VARIATION OF ENZYME ACTIVITIES IN CULTURED CHINESE HAMSTER KUPFFER CELLS

by

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CONTENTS

SUMMARY

The purpose of this study was to examine the variation of enzyme activities between cell lines which were either adult, foetal or Simian Virus 40 (SV40) transformed and possessing a similar genetic and epigenetic background. Utilizing Kupffer cells from the Chinese hamster (Cricetulus griseus), it was possible to isolate cell lines which expressed Kupffer cell functions and survived at least 70 population doublings in culture. A large number of cell lines, each originating from a single Kupffer cell, could be isolated from a single animal. Three adult siblings provided material for the isolation of 130 primary adult Kupffer cell lines and one mid-term foetus was used to initiate 24 primary foetal Kupffer cell lines.

At various stages in culture the Kupffer cell lines were assayed for the following enzyme activities: catalase, arginase, microsomal haem oxygenase, β-glucuronidase, peroxidase, alcohol dehydrogenase, lactate dehydrogenase, isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase. The following points emerged from a study of the primary Kupffer cell lines.

(a) Enzyme activities rapidly declined when Kupffer cells were cultured.

(b) After 26 population doublings for primary foetal and 40 population doublings for primary adult cell lines, all enzyme activities were stable until at least the stage of 70 population doublings. The enzyme activities were less in the primary foetal cell lines.

(c) Each enzyme activity demonstrated highly significant variation between the cell lines. The variation was greatest between the primary adult cell lines.

Both primary adult and primary foetal Kupffer cell lines were transformed by SV40 and assayed for the above enzyme activities. Transformation of 65 primary adult Kupffer cell lines by SV40 resulted in the loss of Kupffer cell functions and all enzyme activities, with the exception of β-glucuronidase and glucose-6-phosphate dehydrogenase were rapidly reduced to a fraction of those observed in primary adult Kupffer cell lines. The method of transformation resulted in the emergence of the transformed

phenotype within a few cell divisions after infection with SV40. All enzyme activities in SV40-transformed Kupffer cell lines were stable for at least 90 population doublings after transformation. Accompanying the change in enzyme activities after transformation by SV40 was an increase in variation between cell lines which, prior to transformation were isolated from material with a genetically identical origin. Four SV40-transformed foetal cell lines were indistinguishable from the SV40-transformed adult cell lines.

A study of lactate dehydrogenase (LDH) isoenzyme patterns in primar adult, primary foetal and SV40-transformed Kupffer cell lines revealed that complexities may underlie a total enzyme activity. While culturing of primary adult or primary foetal Kupffer cells resulted in a decline in total LDH activity, the relative proportions of LDH A and LDH B gene products changed Primary cell lines demonstrated a decreased proportion of LDH B gene product when compared with freshly isolated Kupffer cells. After transformation of adult or foetal Kupffer cells by SV40 the polypeptide coded by the LDH B was not detected.

The variation in enzyme activities between primary Kupffer cell lines of similar or identical origin is interpreted to be an example of epigenetic variation arising as a result of each Kupffer cell's individual response to the culture environment. The increase in enzyme activity variation between cell lines after transformation by SV40 is suggested to be the result of a change in the differentiated state. The phenotypes of primary adult, primary foetal and SV40-transformed Kupffer cell lines are discussed in terms of the differentiated state and in the light of the theory of foetalism in neoplasia.

The heterogeneity in enzyme activities was not paralleled by karyotypic variation. Although there was a gradual transition towards heteroploidy, all cell lines, whether primary or transformed, possessed a diploid karyotype during the stages when variation in enzyme activities was apparent The normal karyotype possessed by cells which demonstrated properties of transformation suggests that transformation by SV40 is not the result of kary otypic change.

Analysis of the data revealed quantitative correlations between severa enzyme activities. Primary adult, primary foetal and SV40-transformed cell lines demonstrated differences in the pattern of the quantitative correlations.

The correlations provide evidence for the existence of a mechanism which regulates the activity of two or more unrelated enzymes. Possible metabolic and regulatory bases for the enzyme activity correlations are considered.

ABBREVIATIONS

ADH	-	alcohol dehydrogenase
AMP	-	adenosine monophosphate
Arg.	-	arginase
BSA	-	bovine serum albumin
Cat.	-	catalase
CF	-	complement fixation
DADCF	-	diacetyl dichloro fluorescein
df	-	degrees of freedom
EDTA	-	ethylene diamine tetra-acetic acid (disodium salt)
em.	-	emission wavelength
ex.	-	excitation wavelength
β-Glu.	-	β-glucuronidase
G6PDH	-	glucose-6-phosphate dehydrogenase
IDH	-	isocitrate dehydrogenase
IMP	-	inosine monophosphate
IU	-	international unit
LDADCF	-	diacetyl dichloro fluorescin
LDH	-	lactate dehydrogenase
MHO	-	microsomal haem oxygenase
NAD(H)	-	nicotinamide adenine dinucleotide (reduced form)
NADP(H)	-	nicotinamide adenine dinucleotide phosphate (reduced form)
PBS	-	phosphate buffered saline
p. e.	-	plating efficiency
Perox.	-	peroxidase
PFU	-	plaque forming units
SD	-	standard deviation
SV40	-	Simian Virus 40

SECTION 1

INTRODUCTION

1.1 Introduction

Multicellular differentiated organisms are characterized by the presence of diverse cell types, which differ qualitatively and quantitatively in their complement of structural and catalytic proteins. These differences in protein complement reflect variable expression of a constant genetic complement (Gelehrter, 1971; Gurdon, 1974). It has become apparent that understanding the regulation of specific gene expression is fundamental to an appreciation of differentiation and development, and to the mechanisms by which a cell responds to a variety of stimuli. The study of enzyme activities provides an approach to the solution of these problems. Not only are enzymes the products of gene expression, but also the catalytic proteins by which structural and functional differentiation are achieved. Hence, the study of enzyme activities is in part an effort to reduce the study of differentiation to experimentally accessible dimensions (Paigen, 1971).

In recent years there have been increasing efforts to find systems more amenable than the intact animal for examining the regulatory events which maintain or change enzyme activity in the mammalian cell. The work described herein was an attempt to utilize the advantages of cell culture and develop a system which may be of value in studying certain aspects of enzyme regulation and the dogma which surrounds cell culture. Suffice it to mention at this stage that the main sphere of interest was the phenotypic variation and co-ordinate behaviour of enzyme activities when cells, presumed to be genetically identical, were faced with the challenge of the culture environment or transformation with Simian Virus 40. The aims of this study are described in more detail in section 1.7.

It is the purpose of section 1 to first introduce some of the theories and concepts of enzyme regulation in the mammalian cell, then to briefly introduce examples of environmental parameters and mutations which affect enzyme activities in the intact animal.

With appreciation of these influences, we will then be in a position to consider enzyme activities and phenotypic variation in mammalian neoplasia and cell culture systems. In many areas, no attempt will be made to provide a thorough survey or "in-depth" review of the vast amount of literature on enzyme activities. The references were selected to serve as illustrative examples of the concepts and to focus attention on the subjects at hand, rather than to provide a complete documentation or to establish priority of discovery.

1.2 The expression of an enzyme activity

Before examining the effects of external stimuli, mutations or neoplasia on enzyme activities, it is necessary to consider the molecular processes responsible for the expression of an enzyme activity.

Two general patterns have been recognized whereby enzyme regulation can be effected, one occuring on a time scale of seconds or minutes, the other in hours or days.

(1) Changes in catalytic activity of enzymes present in the cell. The activity of many enzymes can be increased or decreased by interactions with substrates, intermediates and products so as to permit control of enzyme activity depending on immediate cellular demands (Wyngaarden, 1970).

(2) Changes in levels of enzyme protein. Such regulatory phenomena involve alterations in the rate of synthesis of the enzyme molecules as affected by stimuli, and the control of degradation rates (Schimke and Doyle, 1970).

Distinction must thus be made between enzyme level and enzyme activity. Enzyme level is determined by the number or concentration of enzyme molecules present, which may or may not possess full catalytic activity. Enzyme activity is the physiological

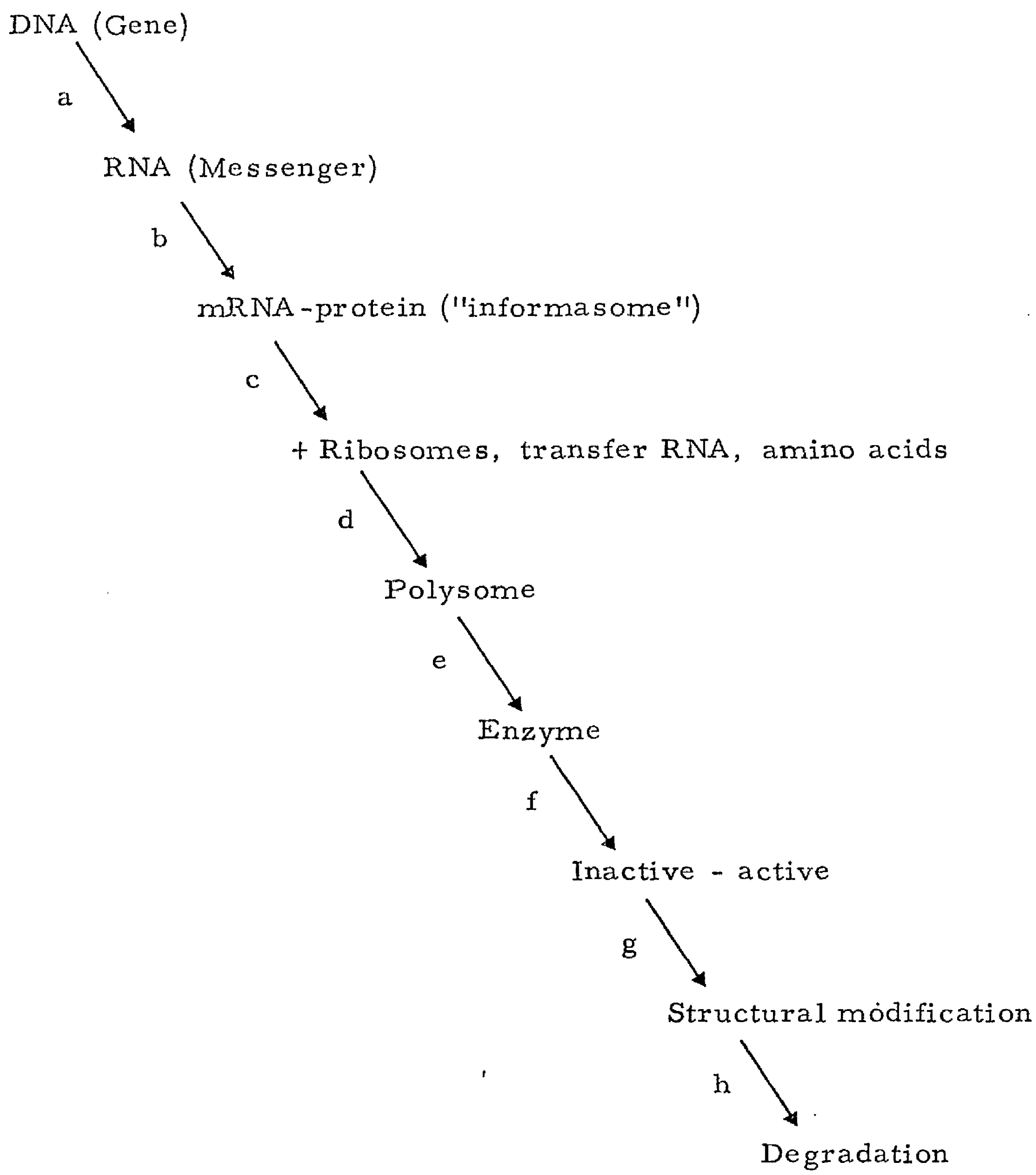

Figure 1.1:- Sequence of events leading to expression of an enzyme activity (modified from Pitot *et al.*, 1971)

consequence of the protein molecules' catalytic activity and need not necessarily be related to the number of enzyme molecules present. Many studies use the term "level" when in fact the measurement was made by catalytic assay.

There will be no attempt to interpret each example of enzyme regulation in terms of specific, molecular mechanisms. The fact that in no case has an exact molecular mechanism been clearly established ensures that for much of this consideration no real evidence exists. The limitations of each system studied, including lack of suitable mutants, differences in cell populations within a given tissue, and complex inter-relationships between nutritional and hormonal variables, have made formulations of mechanisms tentative and often speculative. Potential mechanisms for regulation of synthesis or degradation, with reference to specific examples only when they are particularly germane, are discussed briefly.

Inasmuch as each of the events of protein synthesis and metabolism may be catalyzed by multiple enzymes and may occur in different cellular compartments, or even different cells, and at different times, control of enzyme synthesis and activity could be exerted at several levels. Figure 1.1 outlines the general sequence of events involved in expression of an enzyme activity. The culmination of all these events is the enzyme phenotype. Such a sequence of processes has been termed enzyme realization (Paigen, 1971).

The primary potential sites for the regulation of expression are at the level of mRNA synthesis, a (see Lewin, 1974) and of enzyme synthesis, e (see Pitot et al., 1971). In addition, there are a number of other possible sites of regulation including messenger transportation (b), the formation of actively translating polysomes (c), the availability of ribosomes, tRNA and amino acids (d), and post-translational events (f, g, h), i.e. conversion of an inactive enzyme to an active form, the alteration of enzyme structure and the degradation of enzyme (Pitot et al., 1971). Enzyme activity may also be affected by intèractions with other macromolecules (Paigen, 1971).

