a degree of cell-cycle synchronisation, since the cells were confluent and therefore mostly in the

Go phase of the cell-cycle before seeding into the tissue culture plates. The trypsinizing and seeding of the fibroblasts stimulates mitotic activity which could result in a cell cycle-dependent variation in RNA and DNA synthesis and purine nucleotide metabolism. Nevertheless, variation due to this phenomenon should be minimized by strict adherence to a standard protocol for all experiments. These short term effects are quite different from the very long term effect on the decrease in purine uptake demonstrated in Fig 6, Pg. 47 and Fig 7, Pg. 49 as the cultures aged.

The consequences of these effects are that between individual comparisons of uptake of the purine bases, hypoxanthine and adenine, the amino acids, citrulline and arginine, and perhaps many other substrates, are meaningless unless the differences between two cell lines on any one occasion are greater than an order of magnitude, or the results from many individual experiments are pooled. If such an assay were to be performed in a diagnostic context, for example to test for a partial HPRT deficiency or for a female carrier of a complete HPRT deficiency, there would be a high probability of getting a significantly different result between the control and the test cell lines due to between-experimen variation. Paradoxically this significance is compounded by the low within-experiment (triplication) variation and will be extreme if only one control cell line is used.

The use of ratios of uptake of one substrate relative to another, which should correct effectively for manipulative components of the experimental error, did not significantly improve the results. For example, when using the ratio as a measure to compare uptake of purine between cell lines, there was no improvement in the results. This was due to independent variation in uptake of hypoxanthine and adenine, both in the short and long term, within any one cell line. In the amino acid uptake, the use of a ratio improved the results when compared to protein corrected results, but not when compared to results expressed for cell number .

If substrate uptake by whole cells is to be used in a diagnostic context, it is necessary to use a number of control cell lines of comparable passage number, and to do the experiment on a number of occasions. Since the between individual component of the overall variation was low, relative to the between-experiment variation, it made little difference whether a large number of control cell lines were used on a few occasions, or a small number of control cell lines in a large number of experiments. What was more important was that the test cell line was analysed on several separate occasions, with an absolute minimum of three analyses. The cell lines also had to be free of mycoplasma, which could have had a major effect on the uptake of many substrates, and especially of pyrimidine and purine substrates (Harley E H et al., 1970).

The value of an analysis of variance is that it enables the significance of variation at different levels to be identified. In this present context these levels are those of the replicates, the experiments, the individual fibroblast culture, and the species. The variation at the level of the individual fibroblast cultures were not found to be significant for buffalo or for rhinoceros with the purine uptake. Between individual human cell lines there was a significant variation in the purine uptake (Table 2, Pg. 50) when the total data from all the fibroblast cultures analysed at many different passage numbers was used. However, this was not a consistent feature when subsets for this data were analysed, or when the accumulated data from all three species was analysed (Fig 8, Pg. 52). The only significant variation in the amino acid uptake between individuals was in the rhinoceros, this was in the citrulline uptake and was only significant at the 5% level (Table 11 Pg. 76).

At the between species level there was significant variation for the hypoxanthine and adenine uptake, which on a pair-wise comparison (Fig 8, Pg. 52 and Table 5, Pg.55), was significant between each species pair for each substrate, with the exception of adenine uptake between human and buffalo. The amino acid uptake showed a significant variation in the arginine uptake and in the ratio (Fig 15, Pg. 75 and Table 12, Pg. 78), which on a pairwise comparison (Table 13, Pg.79) was significant for

the human/rhinoceros and buffalo/rhinoceros pairs. There was no variation between the buffalo and human for either of these substrates or between the three species in the uptake of citrulline. Previous studies have shown that chinese hamster cells are deficient in ASS activity (Hooper and Morgan, 1979 and Gonzalez-Noreiga et al., 1980). A pilot study performed in this laboratory (Sadler, 1990) suggested a lower uptake of labelled citrulline in all the animal fibroblasts examined, compared to man. With the more comprehensive approach used in the present study, the decreased ratio in the rhinoceros was found to be a consequence mainly of the high uptake of arginine.