Ultimately, control of specific enzyme synthesis is dependent on activation or inactivation of specific DNA segments to allow for the synthesis of the appropriate RNA species. Explaining the mechanisms whereby such control occurs is one of the central problems of molecular and developmental biology. However, given that a gene is capable of being transcribed there exists a number of possible mechanisms whereby enzyme synthesis can be increased.

Enzyme induction has been observed in mammalian cells, but in relatively few systems has there been convincing evidence of an increase in the rate of synthesis of enzyme molecules. The major established examples of enzyme induction in mammalian cells are hepatic tyrosine aminotransferase (Gelehrter, 1971), alanine aminotransferase (Segal and Yim, 1965), tryptophan pyrrolase (Knox and Greengard, 1965) by glucocorticoids, microsomal aryl hydroxylase by polycyclic hydrocarbons (see Gelehrter, 1971), arginase by arginine (Schimke, 1963), alkaline phosphatase by prednisolone (Griffin and Cox, 1966) and δ-aminolevulinic acid synthetase by steroids (Granick and Kappas, 1967).

There is considerable evidence that alterations of RNA metabolism are involved in enzyme regulation in mammalian cells. A majority of hormonal, drug and nutritionally induced increases in enzyme activity, including those mentioned above, are prevented by the administration of actinomycin D or other inhibitors of RNA synthesis (Schimke, 1973). It seems likely that RNA synthesis is necessary for the initiation of increased synthesis of specific protein, but once that RNA synthesis is accomplished its utilization can take place for some time (Schimke, 1973). Certainly, different classes of genes are regulated independently at the transcriptional level during development (Gurdon, 1974).

There have been several models proposed for mechanisms of transcriptional control (Davidson and Britten, 1971

1973; Georgiév et al., 1972). In general they have all been modifications of, or at least influenced by, the original model proposed for bacteria by Jacob and Monod (1961). Crick (1971) and Paul (1972) propose models not unlike those suggested previously but which specifically consider the organization of eukaryote DNA. Although evidence to support these models, or test their predictions is not yet forthcoming, they provide the framework for subsequent enquiry.

Transcriptional control is obviously instrumental in differentiation and the timing of developmental sequences. However, it would appear that translational control is of great importance in differentiated cells (Pitot et al., 1971). Translational control is probably used to make quantitative adjustments to a pattern of protein synthesis determined by the synthesis of new messages (Gurdon, 1974). Pitot et al. (1971) suggest a number of enzyme systems which probably demonstrate control at the translational level. Strictly speaking, "translation control" in this context refers to "post-transcriptional control". It is the finding of relatively long lives of mRNA (see Tomkins et al., 1969) that has lead to a variety of proposals that regulation can occur at one or more of the many steps which occur subsequent to messenger synthesis. For example, there could be selective stabilization of certain messengers (Fuhr et al., 1969), delayed translation (Newell, 1971), masking of messenger use (Gurdon, 1974) or initiation control (Lingrel, 1974).

Regulation of ribosome function on the basis of variations in transfer RNA acceptor properties (Maenpaa and Bernfield, 1970), phosphorylation of ribosomal proteins (Kabat, 1971) or amino acid availability may also regulate synthetic capacity. Munro (1968) demonstrated the sensitivity of rat liver polysome profiles to amino acid availability and the cyclic variations in tyrosine aminotransferase activity have been explained on the basis of amino acid availability (Schimke and Doyle, 1970). Finally, control of protein synthesis at the level of release of specific peptides has been suggested (Cline and Bock, 1966).

Recent advances in the ability to isolate and utilize mammalian mRNA and specific polysomes (see Schimke, 1973; Paul et al., 1973; Lingrel, 1974) suggest that in the near future it may be possible to examine in more detail the induction or repression of specific enzyme synthesis and to identify the rate limiting step for such a synthesis. Clearly, a number of sites in the sequence of events leading to enzyme synthesis may be rate limiting for any particular enzyme in any given circumstance. There is no reason to consider that all agents and stimuli which alter the rate of specific enzyme synthesis must do so by the same mechanism.

The above discussion considers increases in enzyme activity. The studies of repression in mammalian cells are hampered even more by the diversity and many levels of control in protein metabolism. Hanninen (1971) has reviewed the field of repression of mammalian enzyme synthesis. Although numerous examples are cited, to-date there exists no evidence that this repression is at the level of gene transcription. The only general case of repression would appear to be that of long-term repression due to gene-packaging, a consequence of developmental programming (Hanninen, 1971).

Structural modification of synthesized enzyme molecules may also affect catalytic activity. Three separate mechanisms have been discerned.

(1) Regulation through non-covalent changes (allosteric) in enzyme structure, e.g. aspartate transcarbamylase and glutamate dehydrogenase (Yielding, 1971).

(2) Regulation through covalent changes in structure, e.g. phosphorylase (Krebs and Fischer, 1962).

(3) Regulation through hydrolysis modification of structure, e.g. activation of trypsinogen to trypsin (Ottersen, 1967).

While allosteric regulation and hydrolysis provide for rapid fluctuations in biological activity through changes in side chain environment based on simple equilibia, covalent modification of side chain function permits a slower and more sustained response to regulatory signals. Reversal of this latter response requires another enzyme (Yielding, 1971).

Control of specific enzyme degradation has been shown to be important in regulating a number of enzyme levels (Rechcigl, 1971). An enzyme level is ultimately determined by the opposing rates of synthesis and degradation (Schimke and Doyle, 1970). In a steady state the level of an enzyme is a function of both the rate of synthesis and the rate constant of degradation, and an alteration in either rate can affect the level of the enzyme. The concept of enzyme turnover is emerging as central to understanding enzyme regulation in mammalian cells. In all systems studied, it has been concluded that enzyme is synthesized at a constant rate while a constant fraction of active enzyme molecules present in the tissue is broken down per unit time (Rechcigl, 1971).

Grisolia (1964) and Pine (1967) suggest that enzyme molecules are individually available to a degradative process which is present at all times. Shifting concentrations of substrates and cofactors which occur under various hormonal and physiological conditions, would lead to a variety of effects on specific enzymes, either to stabilize or labilize them. Although there appears to be considerable specificity in the degradation process it is unlikely that degradation of each enzyme requires a specific protein (Schimke, 1973). Rather, the number of types of protease in mammalian cells (Hartley, 1960) performing different functions at different sites and times may provide the necessary specificity.

The only exception to the concept of constant turnover of enzyme molecules has been provided by the studies of Yagil and

Feldman (1969). These workers observed that glucose-6-phosphate dehydrogenase, maleate dehydrogenase and 6-phosphogluconate dehydrogenase molecules were stable entities in cultured cells, and have suggested that the steady-state model employed to describe enzyme regulation in mammalian cells does not apply to these enzymes in cultured cells.

The above brief introduction to some of the current theories of enzyme regulation in mammalian cells suggests that while there is no shortage of models, the specific molecular mechanisms of regulation of enzyme levels and activities still remain unsolved. It is most likely that, given any site at which control can be exerted, examples will be found where such control is exerted. However, the above aspects of enzyme regulation must be borne in mind when we consider the effects of environmental stimuli and the main subject of this dissertation, phenotypic variation of enzyme activities.

1.3 Environmental influences and enzyme activities in the intact animal

A multitude of studies have shown that enzyme activities in mammalian cells can be altered by a wide variety of physiologic, nutritional and hormonal manipulations, administration of various pharmacologic agents, and by circadian rhythms. The list of enzymes so affected is large and includes examples from essentially every major metabolic pathway in one or more tissues (Schimke and Doyle, 1970; Schimke, 1973). Below is a brief consideration of some of these environmental influences, giving examples from which most of our knowledge has been obtained.

1.3.1 Dietary factors and enzyme activity

Dietary change can result in alteration of a considerable number of enzyme activities (see Freedland and Szepesi, 1971).

The bulk of enzyme adaptations due to dietary factors are consistent with deduced physiological requirements. After starvation, the activities of enzymes in the Embden-Meyerhof pathway, gluconeogenesis and the Krebs cycle are increased or maintained with respect to the activities of other enzymes (Freedland, 1967). When starved rats are refed, a number of metabolic alterations occur, such as hyperglycogenesis, the return of a number of enzyme activities to normal and overshoot of normal activity by a number of other enzymes (Freedland and Szepesi, 1971).

Protein restriction results in major changes of activity of the rat hepatic enzymes xanthine oxidase, guanase, uricase, five enzymes of the urea cycle, serine dehydratase and ornithine transaminase (see Wyngaarden, 1970). The studies of Knox and Greengard (1965) demonstrated a direct relationship between the level of many liver enzymes concerned with the metabolism of amino acids and the amount of protein in the diet. The regulation of apoenzyme synthesis de novo via increased transcription has been implicated in the dietary alteration of xanthine oxidase, threonine dehydratase and ornithine transaminase (see Wyngaarden, 1970). These studies suggest that protein restriction and refeeding affects the rate of synthesis of specific mRNA molecules.

There are a number of indications that during dietary induction of enzyme activity, the half-life of some enzymes is shortened, and would facilitate reaching a steady-state in a shorter time (Freedland and Szepesi, 1971). The pattern of independent regulation of synthesis and degradation during dietary change has been especially studied in relation to protein and arginase activity and fat-free diets and acetyl-CoA-carboxylase activity (Schimke, 1973; Majerus and Kilburn, 1969).

Dietary changes can also produce a classical type of repression of enzyme synthesis. Glucose can repress threonine dehydratase and ornithine transaminase synthesis by inhibiting the incorporation of amino acids (Jost et al., 1968). This was the first case of catabolite repression in mammals and as such it resembles the bacterial system, although, it appears that the repression is at the translational rather than the transcriptional level.

1.3.2 The effect of hormones on enzyme activities

In recent years many reports have appeared indicating that hormones are capable of selectively stimulating or inhibiting the *de novo* synthesis of specific enzymes. While all classes of hormone have been shown to affect some enzyme activity (Pitot and Yatuin, 1973), the glucocorticoids, insulin, glucagon and thyroxine have been the most intensively studied. Most contributions to our understanding of the action of hormones in regulation of enzyme level have come from relatively few enzyme systems. These include tyrosine aminotransferase, tryptophan pyrrolase, serine dehydratase and alanine aminotransferase (see review, Rosen and Milholland, 1971). Although certain enzymes are found in several tissues other than liver, it is only in this organ that they are hormone responsive (Rosen *et al.*, 1958). A possible explanation for this phenomenon is the existence of isoenzymes. Different forms of tyrosine aminotransferase and serine dehydratase exist and failure to observe the expected response to a hormone may merely reflect an atypical distribution of the isoenzymes (Rosen and Milholland, 1971).

The qualitative and quantitative enzymatic response to steroid administration is markedly affected by the turnover rates of the enzymes themselves (Berlin and Schimke, 1965; Rosen and Milholland, 1971), the nature, dose, route and duration of administration, and by the age, and hormonal and nutritional state of the recipient animal (Gelehrter, 1971). Thus, it is not surprising that an almost bewildering array of observations have been reported concerning hormone induction of enzymes in intact animals. However, some important observations can be gleaned from, for example, the study of steroid induction of tyrosine aminotransferase, which has been extensively studied as a model of enzyme induction in mammalian cells.

The administration of hydrocortisone to intact or adrenalectomized rats results, after a lag period of 1 to 2 hours, in a rapid 5-fold increase in the activity of hepatic tyrosine aminotransferase (TAT) which is maximal within about 6 hours and then declines with a half-life of about 2.5 hours (Kenny et al., 1968). It has been demonstrated immunochemically that the increase can be attributed entirely to a steroid-mediated increase in the rate of synthesis (Kenny et al., 1968). This steroid induction of TAT is prevented by actinomycin D, and associated with increases in RNA polymerase activity and nuclear RNA labelling which preceeds the increase in TAT activity (see Gelehrter, 1971). However, the magnitude of the increase in RNA synthesis is too large to be interpreted as the increased synthesis of mRNAs for the enzyme (Gelehrter, 1971). It is possible that the increase in RNA synthesis is a separate effect of the steroid treatment, unrelated to enzyme induction. The fact that steroid induction of TAT in cultured cells is not associated with any increase in overall RNA synthesis suggests that ribosomal RNA synthesis is not necessary for hormone action and that the regulation of TAT synthesis occurs at some steps in protein synthesis after gene transcription (Gelehrter and Tomkins, 1967).

The induction of TAT by steroids has been explained in a model presented by Tomkins et al. (1969). In this model, the inducing steroids have a single action – to antagonize a post-transcriptional repressor which both inhibits messenger translation and promotes messenger degradation. It is proposed that the mRNA for TAT is stable, and that the mRNA for the repressor as well as the repressor itself must turn over rapidly. It is interesting to note that both insulin and glucagon also increase the rate of synthesis of TAT (Kenny et al., 1968). These two hormones are usually considered to be physiological antagonists, and Holt and Oliver (1969) have suggested that different isoenzymes are induced by each hormone.

Hormones may also reduce enzyme activities. For example, glutamic-pyruvic transaminase degradation rate was increased by thyroxine, as was that for serine dehydratase (Freedland and Szepesi, 1971). Insulin causes a decrease in gluconeogenic enzyme levels while growth hormone prevents the synthesis of TAT (Hanninen, 1971).

1.3.3 Chemical induced changes in enzyme levels

A vast literature exists on the effects of various compounds on enzyme activities. However, several chemical compounds have demonstrated specificity for certain enzyme activities and have been used for studying enzyme regulation in mammalian cells.

More than 200 drugs, insecticides, carcinogens and other chemicals are known to stimulate the activity of a variety of hepatic microsomal drug-metabolizing enzymes (Gelehrter, 1971). The induction of these enzymes is prevented by puromycin treatment suggesting that the increase in activity reflects de novo synthesis of the enzymes (Gelboin and Blackburn, 1964). Nebert and Gelboin (1970) have shown that the initial phase of microsomal oxygenase induction appears to involve the accumulation of "induction-specific RNA" and that this RNA can accumulate in the absence of protein synthesis.

New insight into the question of enzyme turnover has been obtained through the use of two inhibitors. Allylisopropylacetamide blocks the synthesis of new catalase without interfering with the activity of previously formed enzyme, while 3-amino-1,2,4-triazole irreversibly inhibits catalase enzyme without interfering with its resynthesis (see Rechcigl, 1971).

Compounds known to produce experimental porphyria, such as allylisopropylacetamide, have been shown to induce δ-aminolevulinic acid synthetase to high levels (see Rechcigl, 1971). A review by Granick and Sassa (1971) has considered the use of this system in detail.

1.3.4 Rhythmic changes in enzyme activity

Circadian rhythms have been detected for a number of enzyme activities. In no case has the mechanism been elucidated. The rhythm of TAT activity is perhaps the most studied (Fuller, 1971). It appears that control by any single hormone does not account for the TAT rhythm, nor is there evidence for direct or indirect neural control (Fuller, 1971). Tryptophan pyrrolase (Hardeland and Rensing, 1968), phosphoenol pyruvate carboxykinase (Phillips and Berry, 1969) and hepatic drug-metabolizing enzymes (Fuller, 1971) all demonstrate a circadian rhythm. In 1975, Hardeland described a circadian rhythm in TAT activity in cultured liver cells. Probably because of the physiological complexities, the rhythm of enzyme activities is the least understood aspect of enzyme regulation.

1.4 Mutations which affect enzyme levels

The lack of suitable mutations has limited the rate of progress and depth of knowledge concerning the regulation of enzyme levels in mammalian cells. A small number of mutations affecting enzyme levels have been recognized, and utilized for the study of control of enzyme levels.

δ-Aminolevulinate dehydratase (see Doyle and Schimke, 1969). In mouse liver, the Lv locus has been shown to regulate the rate of enzyme synthesis. Mouse strains homozygous for the Lv^a allele have high liver enzyme activity, while strains homozygous for the Lv^b allele have activities $^1/3$ that of the Lv^a genotype. The levulinate locus controls the amount and not the activity of δ-aminolevulinate dehydratase. Doyle (1971) demonstrated that the enzymes from both homozygous states were similar and suggested that the mutation affecting the rate of synthesis is not a structural mutation.

Catalase (see Rechcigl, 1971). In mouse liver, the *Ce* locus regulates the rate of catalase degradation. Due to a reduction in the rate of degradation, mice which are *cece* possess twice the level of catalase. The mutation is relatively specific for catalase among liver proteins, and no effect could be detected on kidney catalase levels. In contrast to the mutation in humans, the genetic factor regulating the degradation of mouse catalase is probably distinct from the gene specifying the structure of this enzyme. Ganschow and Schimke (1969) have described another mutation in the mouse which appears to affect catalase structure. Thus, in the mouse separate genes determine the structure and the liver concentration of catalase.

β-Glucuronidase (see Paigen *et al.*, 1975). In mice, the *G* locus controls the structure of β-glucuronidase, individuals with genotype *gg* possess approximately $^{1}/14$ of the normal enzyme activity. The *E* locus is distinct from the *G* locus and controls the insertion of β-glucuronidase into endoplasmic reticulum. Mice with genotype $e^{go}e^{go}$ have low levels of liver β-glucuronidase.

The Pd locus. Dagg *et al.* (1964) demonstrated that in mice, the *Pd* gene controls the activities of three distinct enzymes - dihydrouracil dehydrogrenase, dihydropyrimidase and 3-ureido-propionase. Pd^{a} animals possess between $^{1}/8$ and $^{1}/3$ of the normal activities of these three enzymes. Although in some respects, the *Pd* mutation resembles an operator gene mutation in bacteria, this mutation did not attract attention until the studies of Sanno *et al.* (1970). These workers found that only the last enzyme of the pathway, ureidopropionase, is affected by the mutation. There exists fragmentary evidence for a mutation in the mouse which may involve a regulatory gene controlling the levels of glucose-6-phosphatase (Erickson et al., 1968).

There are a number of examples of human genetic defects with differences in enzyme levels as a result of altered rates of synthesis or degradation. Sutton and Wagner (1975) have reviewed aspects of mutation and enzyme function in humans. Mutations of catalase (Matsubara et al., 1967) and glucose-6-phosphate dehydrogenase (Yoshida et al., 1967) also appear to indirectly affect the rate of enzyme degradation by making it more labile *in vivo*. Another interesting example is the elevated level of AMP-pyrophosphorylase which occurs in the red cells of patients lacking the separate enzyme, IMP-phosphorylase, a phenomenon resulting from stabilization of the AMP-phosphorylase during red cell ageing (Rubin et al., 1969). Yoshida et al. (1970) described a patient with a markedly elevated level of glucose-6-phosphate dehydrogenase, which differs from normal enzyme by one amino acid residue, and which is synthesized at a rate approximately four times greater than the normal enzyme.

Krooth (1970) suggests that the rare recessive autosomal defect resulting in orotic aciduria is the result of a mutation with regulatory affect on two enzymes, orotidine-5'-monophosphate pyrophosphorylase and orotidine-5'-monophosphate decarboxylase. Another example of a gene controlling an enzyme level was observed in the mouse by Shows and Ruddle (1968). These workers presented evidence for a regulatory function for a gene *Ldr-1* in controlling the lactate dehydrogenase *b* subunit. Animals homozygous for one allele of the *Ldr-1* gene have subunits in their erythrocyte lactate dehydrogenase and in the lactate dehydrogenase of other tissues. Animals homozygous for the other *Ldr-1* allele have *b* subunits in other tissues, but lack *b* subunits and have only *a* subunits in their erythrocyte lactate dehydrogenase.

That the number of mutations affecting enzyme levels seems to be small is probably due more to limited study rather than to their infrequent occurrence. In the few cases where they have been searched for systematically, such as the screening of 63 inbred mouse strains for variants of β-glucuronidase (see Paigen et al., 1975), they have been found. More such efforts would undoubtedly reveal other mutations affecting the level of specific enzymes.

1.5 Enzyme activities and neoplasia

The appearance, maintenance and decline of specific enzyme activities as part of a developmental sequence and ageing, have been the subject of several reviews (Hermann and Tootle, 1964; Greengard, 1971; Moog, 1971). Enzyme development has also been reviewed in terms of the differentiation of metabolic pathways (Papaconstantiou, 1967) and in relation to functional differentiation (Moog, 1965). More general accounts of the control of gene expression in animal development can be found in Davidson (1968) and Gurdon (1974).

Although it is not proposed to present a semantic argument, it is necessary to define in the context of this dissertation, the term, "differentiation". Differentiation may be considered to be a process whereby cells develop specialized functions at the expense of other potential functions, a process which is presumably irreversible and heritable, breeding true through successive generations of cells. The expression of selected enzyme activities is the matter of differentiation; these enzymes are responsible for the specialized syntheses and functions of the differentiated cell.

The disruption or even apparent reversal of normal enzymic differentiation which occurs in neoplasia has been the subject of much attention. Normal enzymic differentiation leads to the characteristic quantitative pattern of enzymes possessed by each adult tissue

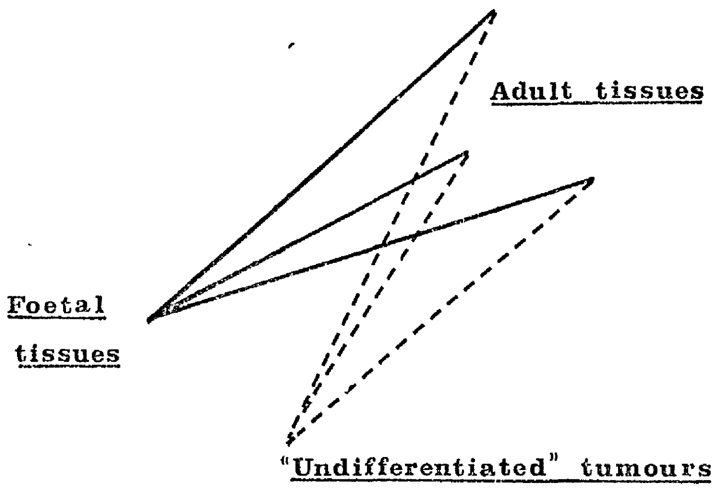

Figure 1.2:- Diagram of the relationships between 3 similar enzyme patterns in foetal tissues, their divergence to the differentiated patterns characteristic of three different adult tissues and the convergence of undifferentiated tumours in neoplasia.

The convergence is in the direction of increasing likeness to foetal tissue (from Knox, 1972).

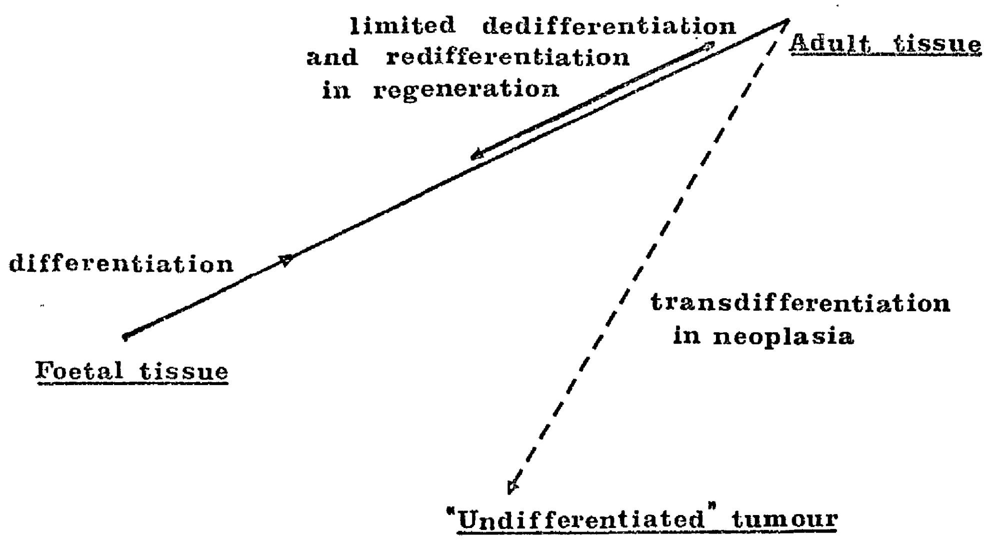

Figure 1.3:- The relationships between various types of differentiation sequence involved in regeneration or neoplasia in a particular tissue.

(Knox, 1972). The widely different patterns of composition characteristic of normal tissues are each changed in neoplasia, more or less, and in different ways in particular tumours, but possibly all in the direction toward the common pattern shared by the most undifferentiated tumours from any origin. It is possible that a series of tumours could be classified in such a way that their enzymic compositions form a convergent series between the divergent patterns of the normal tissues and the converged or common patterns of the most undifferentiated tumours. Figure 1.2 illustrates the possible simplified relationship between differentiation of foetal cells in the direction of normal adult tissues and neoplastic change. The foetal tissues with similar enzyme patterns differentiate from one another into adult tissues that are dissimilar. In the process of neoplasia, this differentiated state is altered and the emerged enzyme patterns tend to be tangential to the direction of normal differentiation. While some tumours appear undifferentiated with respect to their tissue of origin, others occur in a series of intermediate tumours, less differentiated than their tissue or origin, that converge in type on the most undifferentiated fast-growing tumours (Knox, 1972, 1974). These converged tumours exhibit certain aspects of gene expression and regulation which appear identical to those in foetal tissues, but differ in many other properties (Wu, 1973).

Figure 1.3 illustrates the relationships between the various types of differentiation involved in regeneration or neoplasia in a particular tissue. In the usual context, the developmental sequence of a determined embryonic cell to produce a tissue with a characteristic histotypic phenotype is considered to be differentiation. The notion that a neoplasm should be considered as arising by differentiation has, with few exceptions, escaped serious attention (Markert, 1968; Pierce, 1970). In all probability, we may have been misdirected by the lack of overt manifestation of histotypic differentiation of most tumoúrs, which has lead to the doubtful concept of "dedifferentiation". In the sense that a neoplasm possesses stable and heritable properties, it must qualify as a differentiated tissue. "Dedifferentiation" implies a reversal of the differentiation sequence, a phenomenon which occurs only to a limited extent during regeneration and tissue repair, the cells involved then redifferentiate along the sequence followed by the previous differentiation (Coggin and Anderson, 1974). Neoplasia in all its forms is a special expression of abnormal programming of gene functions during cell differentiation (Markert, 1968; Weinhouse, 1974) and as such can be considered to be transdifferentiation. Such a phenomenon has also been called by the more limiting terms "derepressive dedifferentiation", "retrogenic expression" (Anderson and Coggin, 1971) and "retrodifferentiation" (Gold, 1971).