It was initially hoped that between-species differences in uptake of these substrates might provide a useful means of differentiating between or characterising different mammalian cell strains. However, the wide experimental range of the results, most marked for hypoxanthine and arginine uptake in the rhinoceros fibroblasts and the citrulline uptake in human fibroblasts (Fig 8, Pg.52 and Fig 15, Pg. 75) diminishes the usefulness of this approach. Nevertheless it is evident that there is a significant difference between species in the mean flux of substrates through pathways catalysed by " house keeping" enzymes of purine salvage and in some of the enzymes of the urea cycle. It would be interesting to know whether these changes in biochemical flux relate to more general physiological adaptations. This is a little explored area of comparative metabolism at the

level of the cultured cell line but one which deserves further study since our starting-point null hypothesis (that all mammalian skin fibroblasts should show no significant differences in these experiments), has been invalidated. The way in which these different fluxes are integrated into the overall metabolic control of the cell, and how and why they vary is poorly understood.

The decline in uptake of purine bases with passage number in human fibroblasts is similar to results previously reported (Paz et al., 1977). Fibroblasts from the oldest donor senesced at 38 population doublings and the fibroblasts from the youngest donor senesced at 45 population doublings which is in agreement with Hayflick, (1980) who reported that the number of population doublings achievable by cultured normal human fibroblasts are inversely proportional to donor age. The decline in the uptake of hypoxanthine and adenine with increased population doublings did not appear to have any relation to the physical age of the donor since the one year olds' fibroblasts declined more rapidly then the 72 year olds' fibroblasts, whereas the 27 year olds' fibroblasts showed hardly any decline at all (Fig 7, Pg.49). Two of the eight rhinoceros fibroblast cultures reached 16 population doublings before senescing, the other six senesced at between 13 and 15 population doublings. Since there was a rapid decline in the uptake of hypoxanthine and adenine as the fibroblasts aged, it was important to obtain comparative

results within the first four passage numbers. The ages of the rhinoceroses where unknown at the time of biopsy. As a consequence of the effect of passage number on the purine uptake, the amino acid uptake experiments were also analysed at similar passage numbers.

Purine base uptake between transformed and untransformed cells was markedly different. It was interesting to note that where there had been a highly significant variation between human and rhinoceros fibroblasts in ·the uptake of hypoxanthine, there was no significant variation between these same fibroblasts after they had been transformed (Table 9, Pg. 66). The level of significance of the adenine uptake difference between the transformed and untransformed fibroblasts of these two species decreased from 0.5% to 5% level of significance (Table 4, Pg. 54 and Table 9, Pg. 66). Bavard et al., (1992) showed an increase in HPRT activity in rabbit SV40 transformed chondrocytes which was also found in the transformed fibroblasts used in this study. This is instructive in demonstrating the capability of transformed fibroblasts for upregulating flux through pathways contributing to nucleic acid synthesis. For hypoxanthine uptake, increased activity of HPRT contributes to this effect, but the lack of a precisely proportional increase in uptake to cell free HPRT activity showed that there were multiple components to this regulation.

The fact that all the human transformed fibroblasts senesced was interesting, and that they senesced in the same order as their parent fibroblasts. The oldest donor senesced at 38 population doublings for untransformed fibroblasts and at 44 population doublings for the transformed fibroblasts, whereas the youngest donor senesced at 45 population doublings in untransformed fibroblasts and at 57 population doublings in the transformed fibroblasts.

The persistence of senescence in the transformed fibroblasts is consistent with the observations of Sugawara et al., (1991) that senescence is dominant over immortality and suggests that in these transformed fibroblasts the senescent-inducing genes, if such exist, are still functioning (Sugwara et al., 1990; Klein et al., 1991).

5 CONCLUSION

Great care was taken in this study to standardize and perform the experiments in an identical manner for both the purine base uptake and the amino acid uptake. The cell density, the medium used, incubation time for the double label experiment as well as the incubation time prior to the seeding of the cells was standardized. Skin fibroblasts were used from all three species and these did not noticeably vary morphologically. The analyses from all the experiments were performed at similar passage numbers. Despite the above precautions, the predominate variation was found to be at the between-experiment level. This has serious implications for attempts to determine significant differences in substrate uptake at the between-individual level. Therefore, when using this technique as a diagnostic tool, as, for example, when screening for partial enzyme deficiencies, double label uptake experiments would have to be repeated on several different occasions and with several controls. If screening for a partial enzyme deficiency, the results would have to be confirmed with more specific cell free enzyme assays, such as HPRT, APRT, ASS, ASL and arginase assays in the present context.

The results from the between-species comparisons suggest that there is a significant variation in the uptake of hypoxanthine , adenine and arginine, inspite of the significant experimental variation. No significant variation

was found between the buffalo and human in adenine uptake or in either of the amino acid uptakes

Transformation of fibroblasts appears to delay senescence by +/- 10 population doublings and results in a marked increase in uptake of some substrates, which is not always a simple consequence of an increase in activity of a single enzyme.

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