The phenomena of redifferentiation and transdifferentiation need not be restricted to cells at the ultimate point in their differentiation. The precursor cell of a neoplasm may arise from a fully differentiated cell (Markert, 1968; Farber, 1973) or may arise from a relatively undifferentiated stem cell (Pierce, 1970). Specifying a cell as being "more" or "less" differentiated is only with reference to properties which can be recognized - the mere absence of recognizable properties does not necessarily imply a lesser degree of differentiation. However, in this dissertation, differentiation will only be used in an operational sense where a neoplastic cell which is less differentiated, or undifferentiated, is so only with respect to the histotypic properties of the tissue of origin.

Coggin andAnderson (1974) suggest several principles of differentiation which may be of relevance when considering neoplasia. These are that (a) genes are turned on and off in sets (not necessarily genetically linked), (b) a given gene may appear in more than one gene set, (c) exclusion rules exist for certain genes or perhaps gene sets which forbid cotemporal expression, and (d) the sequence of activation and inactivation can only proceed in one direction. Pierce (1970) suggests that the normal genome contains all the information necessary for the phenotypic expression of neoplasia, which occurs by activation of the appropriate parts of the normal genome through removal of repressors.

The evolution of the tumour phenotype is in part associated with varying degrees of suppression of the normal phenotype. The sets of genes functional for specific differentiated properties in the tissue of origin are apparently more or less randomly shut off as the neoplastic process evolves. As exemplified by cases of human neoplasia, e.g. insulinomas, melanomas and plasmacytomas, the tumour phenotype may exhibit a variety of levels, reflecting the special characteristics of the cells at a given level of repression (Busch, 1974). The loss of certain enzyme activities in neoplasia, however, may not be an entirely random process. Knox (1967) believed that there exists a minimal enzyme pattern of neoplasms and laterly (1972, 1974) suggested it to be the foetal enzyme pattern for the tissue in question.

Greenstein (1956) was the first worker to suggest that many neoplastic tissues possessed similar patterns of enzymes. This generalization has been questioned by several workers, and more recently by Pitot et al. (1974) who observed that individual tumours of a given tissue exhibit a considerable degree of heterogeneity in enzyme activities. These workers also noted that considerable variation in enzyme patterns has been reported amongst mammary carcinomas, myelomas and thyroid tumours. Bresnick et al. (1971) and Reynolds et al. (1971) found that the enzymic pattern or biochemical phenotype of both spontaneous and transplanted rodent hepatomas is stable and unique to the particular lesion. Knox (1972) has demonstrated that in the case of hepatomas, many enzyme activities present in the tumour depend on growth-rate. Fast-growing hepatomas contained foetal type enzymes and were deficient in the adult type enzymes. Slow-growing hepatomas also contained foetal type enzymes but were less deficient in adult type enzymes. Whilst Knox (1972) does not dispute uniqueness in tumours, he has demonstrated by statistical analyses that there is a tendency for convergence of enzymic properties in neoplasia. Knox's (1972) conclusions are based on collation of data for 9 enzyme activities in 18 transplanted rat tumours and a larger collation of data for 161 enzyme activities in 17 rat tissues and hepatomas. In addition, 22 non-enzymic components were considered. An extended study (Knox, 1974) also supported the earlier conclusions. The difference in emphases taken by these two groups (Knox, 1972; Pitot et al., 1974) can best be attributed to the level of generality which they consider. While Knox (1967, 1972, 1974) has attempted to construct a unifying theory of neoplasia based on enzyme activity patterns in a large spectrum of tumours, Pitot et al. (1974) have been mainly considering specific enzyme activities in rat hepatomas and describing how these respond to external stimuli (e.g. nutrition and hormone induction).

Weber and Lea (1967) and Weber (1974) have also considered the phenotypic heterogeneity of rat hepatomas. In these studies Weber has demonstrated a large number of biochemical alterations that correlate with hepatoma growth-rate: included are discriminants of carbohydrate, nucleic acid, protein, amino acid and other aspects of metabolism. The rodent hepatomas represent a class of diverse tumours in which some deviate only slightly from normal hepatocytes, while others are highly malignant. There exists a fine gradation in metabolic pattern from near normal to very deviant. Underlying the superficial heterogeneity in expression of malignancy and growth-rate between hepatomas there exists the operation of ordered and correlated expressions of morphological, biological and metabolic behaviour (Weber and Lea, 1967). It must be emphasized that the conclusions reached by Knox (1972) and Weber (1974) do not preclude the existence of phenotypic variation between neoplasms.

The basis for phenotypic variation between neoplasms of similar origin is not understood. While tumours may produce proteins or possess antigens which are not found in their tissue of origin (Bower and Gordon, 1965; Gold, 1971; Hellstrom et al., 1971; Coggin and Anderson, 1974) and exhibit different enzyme activities (Shonk and Boxer, 1967), evidence that this is the result of a specific genetic alteration has not yet been forthcoming.

In neoplasia, antigens specific to the adult, differentiated cells disappear, while new tumour-associated antigens appear; in many instances these neo-antigens are also present in embryonic tissue (Weinhouse, 1974). Foetal isoenzyme patterns also emerge at the expense of adult patterns. Many well-differentiated hepatomas have largely lost isoenzymes of the differentiated hepatocyte but do not exhibit a resurgence of the foetal isoenzyme. When resurgence of the foetal form does occur, there is always a loss of the normal hepatic isoenzyme (Weinhouse, 1974). These observations suggest that certain genes may have to be switched off before others are switched on. However, in general the alterations seem to be sporadic and unpredictable, and therefore suggest a disordered rather than a programmed mechanism of gene activation. The disorder in pattern of gene expression is well illustrated by the number of reports of ectopic hormone production by non-endocrine tumours (Omenn, 1970).

In the light of the above observations, the view expressed by Markert (1968), Pierce (1970) and Weinhouse (1974) that neoplasia is a disorder or aberration of normal differentiation appears attractive. Neoplasia is the result of an impairment of a regulatory mechanism responsible for the highly selective control of gene transcription in normal differentiated tissue. Once the control is altered or lost, many of the familiar patterns of neoplasia would inevitably follow; chromosome disorder, loss of antigens, alterations in surface properties and all the other characteristics of tumour progression could be envisioned to result from this initial injury.

Since we do not know which cells in a given tissue give rise to a particular tumour, it has not been possible to trace the cellular events leading to the development of a tumour. Pierce (1970) believes that the target for neoplasia is the stem cells of normal tissues, and what has been interpreted as "dedifferentiation" is in reality an abortive attempt at differentiation by the neoplast stem cell. Farber (1973) has discussed aspects of cellular evolution in the development of a tumour and stresses the possibility of selection acting on mixed cell populations as being vital to the progression of the tumour.

At one time it was thought that disorders of the chromosomes were responsible for the development of neoplastic disease. Cytogenetic abnormalities do not totally explain either neoplasia or phenotypic variation between tumours. There are numerous examples of normal diploid karyotypes seen in neoplasms, both rodent and human, of differing degrees of malignancy and differentiation (Nowell and Hungerford, 1961; Sandberg et al., 1961; Stevens and Bunker, 1964; Nowell et al., 1967; Mark, 1969; Nowell and Morris, 1969; Potter et al., 1969; Tseng and Jones, 1969; Granberg, 1971; Mitelman et al., 1975). Presumably in such cells, few if any changes had occurred in the DNA, although point mutations and small re-arrangements cannot be excluded. Thus, while it is true that most neoplasms exhibit cytogenetic abnormalities (Sandberg and Sakurai, 1974) it appears that the karyotypic changes are the result of neoplasia and not its cause. Similarly, karyotypic changes may contribute towards, but do not appear to be essential for phenotypic variation.

It appears that the major contribution towards phenotypic variation between neoplasms of similar origin may be from the translational process. Pitot et al. (1974) demonstrated that enzyme mRNA template lifetime was different between hepatomas and suggest that phenotypic variability is the result of template lifetime variability. Previously,

Table 1.1 Variations in selected enzyme activities in rat hepatomas with slow or fast growth-rates

The activities are expressed as a percentage of that found in normal rat liver. The slow growth-rate hepatomas were Reuker, H35, and Morris 5123D, 7800, H-35, 5123tc, 7299C, 7288B whilst the fast were Morris 3683, 3924A, Dunning, Novikoff and DAB-induced.

References: (1) Weber, 1963; (2) Wu et al., 1965; (3) Levintow, 1954; (4) Pitot et al., 1963; (5) Bottomley et al., 1963; (6) Otani and Morris, 1965; (7) Bresnick, 1964; (8) Kizer and Chan, 1961; (9) Weinhouse, 1966.

Enzyme	Enzyme activity % of normal liver		Reference
	Slow	Fast	
Glucose-6-phosphatase	<1-56		1
Fructose-1,6-diphosphatase		<1-57	1
Phosphoglucomutase		7-69	1
Glutamine synthetase	135	<15	2
Glutamine synthetase (mouse)		2-1,500	3
Tyrosine aminotransferase	150-960	<10	4
Threonine dehydratase	0-1,700		5
Aspartate aminotransferase	200	>20	6
Aspartate carbamoyl transferase	100-150	200-500	7
Hydroxy tryptophan decarboxylase	140	<2	8
Pyrroline-5-carboxylate reductase	31-310		4
Monoamine oxidase	83	17	8
Hexokinase	5-261		9
Aldolase	25-133		9

Moyer and Pitot (1972) had demonstrated that certain membrane proteins of the endoplasmic reticulum from neoplasms have different decay characteristics to the presumed homologous protein from normal tissue. Thus, they proposed that these membrane protein changes and phenotypic variability were associated with the stability of the membron, the stable template complex with polysomes and intracellular membrane (see Pitot et al., 1974; Shires et al., 1974).

While our present knowledge of molecular biology is rapidly increasing, relatively little is known about what differentiates the neoplastic cell from other cells and what is the basis for the phenotypic variation. With respect to enzyme activities, the variation has only been described, with little indication as to the mechanism of this variation. Although many neoplasms possess enzyme activities found in the tissue of origin there may be considerable variation in activity of specific enzymes in a given class of neoplasm (Shonk and Boxer, 1967). Table 1.1 presents the variation of selected enzyme activities found in rat hepatomas. These hepatomas have been divided into those with either slow or fast growth-rates. Apart from a difference in enzyme activities between slow and fast growth-rate hepatomas, there exists for several enzymes a considerable range in activity for hepatomas of a given classification. In a number of cases, enzyme activity was elevated several fold above that found in normal rat liver. In other cases there has been a dramatic reduction in enzyme activity below that found in normal liver. Such phenotypic variation between rat hepatomas is also demonstrated by the response of tryptophan pyrrolase activity to induction by tryptophan (Dyer et al., 1964). The more recent studies of Potter et al. (1969) also reveal several fold differences in tyrosine amino-transferase, serine dehydratase, glucose-6-phosphate dehydrogenase and citrate cleavage enzyme activities between Morris hepatomas. In earlier studies Pitot (1960), Potter et al. (1960) and Rechcigl et al. (1962) demonstrated that in hepatoma 5123, a number of enzymes whose activity had been previously very low or absent in other Morris hepatomas were present at very high levels.

Table 1.2 Relative enzyme patterns of 10 human hepatomas

(data extracted from Shonk and Boxer, 1967)

Enzyme abbreviations:- GK-glucokinase; PFK-phosphofructokinase; ALD-aldolase; GAPDH-glyceraldehyde-3-phosphate dehydrogenase; PGK-phosphoglycerate kinase; PGM-phosphoglycerate mutase; ENO-enolase; PK-pyruvate kinase; LDH-lactate dehydrogenase; GPDH-glycerol phosphate dehydrogenase; FDPase-fructose-1, 6-diphosphatase; PGluM-phospho glucomutase; G6PDH-glucose-6-phosphate dehydrogenase; MDH-maleate dehydrogenase; ICDH-isocitrate dehydrogenase.

Relative enzyme activity (%)

	GK	PFK	ALD	GAPDH	PGK	PGM	ENO	PK	LDH	GPDH	FDPase	PGluM	G6PDH	MDH	ICI
Liver[a]	100	100	100	100	100	100	100	100	100	100	100	100	100	100	10
Hepatomas 1	164	96	42	138	64	125	160	214	47	7	5	17	4	38	2
2	91	56	85	141	105	87	96	110	115	95	95	56	92	96	5
3	236	148	61	161	113	84	133	152	160	16	68	45	183	78	4
4	191	172	88	127	215	69	129	514	73	<1	1	8	25	25	
5	91	32	33	42	31	45	31	129	18	4	14	11	25	7	
6	191	92	58	91	56	75	71	90	53	11	14	16	17	46	
7	91	12	39	277	142	76	129	133	144	6	18	5	42	-	
8	109	4	36	71	85	56	87	124	59	19	159	<1	625	116	
9	464	168	148	168	231	-	100	362	135	n.d.	45	-	300	-	
10	64	92	48	135	75	142	67	95	31	13	5	45	142	78	

a - average pattern observed in seven surgical biopsies of human liver, n.d. - not detected

The data in Table 1.2, extracted from Shonk and Boxer (1967), present 15 enzyme activities in 10 human hepatomas relative to those found in normal human liver. While Shonk and Boxer (1967) and Bosmann and Hall (1974) have shown that human colon carcinomas exhibit a uniform enzyme pattern, human hepatomas demonstrate considerable variation in enzyme activity. Data presented by Knox (1972) suggest that rat mammary carcinomas are considerably less variable than rat hepatomas. The enzyme activities which show greatest alteration in the hepatomas are those which are involved in some of the specialized functions of the liver. It appears likely that hepatomas exist in diverse gradations and are considerably more variable than most other neoplasms. This may be partly due to a lack of cellular uniformity of the developed hepatoma and variations between hepatocytes in the pre-neoplastic stage. Rappaport (1963) describes regional differences in properties and functions in rat liver.

The phenotypic variation demonstrated amongst neoplasms may be reduced or exaggerated by a number of environmental and physiological factors. The enzyme patterns of neoplasms, and especially hepatomas may be altered, depending on the time of day when the animals were killed, in relation to fasting or feeding, by the level of protein in the diet (Potter et al., 1969) or by the injection of hormones, theophylline or adrenalectomy (Watanabe et al., 1969).

The above brief consideration of neoplasia and enzyme activities indicates that while there is no shortage of descriptions of phenotypic variation, the basis of this variation and in fact that of the neoplastic transformation itself is not understood. Studies of neoplasia in the intact animal have been hampered to a considerable extent by the fact that the cells which give rise to the tumour cannot be recognized, and that various physiological and environmental influences can greatly affect the variation. The use of cell culture techniques makes it possible to greatly reduce this environmental variation, as well as to study the progeny of single cells.

1.6 Enzyme activities in cultured cells

1.6.1 Cell cultures and enzyme regulation studies

Cultured cell systems offer many potential advantages over the intact animal for studies of enzyme regulation. The investigator can utilize single-cell species and isolate more effectively single parameters for control, such as specific nutrients, drugs and hormones, and phases of cell growth. In addition, use of clonal cell populations allows for the development of mutants, the use of which has been vital in understanding enzyme regulation in microbial systems. Schimke (1969) lists 20 enzymes whose levels can be altered in cell culture and which have been the subject of enzyme regulation studies. The types of stimuli that affect enzyme levels in these systems is highly varied, ranging from viral infection to alterations in the cell or culture cycle, or alterations in added nutrients and drugs (see Schimke and Doyle, 1970).

Whether cell lines show histotypic differentiated properties or are relatively undifferentiated, "fibroblast-like" cells, they have been shown to exhibit a variety of enzyme regulatory phenomena. All the systems studied in intact animals have been found in certain cell lines and their study has contributed greatly to our knowledge. The use of cell cultures in such studies has been considered by Schimke (1969) and Rechcigl (1971). Apart from studies on regulation of enzyme activity, or induction and repression, a contribution of cell culture studies has been to allow examination of enzyme activity variation during the cell cycle.

In culture, the stage of both growth phase and cell replication cycle influence a number of enzyme activities. The activities of thymidine kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase and deoxyribonuclease show significant changes during the cell cycle (see Schimke, 1969). Klevecz (1969) observed intermittent changes in lactate dehydrogenase activity during the cell cycle and attributed them to changes in synthesis with a relatively constant rate of degradation. Berg et al. (1975) found that lysosomal enzymes did not show fluctuations of enzyme activity during the cell cycle. Another interesting observation is the discovery that tyrosine aminotransferase is inducible at a certain stage of the cell cycle, whilst being constitutive at other stages (see Gelehrter, 1971).

Certain enzyme activities tend to remain the same during the phase of rapid and slow proliferation, while others tend to increase significantly during the phase of slowed growth or confluence. In human fibroblast cultures, the specific activities of glucose-6-phosphate dehydrogenase, galactose-1-phosphate uridyl transferase (see Mellman, 1971), catalase (Sun et al., 1975), galactosidase and glucosidase (Galjaard et al., 1974a) and acid phosphatase (Ryan et al., 1972) increase as mitotic activity of the culture decreases. Certain other enzymes tend to show little or no difference in activity between pre-confluent and early growth, but activity decreases as cells are allowed to remain in post-confluent culture. Lactate dehydrogenase, hexokinase, phosphofructokinase and galactokinase appear to lose activity during the post-confluent phase of the culture (Mellman, 1971). The precise mechanism of this loss of activity is uncertain; instability of the enzyme molecule, depletion of stabilizing substrates, or allosteric effects of metabolic products are possibilities.

Although as yet there have been few studies which have exploited cell cultures to examine this aspect of enzyme activity variation, the future can be expected to see greater use being made of the advantages of such a system. In existing studies, there is difficulty in the interpretation of specific activity as a function of the time that cells have proliferated undisturbed in a culture vessel. Miedema and Kruse (1965) demonstrated that cell protein content may change during the culture cycle. Thus, it is reasonable to suspect that some of the published specific activity differences may not always correctly reflect the enzyme content of cells. Cell cycle parameters also may change during the period of exponential growth, and frequency of medium change greatly influences these parameters (Kimball et al., 1974; Ryan et al., 1972).

1.6.2 Loss of functions and enzyme activities in cultured cells

Most reviews on the use of cell culture systems begin with lamentations on the well documented phenomenon of loss of enzyme activities during explantation, or the extremely low, or absence of many enzyme activities in cultured cells (see Eagle, 1965; Davidson, 1964; Schimke, 1969; Green and Todaro, 1967). Loss of differentiated functions characteristic of the tissue of origin has often been noticed in cell culture (Davidson, 1964; Wigley, 1975), and the similarity between many of the cell lines derived from normal tissue and some tumour tissue noted. If we follow the loss of specialized syntheses by cells in culture, we see that each of these functions may be lost at its own individual rate, in other words this loss of expression of the differentiated state is not an all-or-none phenomenon (Terzi, 1974).

Probably the most extensive and detailed descriptive studies of the process of transformation from tissue cell to cultured cell in the literature are still those of Champy carried out mainly between 1912 and 1920 (see Champy, 1920). It was this worker who first applied the term "dedifferentiation" to the events characteristically occurring when a culture is initiated. The main contributions of the early tissue/cell culture work were to establish that (a) loss of overt differentiated form and function occur when a culture is initiated, (b) this process occurs with extreme rapidity, sometimes even preceding the initiation of mitotic activity, (c) at least in some cases the actual parenchymal of other histotypic cells are the ones whose descendents constitute the derived cell culture, though often being overgrown by "fibroblast-like" cells (see Champy, 1920; Davidson, 1964).

Three main hypotheses have been proposed to explain the loss of overt differentiated functions and enzyme activities in cell culture. These are:-

(1) Overgrowth in a heterogeneous population of the cell type of interest by another cell type more suited to proliferation in the culture environment (Schimke, 1969).

(2) Inadequate nutrition, accessary co-factors or environmental stimuli may result in decline and loss of differentiated functions and associated enzyme activities (Green and Todaro, 1967).

(3) There is a genuine decline in degree of differentiation although environmental conditions are adequate for its expression. Such an irreversible decline could be analogous to dedifferentiation in regeneration or a transdifferentiation in neoplasia.

The cell's environment in vivo differs profoundly from that in culture, in terms of spatial arrangements, extracellular factors and even mechanical forces, and viewed in this light it is not surprising that differentiated functions tend to be lost in culture (Terzi, 1974). If these differentiated functions are dependent on the maintenance of complex interactions they might frequently be lost in culture due to lack of, or disruption of, the proper connections. However, in the long term the patterns compatible with growth tend to be similar and, in spite of the differences in tissue of origin, the cells tend to have similar enzymic constitutions (Terzi, 1974).

Regardless of the tissue or species of origin, a "fibroblast-like" cell type usually predominates when new cultures are isolated from tissues, and it has been suggested that the specialized or differentiated cells of the tissue are lost at an early stage (Sato et al., 1960; Franks and Cooper, 1972). In studies on rat liver cells in culture, Sato et al. (1960) concluded that the proliferating cells did not derive from hepatocytes but from a minority population which was at an advantage in culture conditions and hence outgrew the hepatocytes. Franks and Cooper (1972) described the ultrastructural characteristics of cell lines from various tissues of mice and suggested that the cell lines were derived from primitive mesenchymal cells of vascular origin, which may adopt either of two morphologies in culture and which occasionally may express specific differentiated functions in culture. The selection hypothesis does not exclude the fact that specialized cells may lose their functional capacity during the time that they survive culture conditions but suggests that they do not contribute to the rapidly dividing population which eventually predominates.

The selection hypothesis must account for the great diversity of differentiated functions exhibited by a large number of established cell lines (see Wigley, 1975). If this diversity is derived from cells of identical origin (Franks and Cooper, 1972), then the cell culture environment results in a great range of aberrant gene expressions and ectopic production of a wide range of proteins e.g. fibroblasts may be found that synthesise myosin (Terzi, 1974). Although this may be a satisfactory explanation, it simply begs more evidence before it can be accepted as a general phenomenon. The great problem is that it is impossible to estimate the proportion of cell lines which demonstrate aberrant gene expression. The reported cases, although numerous (Wigley, 1975), probably represent only a fraction of all cell lines initiated. However, since no systematic survey has been conducted, this question cannot be answered.

The problem of inadequate nutritional or environmental factors is central to the techniques of cell culture. Recent years have seen major developments in the composition of culture media, and this progress is demonstrated by the increasing success in culturing cells of diverse origins and properties (see Wigley, 1975). However each cell type is likely to have its own optimum conditions for maintenance and growth, and thus there probably exists a number of unidentified factors which are responsible for the maintenance and expression of differentiated functions in the intact animal. Culture conditions constitute an environment which is predictably inadequate for the maintenance of the full range of differentiated cellular characteristics. It thus seems attractive to consider that deficiencies in the culture conditions are responsible for the loss of most differentiated functions and associated enzyme activities. The loss of function can often be reversed when the proper conditions are restored in culture (Cahn and Cahn, 1966; Coon 1966) or when the cells are restored to the animal (Priest and Priest, 1964; Finch and Ephrussi, 1967). Thus, in these examples the cells appear to have retained their differentiated state but require an external stimulus before this is expressed. Unless the culture conditions are known to be adequate for stable support of the expression of a differentiated function, absence of the end product does not necessarily signify any basic loss in specialization.

The third hypothesis that a genuine decline or loss of differentiated function occurs when a cell is transferred to the culture environment, although attractive, is difficult to verify. The ability of mouse fibroblasts to synthesise collagen or hyaluronic acid decreases with age in culture (Green and Todaro, 1967). However, the time span involved in these observations, the many years that L cells have been in culture, is different from the few days or weeks normally associated with loss of differentiated function in culture. The reduction in collagen and hyaluronic acid synthesis over many years in cultured L cells is thus likely to be the result of some other phenomenon. The most convincing evidence of a change in differentiated state is the fact that isoenzyme patterns of liver cells in culture are similar to those of poorly differentiated tumours and foetal liver but vastly different from those of liver cells in situ (Nitowsky and Soderman, 1964; Yasin and Goldberg, 1966; Weinhouse, 1974). However, before such observations can be accepted as evidence of a change in the differentiated state of the cultured cell, it is necessary to be sure of its identity. Hence, these observations do not distinguish between the selection hypothesis and genuine changes in the differentiated state.

The main evidence to suggest that changes in differentiated functions may occur in cultured cells is derived from experiments with oncogenic viruses. Viral transformation of the cells in culture may result in the loss (Green, 1970) or gain (Baluda, 1962) of differentiated functions characteristic of a cell type as well as the emergence of other specific properties (Green, 1970). Green and Todaro (1967) consider it doubtful that spontaneous changes in the reverse direction of differentiation occur in cultured cells, and consider that contamination by other cell types may be the cause in reported changes. It should be noted that the expression of differentiated function in cultured cells may be inhibited by bromodeoxyuridine (Silagi and Bruce, 1970) or hybridization with fibroblastic cells (Rintoul et al., 1973).

An additional theory to explain loss of specific enzyme activities and differentiated functions in cell culture proposes that the process of mitotic proliferation is fundamentally antagonistic to the differentiated state and/or function. This theory has been critically examined by Davidson (1964). In recent years data have been collected which show that many types of differentiated cell divide in vivo, in situ and in culture. However, proliferation of the differentiated cell may be less rapid than that of less differentiated cells, and as a result, overgrowth will occur (Green and Todaro, 1967).

The available evidence makes it impossible to distinguish between the hypotheses. However, it seems likely that no single hypothesis will be universally true. Individual cases of loss of differentiated function (or rather its expression), or specific enzyme activities will have individual explanations which will depend on the circumstances, cell type and function or activity considered.

1.6.3 Differentiated cells in culture

The relatively few cell lines in culture which have been shown to be derived from a specialized cell of any type have usually originated from tumour tissue (Wigley, 1975). Terzi (1974) asserts that if tumour cells in vivo retain differentiated functions, they are more likely to express these functions in culture than their normal counterparts. However, the problems of origin of cell lines in culture are also illustrated by those derived from tumour tissue. Many tumour-derived cell lines in culture in fact consist of normal stromal cells present in the original cell inoculum which have been preferentially selected (Wigley, 1975).

The few examples of apparently normal, specialized mesenchymal cells in culture have been listed by Wigley (1975). These include cell lines of chondrocytes, myoblasts, osteoblasts and corneal epithelium. Mesenchymal cells, like some tumour cells, appear to have an inherent ability to survive and grow in tissue culture, a property not shared by most normal epithelial cells. Specialized mesenchymal cells are subject to the same problems as all cell cultures in the potential overgrowth of the culture by less specialized "fibroblast-like" cells. The true fibroblast must be considered to be a specialized cell and is capable of synthesizing collagen. Franks and Cooper (1972) suggest that the cells responsible for overgrowth of cultures are not fibroblasts but "primitive" cells with "fibroblast-like" appearance and probably share many properties with tumour cells.

Cells derived from the epithelium, such as glandular parenchymal cells, have been the most difficult to establish in culture. It has not proved possible to isolate and propagate normal epithelium for any reasonable length of time in culture. Those reports describing long-term cell lines of supposedly epithelial origin usually put forward inadequate criteria for their identification (Wigley, 1975). "Epithelium" is used in this sense to describe cell types which cover or line surfaces or glands, not in the misleading morphological sense often used in the cell culture literature. This morphology is a contiguous pavement-like sheet of polygonal cells and is often assumed to indicate a true epithelial origin. Grisham et al. (1975) hold the view that normal, fully differentiated hepatocytes with their myriads of functions have never been obtained in proliferating culture. When hepatocytes are cultured they appear structurally simple and maintain only some limited but specific properties of hepatocytes in situ (Grisham et al., 1975). The fact that these studies, and those of Chessebeuf et al. (1974, 1975) yield cells in culture, isolated from liver cell suspensions, which simultaneously demonstrate a number of hepatocyte specific functions, suggests that the cell lines may have been derived from hepatocytes which subsequently lost some of their in situ properties.

1.6.4 Phenotypic variation in cultured cells

The impression is often presented in the literature that cell lines tend to possess similar enzymic constitutions (e.g. Davidson, 1964;

Terzi, 1974). It is suggested that long-term patterns compatible with growth in culture tend to be similar and thus the cell lines show convergence of properties (Terzi, 1974). That such a view can be elevated to generality is not possible. The variability which abounds between normal cell lines catalogued by Wigley (1975) indicates that for many cell lines, convergence of properties does not occur, even after considerable periods in culture. Thus, while the cell lines discussed by Wigley (1975) represent a class which shows considerable phenotypic variability, it is not possible to estimate the extent of this phenomenon. However, it is undoubtedly true that a large number of cell lines from apparently different origins show convergence of their properties. Such a situation is analogous to that described for tumours in the previous section. While a pattern of convergence of properties may be evident, considerable phenotypic variability can be discerned when specific differentiated functions are considered.

In some ways the problem has been confounded by the introduction of the concept of "household" and "luxury" functions (Ephrussi, 1972). This concept distinguishes between "ubiquitous" functions, i.e. metabolic functions essential for the maintenance and growth of any cell, and "differentiated" functions which must be necessary for the survival of the multicellular organism and of the species, but not for that of the cell. It is doubtful whether any cell in culture has only "ubiquitous" or "household" functions and by the considerations mentioned in the previous section, cells in culture must be regarded as being differentiated in some sense. The concept suggests that when differentiated cells are explanted and placed in culture, selection operates and the specialized syntheses become useless or even detrimental and thus tend to be lost (Terzi, 1974). Such a phenomenon would result in cell lines possessing only "ubiquitous" functions, i.e. their properties converge. Although the concept may possess some theoretical value when considering problems of differentiation, at this stage evidence does not appear to enforce an operational value.

The finding by Franks and Cooper (1972) that the same cell type is established in culture from a number of tissues, suggests caution in interpreting data on convergence of cell line properties. If many cell lines from separate tissues in fact come from the same cell type, convergence of properties would be expected. However, with this in mind the data reviewed by Davidson (1964) does suggest a striking similarity in enzyme patterns between some long-term cell lines. The pessimistic view that this similarity is the result of contamination by one of the cell lines under study, e.g. HeLa (see Lavappa et al., 1976) should not be excluded. As with hepatomas, examples exist of cell lines which show vastly different activities of the same enzyme. The variations in enzyme activities in many of the neoplasms mentioned in the previous section are often manifested when these neoplastic cells are cultured (Aviv and Thompson, 1972; Kulka et al., 1972; Richardson et al., 1974; Tashjian et al., 1975).

Non-neoplastic cells may also demonstrate phenotypic variability (Cristofalo et al., 1967). For example, alkaline phosphatase activity may vary 100-fold between human cell lines of diverse origins (Davidson, 1964). Various clones of a mouse liver cell line possess 20-200 times the β-glucuronidase activity characteristic of normal liver cells (Kuff and Evans, 1961) and induction of arginase by arginine shows considerable variation between certain human cell lines (Davidson, 1964).

Nitowsky and Herz (1961) demonstrated a large amount of intra-strain variation of alkaline phosphatase activities in HeLa cell lines. This variability was also apparent between clones derived from given strains (Bottomly et al., 1969). In these studies, Bottomly et al. (1969) examined HeLa strains and observed a 200-fold variation in alkaline phosphatase activity, 12-fold for glucose-6-phosphate dehydrogenase, 8-fold for lactate dehydrogenase and 2-fold for 6-phosphogluconate dehydrogenase. In addition, 3 out of 7 strains possessed alkaline phosphatase activities which were inducible by hydrocortisone. These authors suggested that mutation and karyotypic variability were the cause of this large variation in enzyme activities.

Data collated by Davidson (1964) on variation between four clones descended from one C3H mouse cell demonstrate considerable phenotypic variability. In addition to karyotypic variability, the activities of hexokinase, phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase varied 4-fold, 9-fold and 6-fold respectively. β-Glucuronidase and arginase activity also showed marked variation from clone to clone in a larger study of 17 clones.

These early studies suggest that although derived from genetically identical material, each clone is unique if all its properties are considered together. Davidson (1964) believes that the divergence between clones is the result of mutations affecting the functions considered. The data could be viewed as a record of divergent population evolution under identical environmental circumstances. Although Davidson (1964) cites cases of mutation which affect enzyme activities and nutrient requirements of cultured cells, the evidence that the phenotypic variation of clones of identical origin is the result of mutation is non-existent. As in the case of phenotypic variability of neoplasms, recourse could be made to explanations based on epigenetic variations. In 1960, Scott et al. suggested that enzyme variations between C3H clones was due to epigenetic variation.

The phenotypic variation of cell lines is also demonstrated by the studies on those initiated from the same tissue sample. This variation is not due to variation in enzyme assays, culture conditions or the stage at which the cells are harvested. In a study of several cell lines from one human skin biopsy, Milunsksy et al. (1972) observed a 60-500% variation in the activities of five lysosomal enzymes. These workers suggest that fibroblasts from the same individual exhibit considerable heterogeneity in enzyme constitution. Such variability was previously suggested by Felix and Demars (1971). There also exists a wide range of propionyl CoA carboxylase (Gompertz et al., 1975) and acid hydrolase (Hultberg et al., 1973; Young et al., 1975) activities in cell lines which were initiated from the same human skin biopsy. Although epigenetic variations in the same cell type may account for this variation, there are several studies which suggest that the variation may be the result of comparing cell lines derived from different cell types.

Martin (1966) demonstrated a great variation in enzyme activities between related clones of human skin fibroblasts. He suggested that while the fibroblasts themselves may have been heterogeneous, it was possible that very different types of cells occasionally become established in culture and appear as non-specific "fibroblasts". Such cells could include smooth muscle cells, endothelial cells, hair follicle epithelial cells, eccrine and apocrine epithelial cells, schwannian cells and fat cells. Papayannopoulou and Martin (1966) demonstrated heterogeneity of cells migrating from a human skin explant. Constitutive cells with high activities of alkaline phosphatase were found at a frequency of 2×10^{-2}. Recently, Martin et al. (1974) have demonstrated the emergence of a number of cell types from a skin biopsy, and suggest that these contribute to the heterogeneity found in cultures. Amniotic fluid cells in culture are probably derived from a heterogeneous population (Nadler, 1972). Melancon et al. (1971) demonstrated that histidase activity in cells from the same sample of amniotic fluid was only found in those with "epithelial" morphology and not cells with "fibroblast" morphology.

As in the case of neoplasia, chromosomal "disorder" has been suggested to be the cause of both phenotypic variation and the loss of specific functions in culture. Almost all established cell lines are heteroploid in karyotype, though diploidy may persist for a long period under appropriate conditions (Hayflick, 1965). It is not clear whether chromosomal abnormalities occur as mitotic errors gradually accumulated in culture as time goes on, or whether they appear suddenly in high frequency after a certain period in culture has elapsed. The early observations of Champy (1920) suggest that loss of differentiated status can occur prior to the first mitotic division in culture, and as such is unlikely to be the result of chromosome abnormalities occurring in culture. Since there exist a number of both short-term (Davidson, 1964) and long-term (Hayflick, 1965) diploid cell lines, it appears that a normal karyotype is not incompatible with proliferation in culture. However, in a number of cases, mitotic irregularities and the development of chromosomal "disorder" occurs at nearly the same time as the loss of differentiated cell characteristics (Davidson, 1964) although there is no evidence to suspect a causal relationship. An interesting idea has been entertained by Terzi (1972a) who suggests that cells in culture rapidly become cytogenetically polymorphic and that the variation in karyotype represents a loss of selection which may operate against many abnormal chromosome constitutions in vivo. Thus, provided they do not affect viability in the relatively constant culture environment, chromosome "disorders" will be tolerated.

Human biopsy material maintains a stable chromosome complement in culture (Hayflick, 1965). Unless the original tissue was chromosomally aneuploid, the cell culture that develops is essentially diploid (Mellman, 1971). An interesting association between chromosomal variability and enzyme activity variation in human cells has been suggested by Decarli et al. (1963). These workers found that alkaline phosphatase activity was correlated with the number of small acrocentric chromosomes in a heteroploid cell line. However, this observation has not been confirmed in cells exhibiting trisomy-21 (Cox, 1965) and Cristofalo et al. (1967) found that heteroploidy and cell morphology change was not associated with alkaline phosphatase activity. The idea has recently been resurrected by Nose and Katsuta (1975) in their studies on constitutive variants for alkaline phosphatase in CHO cells. These workers suggest that chromosomal change may have resulted in the constitutive variants. The studies are, however, not strictly comparable because Decarli et al. (1963) were studying a more-or-less continuous distribution of activity whereas Nose and Katsuta (1975) studied rare variants which demonstrated a 300-fold increase in activity. However, it seems likely that certain chromosome "disorders" render cells more susceptible to neoplastic transformation (see Mellman, 1971).

1.6.5 Phenotypic variation and medical diagnosis

Cultivated human cells are being used with increasing frequency for the detection and investigation of familial metabolic disorders. Cell culture methods for identifying individual heterozygotes for recessive mutations are important in genetic counselling and also in testing amniotic fluid cells of an early foetus threatened with a defined genetic disease. This aspect of somatic cell genetics has been reviewed by Hsaia (1970), Krooth and Sell (1970), Mellman (1971), Milunsky and Littlefield, (1972) and Nadler (1972), and at present the enzyme defects characteristic of various hereditary metabolic diseases of humans have now been observed in skin fibroblasts in culture in over 50 different diseases. Nowhere is phenotypic variability of cultured cells more apparent and important than in the area of medical diagnosis. The use of cultured cells in diagnosis assumes that the phenotype in culture reflects the phenotype of the individual, from which the genotype is often deduced.

The causes of phenotypic variability in medical diagnosis are the result of the factors discussed above. Accurate diagnosis requires strict control of culture and assay conditions, and the cells must be taken from the same stage of the culture cycle. A few recent studies have demonstrated that heterozygotes may not be detected for a number of diseases. In a number of cases, the heterozygote is expected to possess an enzyme activity intermediate between the normal and affected homozygotes, but cannot be accurately diagnosed because of phenotypic variability. Such cases include hypercholesterolemia (HMGA reductase; Goldstein et al., 1974), Gaucher's disease (β-glucosidase; Beutler et al., 1971), glycogen storage diseases (phosphorylase kinase; Migeon and Huijing, 1974), infantile metachromatic leukodystrophy (aryl suphatase A; Kaback and Howell, 1970) and combined immunodeficiency disease (adenosine deaminase; Chen et al., 1975). Mellman (1971) considers the cases where heterozygotes can be unambiguously identified. The lower activity (Kaback and Howell, 1970) and considerable variation (Gerbie et al., 1972) of many enzyme activities in cultured amniotic fluid cells makes diagnosis even more difficult. It is possible that such difficulties may be overcome after identification of cell type, and phenotypic variation has been described for each particular enzyme activity considered.

1.7 Purpose of this study

The above considerations demonstrate that while several studies have specifically considered phenotypic variation in normal and neoplastic tissues, the extent and mechanism of this phenomenon is not understood. Intact animal studies have often proved too complex and have failed to dissect constitutive, epigenetic variation from environmental effects. It is with this problem in mind that recourse was made to cell culture systems for the study of phenotypic variation in normal and neoplastic cells.

Previous studies have been hampered by doubts as to the origin and homogeneity of the material in question. These studies have been based on cells whose determined and differentiated states before culture or neoplastic transformation have been unidentified. It was the initial purpose of this study to develop a system whereby cells of known identity and differentiated potential can be isolated in culture. These cells, Kupffer cells, were to be isolated from the Chinese hamster whose distinctive diploid karyotype (2n = 22) facilitates cytogenetic study.

Once identified, cell lines possessing histotypic differentiated functions would be suitable material with which to study phenotypic variation. The heterogeneity with respect to several enzyme activities was to be studied in a large number of cell lines initiated from identical, identified material. The aim of this approach was to yield insights into the loss of differentiated functions and enzyme activity, and to investigate the extent and distribution of enzyme activity variation. At present it is not possible to distinguish whether the variation described in previous studies was the result of a continuous distribution or the detection of rare variants, perhaps mutants, selected from a discontinuous distribution of enzyme activity. If the cited variation is in fact due to rare variants, it would be possible to estimate their frequency and utilize them for genetic regulation studies. Enzyme activity variation was also to be described in foetal Kupffer cell lines.

The previous sections have eluded to several factors in common between the neoplastic cell and the normal cell in culture. In both types there is a suppression of the normal phenotype, alteration in differentiated state, selection for proliferative capacity and the emergence of common, often foetal, enzyme patterns. Thus, it may be of value studying the normal cell in culture to give insight into the possible regulatory changes which may occur in neoplasia. These changes were to be compared with those in cultured normal cells which had been transformed with an oncogenic virus and thus presumed to possess malignant potential.

Section 1.5 has presented evidence to suggest that considerable variation arises out of the neoplastic process, with the result that a number of tumours and their derivative cell lines possess "anomalous" enzyme activities. It is possible that neoplastic transformation of cells in culture may increase phenotypic variation, and thus increase the probability of isolating cell lines with "anomalous" or constitutive enzyme activities. In this study it was proposed to transform Kupffer cells with Simian Virus 40, examine the variation of a number of enzyme activities, and to estimate the frequency of the "anomalous" enzyme activities.

The final objective was to analyse enzyme activity data from the primary, transformed and foetal cell lines with a view to examining the possibility of co-ordinate inter-relationships between enzyme activities.

1.8 Terminology

The terminology adopted in this dissertation is in accordance with that proposed by the Tissue Culture Association (see Federoff, 1967), the only exception being in the use of the term "primary cell line". The term "primary culture" was originally used by Hayflick and Moorhead (1961) to describe the stage of culture which terminates with the formation of the first confluent monolayer and subculture. The implication is that until this stage the cell population was heterogeneous, and after the first subculture there is greater selection for those cell types suited to proliferation in culture. There appears to be no precedent in terminology for the cell lines utilized in this study. Each cell line was derived from a single cell, freshly isolated from the animal and was never allowed to reach confluence during the study period. Thus, each cell line was homogeneous with respect to origin. The term "primary cell line" will be used to designate such cultures, and will be distinct from "transformed cell line", which in this study is a primary cell line transformed by the oncogenic virus, Simian Virus 40. The primary cell lines will be derived from either adult or foetal material.

SECTION 2

GENERAL MATERIALS AND METHODS

2.1 Solutions

Solutions were prepared with chemicals of analytical grade or high purity and sterile, metal distilled water.

Phosphate buffered saline (PBS):

Solution A (PBS/A) pH 7.2	Sodium chloride	170 mM
	Potassium chloride	3.4 mM
	Disodium hydrogen phosphate	10 mM
	Potassium dihydrogen phosphate	2 mM
Solution B	Calcium chloride	6.8 mM
Solution C	Magnesium chloride	4.9 mM

Complete PBS consists of solutions A, B C (8:1:1, v/v).

Versene solution:

PBS/A containing 0.6 mM EDTA (disodium salt) and 0.0029% (v/v) phenol red, pH 7.2.

Trypsin solution: 0.25% Difco trypsin in tris saline.

Tris saline (pH 7.4)	Sodium chloride	140 mM
	Potassium chloride	5.1 mM
	Disodium hydrogen phosphate	0.7 mM
	Glucose	5.5 mM
	Tris (hydroxymethyl) amino methane	25 mM
	Phenol red	0.0015%
	Penicillin (Glaxo)	1000 units/litre
	Streptomycin (Glaxo)	0.1 g/litre

The above solutions were sterilized by passage through a 0.22 μ Millipore (13 mm diameter) filter.

2.2 Cell culture methods

General cell culture techniques have been described by Kruse and Patterson (1973) and Paul (1975). The substrates upon which the cells were grown are indicated in the relevant sections. These substrates were either glass coverslips (Chance #1, Macfarlane Robson Ltd.), plastic petri dishes or Linbro wells (Nunclon or Biocult Ltd.), or glass bottles. Cultures grown in plastic wells or petri dishes were incubated at 37°C in humidified incubators containing an atmosphere of 95% air; 5% CO_2. Cultures grown in glass bottles were grown at 37°C after gassing with the above mixture. Coverslips for cell culture purposes were acid washed (0.5 N HCl, 60 mins, 100°C followed by distilled water rinse and 0.5 N NaOH, 60 mins, 100°C), rinsed overnight in running tap water followed by several changes of distilled water. After a final rinse with absolute ethanol, the coverslips were air-dried and sterilized in a hot air oven. Cells attached to coverslips were cultured in plastic petri dishes.

2.3 Medium

All cells were cultured in Glasgow modified Eagle's minimum essential medium (Macpherson and Stoker, 1962) supplemented with non-essential amino acids (glycine, alanine, aspartic acid, asparagine, glutamic acid, each 0.1 mM; proline, serine, both 0.2 mM), nucleosides (adenosine, guanosine, cytidine, uridine, each 30 μM; thymidine, 10 μM) and 1 mM sodium pyruvate. This medium was also supplemented with foetal calf serum (17% v/v; Gibco-Biocult Ltd.). Several different concentrations of foetal calf serum were used in the studies described in Section 3.3.2. The volume of medium used for culturing in each type of vessel was constant-Linbro wells, 1 ml; 35 mm petri dish, 2 ml; 50 mm petri dish, 5 ml; 90 mm petri dish, 12 ml; 4 oz. bottle, 10 ml; 8 oz. bottle, 20 ml.

2.4 Subculture of cells

Cells were detached from the substrate using trypsin and versene solutions (see section 2.1) in the proportion of 1:2. Cell monolayers were briefly washed with a small volume of this solution, incubated in approximately 50 $\mu l/cm^2$ substrate surface area of fresh solution for 10 mins at 37°C in a humid atmosphere, and resuspended in two volumes of culture medium. The subculturing routine for all cell lines is described in section 3.2.3 .

2.5 Mycoplasma contamination

Routine checks were made for mycoplasma contamination using the method of Fogh and Fogh (1964). Cells were grown on coverslips until 50% confluence. The medium was replaced with 3 ml of 0.6% sodium citrate which was then slowly diluted with distilled water to 0.45%. After 10 mins of this hypotonic treatment, the cells were fixed with glacial acetic acid/methanol (1 : 3) fixative for 10 mins. The coverslips were air-dried and stained for 5 mins with orcein (2%, Gurr's orcein in 60% acetic acid). After drying, the coverslips were mounted and examined for the presence of darkly stained granules scattered around the cell periphery.

2.6 Protein determination

The protein concentration of cell extracts was determined using the dye-binding fluorescence technique developed by Hiraoka and Glick (1963) and modified by Bade (1973). Standards were established by substituting known amounts of BSA (Fraction V, Armour Pharmaceuticals). Using micro-cuvettes it was possible to obtain a linear relationship between fluorescence and protein for 10^{-9} g to 10^{-4} g in the cuvette. The dye used for this determination was eosin Y (Gurr). The assay volumes were decreased proportionately below those described by Hiraoka and Glick (1963) and Bade (1973) when only small volumes of cell extract were available.

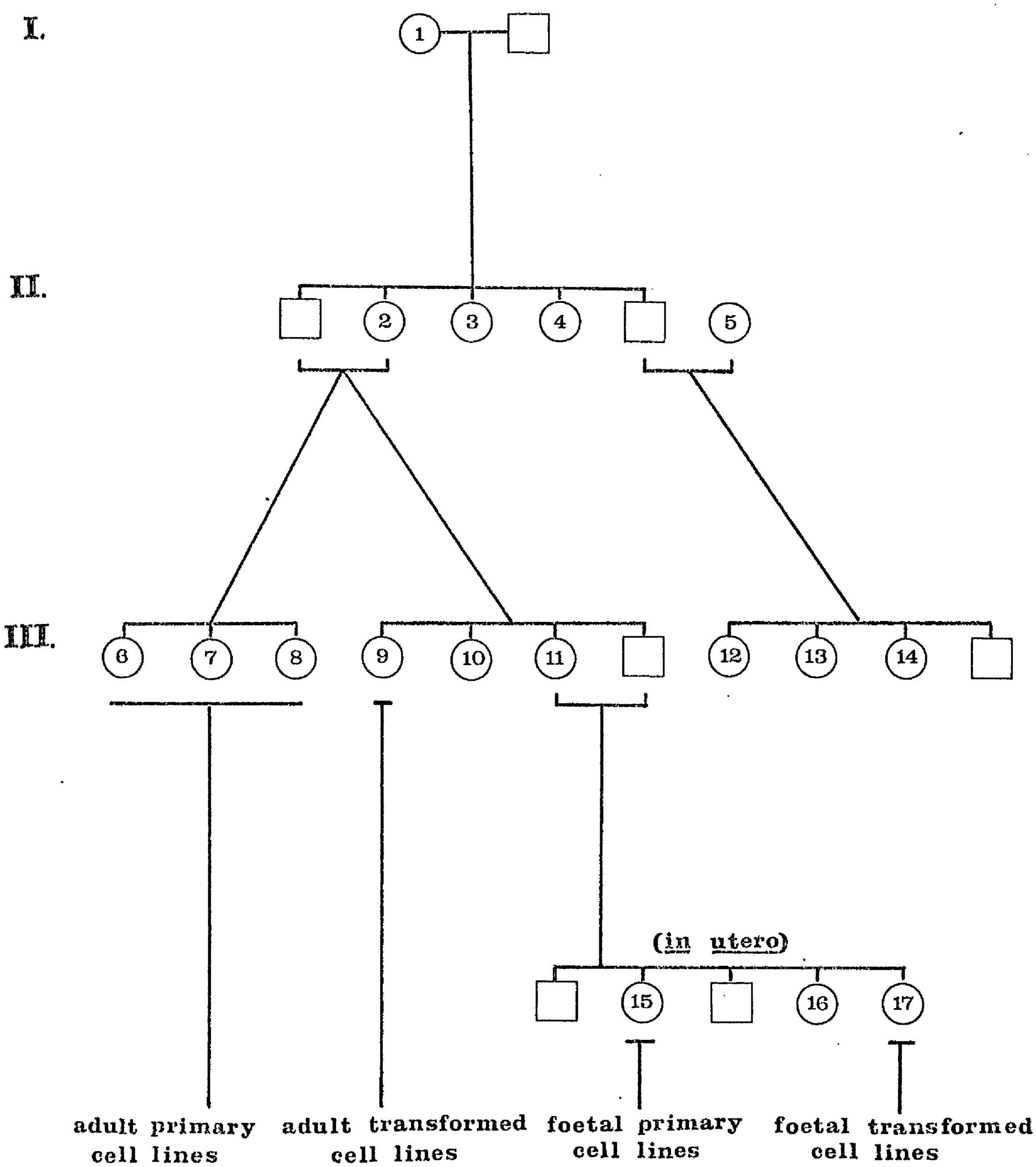

Figure 2. 1:- The pedigree of Chinese hamsters from which the Kuppfer cell lines were isolated.

The female animals used in the various studies are identified by number.

2.7 Glassware and instrumentation

All glassware for enzyme assays and protein determination was acid washed as described in Lowry and Passonneau (1972). Glassware for the preparation of cell extracts was coated with "Repelcote" (Hopkin and Williams Ltd.). Most enzyme activities were assayed utilizing fluorometric techniques, the principles of which have been discussed by Udenfriend (1969) and Lowry and Passonneau (1972). Fluorescence determinations were made with a Baird-Atomic "Fluoripoint" recording spectrofluorometer, and catalase activity determined with a Unicam SP 1800 spectrophotometer.

2.8 Animals and pedigree

Chinese hamsters were obtained from the Institute of Virology, University of Glasgow and had been maintained on standard pellet food. The pedigree relationships between animals from which cell lines originated are shown in Figure 2.1. Cell lines were only initiated from female animals. Animals utilized for the various studies have been identified with numbers which are referred to in the relevant sections.

SECTION 3

THE ISOLATION, CHARACTERIZATION AND KINETICS OF ADULT KUPFFER CELLS IN CULTURE

3.1 Introduction

In order to study phenotypic variation in cultured cells, it is essential to utilize initial material which is both homogeneous and of known origin. While the mammalian liver provides the largest, accessible source of material from a single tissue, this organ contains a variety of cell types. The hepatocytes constitute 90-95% of hepatic cellular weight but only 60-65% of total liver cell population, and the reticuloendothelial cells constitute 5-10% of liver weight, or approximately 35% of the total cellular population. Bile duct, connective tissue and blood vessel wall cells constitute a small percentage of total cell population and a minor component of liver mass (see Lentz and DiLuzio, 1971). The development of reliable enzymatic tissue dissociation techniques (Howard and Pesch, 1968; Berry and Friend, 1969; Haung and Ebner, 1969) has enabled the separation of parenchymal and non-parenchymal cells from liver (Mills and Zucker-Franklin, 1969; Lentz and DiLuzio, 1971; Berg et al., 1972; Bissell et al., 1972; Berg and Boman, 1973). Although the major cell types of liver can now be separated for more detailed study, this investigation is restricted to the separation and culture of a class of hepatic reticuloendothelial cell - the Kupffer cell.

Kupffer cells are mononuclear phagocytes lining the liver sinusoids (Aterman, 1963) and are believed to be unrelated to sinusoid endothelial cells (Wisse, 1974b). The problem of divergent nomenclature of Kupffer cells reflects the uncertainty of their origin. These cells have also been termed macrophages, histiocytes or phagocytes which are either sinusoidal, littoral, stellate or reticuloendothelial (Wisse, 1974a). The physiological function of the Kupffer cell is related to the marked phagocytic capacity of this cell and to its role as a filter for particulate material. Materials phagocytized include bacteria, virus, effete erythrocytes, lipids, cholesterol (Benacerraf, 1963), colloidal particles (Bissell et al., 1972), endotoxins (Trejo and DiLuzio, 1973) and denatured proteins (Buys et al., 1973).

Although the cytology of the Kupffer cell has been well documented, the functional relationships with hepatocytes and endothelial cells have not been established. In the liver, Kupffer cells have variable but often stellate shape, fuzzy surface coat, well developed vacuolar apparatus and an ultrastructure similar to that of macrophages (Wisse, 1974a). The mitotic frequency of Kupffer cells in mouse liver has been estimated to be 0.7% (Shorter and Titus, 1962). A characteristic of the Kupffer cell is the presence of high peroxidase activity localized in the endoplasmic reticulum, perinuclear cisternae and annulate lamellae (Fahimi, 1970; Widmann et al., 1972). The activity of peroxidase in liver reticuloendothelial cells may be 30 times that found in hepatocytes (Berkel, 1974) and may be related to the elimination of micro-organisms from the blood (Widmann et al., 1972).

Kupffer cells are also important in haemoglobin catabolism (Bissell et al., 1972). The existence of microsomal haem oxygenase (MHO) activity in Kupffer cells distinguishes them from sinusoid endothelial cells (Bissell et al., 1972). This enzyme catalyses fission of the protoporphyrin ring of haemoglobin to biliverdin, which is then converted by NADPH-dependent biliverdin reductase to bilirubin; the microsomal haem oxygenase being rate limiting (Tenhunen et al., 1969).

Some biochemical properties of liver reticuloendothelial cells have been described. Although few studies have specifically considered Kupffer cells, the data give indications as to their properties. Relative to hepatocytes, liver reticuloendothelial cells possess high activities of glycosidases (Scamman et al., 1975), β-glucuronidase (Wachstein, 1963), acid deoxyribonuclease (Berg and Boman, 1973), ribonuclease and cathepsin (Wattiaux et al., 1956). Liver reticuloendothelial cells contain the "liver specific" enzymes hexokinase (Sapag-Hager et al., 1969) and aldolase B (Crisp and Pogson, 1972). They also contain different isoenzyme forms or distributions of tyrosine-α-ketoglutarate transaminase (Civen and Brown, 1973), pyruvate kinase (Berkel et al., 1972) and lactate dehydrogenase (Berg and Blix, 1973) to those found in hepatocytes.

The three criteria which serve to distinguish Kupffer cells from all other known liver cells are (a) marked phagocytic activity (Bissell et al., 1972), (b) peroxidase activity (Widmann et al., 1972) and (c) microsomal haem oxygenase activity (Bissell et al., 1972). Cells isolated from liver which demonstrate all three of these properties are thus Kupffer cells.

The existence of criteria for the identification of Kupffer cells makes them potential material for the study of identified cells in culture. The first attempt to culture Kupffer cells was that of Beard and Rous (1934). In their study no attempt was made to distinguish the cultured cells from other reticuloendothelial cells. Bennet (1966) described the isolation and cultivation of phagocytic cells from the mouse liver. Melly et al. (1972) established short-term cultures of liver cells which exhibited diverse phagocytic capabilities. Recently, Munthe-Kaas et al. (1975) isolated and, for a short period, maintained in culture large numbers of Kupffer cells from rat liver. Sandstrom (1965) and Williams et al. (1971) both described contaminating cells of probable reticuloendothelial origin in liver cell cultures with an "epithelial-like" morphology. To date, attempts at long-term culture of Kupffer cells have not been successful. In the above attempts to culture Kupffer cells, phagocytic activity was the only criteria for establishing identity of the cells. Since sinusoid endothelial cells may demonstrate limited phagocytic activity (Wisse, 1974b), this property may not be adequate for unequivocal identification of cell type.

The fact that Kupffer cells can be identified by three criteria and established in culture makes them potential material for the study of phenotypic variation in culture. This section describes the isolation of Kupffer cells by modifications of existing methods and the establishment of these cells in long-term culture. Cells which originated from the liver reticuloendothelial cell fraction and initially exhibited the properties of phagocytic activity, peroxidase activity and microsomal haem oxygenase activity in culture were considered to be of Kupffer cell origin.

Once this identity had been demonstrated, a description of phenotypic variation in culture could be undertaken.

3.2 Materials and Methods

3.2.1 Isolation of Kupffer cells

The procedure for isolating Kupffer cells was developed from several liver cell isolation techniques (Gallai-Hatchard and Gray, 1971; Melly *et al.*, 1972; Berg and Blix, 1973; Bissell *et al.*, 1973; Howard *et al.*, 1973). All solutions for perfusion and incubation were prepared with double glass-distilled water and briefly gassed with a 95% air/5% CO_2 mixture. Aseptic techniques were used throughout and all solutions filtered through a Millipore unit (0.45 μm pore size).

Cells were isolated from ten week old female Chinese hamster siblings at 10:00 hours. Immediately after sacrifice of the animal by cervical dislocation, the liver was twice perfused with 10 ml of warm Ca^{++}-free Eagle's minimal medium containing 1 mM sodium pyruvate, 0.1% protease (Type V, Sigma), 0.1% collagenase (Type I, Sigma) and 0.05% hyaluronidase (Type I, Sigma). Omission of Ca^{++} enhanced dissociation and reduced phagocytosis (Ryder *et al.*, 1975). Perfusion by syringe and hypodermic needle (22 or 24 guage) was via the portal vein, and the superior vena cava outflow from a nick above the renal vein was discarded. The blanched liver was removed and the gall-bladder, large veins and capsule discarded. Using scalpels, the remaining tissue was minced in 2 ml of incubation medium to yield approximately 1 mm^3 pieces. After brief agitation the supernatant was discarded and the tissue piece transferred into two 50 ml conical flasks containing 10 ml of incubation medium. Incubation medium was Ca^{++}-free Eagle's minimal medium containing 0.1% collagenase and 1 mM pyruvate. The mixture was briefly gassed with a 95% air/5% CO_2 mixture, sealed and incubated for 20 mins. at 37°C with gentle agitation. The resulting suspensions were pooled and filtered through three thicknesses of gauze

(0.5 mm mesh) into 2 ml of foetal calf serum. The remaining pieces of liver were digested for a further 10 mins. with 0.25% protease in Ca^{++} -free Eagle's minimal medium containing 1 mM pyruvate, and the filtered supernatant added to the previous filtrate. The suspension was centrifuged (70g, 2 mins., 10°C) in order to remove parenchymal cells (Bissell et al., 1973). The resulting supernatant was centrifuged (500 g, 5 mins., 10°C) and the pellet resuspended and incubated in 10 ml of Ca^{++} -free Eagle's minimal medium containing 1 mM pyruvate and 0.25% protease for 20 mins. at 37°C with gentle agitation. The resulting cell suspension was centrifuged (500 g, 5 mins., 10°C), the pellet resuspended and washed in 5 ml of culture medium, centrifuged and reconstituted to 1 ml with fresh medium. The culture medium has been described in section 2.3.

3.2.2 Establishing primary Kupffer cell lines

The cell suspension obtained from enzyme digestion was plated into two 50 mm plastic petri dishes containing culture medium and unidentified "fibroblast-like" cells allowed to attach. After 1 hour, the supernatants were removed with gentle agitation and replated into 50 mm plastic petri dishes containing either glass coverslip fragments (approximate area, 4 mm^2) or 13 mm glass coverslips and culture medium containing 0.2 mg collagenase/ml. Approximately 8 hours after the second plating, the supernatant was replaced by fresh medium. The removal of "fibroblast-like" cells by differential attachment is based on the method described by Williams et al. (1971). The attached cells were inspected at 24 hour intervals and those which failed to divide were removed by micro-pipette and the glass fragment or coverslip gently washed in fresh medium. Only colonies arising from single cells were allowed to remain. After six days glass fragments which supported a single colony were transferred to plastic culture wells (Linbro). At this stage the colonies contained approximately 50-100 cells. The cells were removed by a drop of trypsin/versene solution (see section 2.4), the glass discarded and fresh medium added to the well. Each well contained a single clone which was used as the origin of a primary cell line. The medium

Figure 3.1:- Procedure for sub-culturing and harvesting cell lines

Details of the culture kinetics can be found in the text. (p.e. - plating efficiency).

Stage:-

1. Freshly dissociated cells seeded onto glass fragments and grown to colonies of approximately 100 cells.

↓

2. Transfer cells (p.e. 20%) to plastic culture wells and grow to approximately 5×10^4 cells.

↓

3. Seed 4×10^4 (p.e. 25%) into 50 mm petri dish and grow to a density of 525 cells/mm^2 (10^6 cells). Prepare cell extract for examination (I).

↓

4. Seed 2×10^4 cells into 50 mm petri dish (p.e. 29%) and grow to a density of 525 cells/mm^2. Prepare cell extracts for examination (II).

↓

5. Seed 2×10^4 cells into 50 mm petri dish (p.e. 35%) and grow to a density of 525 cells/mm^2. Prepare cell extracts for examination (III).

↓

6. Seed 2×10^4 cells into 4 oz. glass bottle (p.e. 43%) and grow to a density of 525 cells/mm^2 (2.4×10^6 cells).

↓

7. Subsequent sub-culturing based on an inoculum of 4×10^4 cells into 4 oz. glass bottle and grown to the above density.

Estimates of cumulative cell population doublings, time in culture, and population doubling time at the end of each stage.

Stage	Cumulative population doublings in culture	Cumulative time in culture (days)	Population doubling time (hrs.)
1	7	8	20.3
2	19	17	17.1
3	26	21	15.3
4	33	25	14.8
5	40	30	15.1
6	48	35	14.7
7+	+7	+4.5	

was changed every day for the first seven days after isolation from the liver and thereafter every three days. Collagenase was included for only the first two days, thus assisting in the removal of any contaminating fibroblasts.

3.2.3 Subculturing routine

Cells were grown and harvested under conditions of medium composition, pH and handling kept as uniform as possible. In Figure 3.1 appears the schedule for subculturing and preparing cell extracts from the cell lines. The number of cell population doublings are estimates based on data for doubling times presented in section 3.3.5. Cells were harvested when the density was 525 cells/mm^2 calculated over four randomly selected 1 mm^2 fields and corresponding to 10^6 cells in the 50 mm petri dish or approximately 50% confluence. This procedure made it possible to minimize variation due to harvesting cell lines at different stages of the culture cycle. At the first examination stage a small number of cells were plated onto coverslips and phagocytic and peroxidase activities determined as described below.

After three subculturings in petri dishes, all cell lines were transferred to 4 oz. glass bottles for further culturing. Extracts from cells grown in 4 oz. bottles were prepared at the stage of 525 cells/mm^2, equivalent to a total of 2.4×10^6 cells. After inoculation with 4×10^4 cells, this density was achieved in approximately 5 days. The transition from petri dishes to glass bottles was achieved with an inoculum of 2×10^4 cells. A strict routine required subculturing the cells every five days and seeding 4×10^4 cells into a fresh bottle. With the exception of a lag period after plating, the cell population was maintained in exponential growth and not allowed to attain confluence.

Harvesting the cells for subculturing and enzyme assays was accomplished with trypsin/versese solution (see section 2.4). The cells were dislodged, gently dissociated in 5 ml of fresh medium, centrifuged (500 g, 5 min., 10°C) and resuspended in 0.9 ml of PBS/A containing 0.1 mg/ml BSA (Fraction V, Armour Pharmaceutical). Cell concentration was determined by haemocytometer and a suitable aliquot containing either 2×10^4 or 4×10^4 cells was used to continue the cell line in a petri dish or glass bottle, respectively.

3.2.4 Demonstration of phagocytic activity

In order to demonstrate phagocytic activity, living cells were incubated at 37°C in 1 ml of culture medium containing 1 μg of colloidal carbon/10^3 cells (colloidal graphite in water, "Aquadag", Acheson Colloids, Plymouth, U.K.). After 30 mins. the cells were examined by phase-contrast microscopy for the presence of clumps of ingested carbon in the cytoplasm. Within this period uptake of carbon by other cell cytpes was negligible. Clones in which carbon uptake was demonstrated in greater than 95% of cells were regarded as being phagocytic. The phagocytic activity was always determined with reference to a control cell line derived from "fibroblast-like" cells isolated from the first plating of liver cell suspension, and at no time demonstrated Kupffer cell properties. Small quantitative differences in carbon uptake occurred between medium supplemented with different batches of foetal calf serum. This variability in no way affected the qualitative classification of cells for phagocytic activity. Variation in serum electrolyte concentrations may account for different phagocytic capacities (Ryder et al., 1975).

Kupffer cells were also labelled before dissociation from the liver. This was achieved by perfusion of the liver with 50 ml of warm culture medium containing 100 μg colloidal carbon/ml. Perfusion was performed for 10 mins., re-using the labelled medium at the rate of approximately 20 ml/min. The liver was then dissociated as described above.

3.2.5 Histochemical detection of peroxidase activity

Cells were fixed for 20 seconds with cold 1.5% gluteraldehyde in PBS/A (pH 7.4), and washed in 0.5 M Tris-HCl (pH 7.4) containing 5% sucrose for 2 mins. at 4°C. The staining method of Wisse (1974a) was employed using 0.05% diamino-benzidine (Sigma), 0.02% hydrogen peroxide ("Aristar" 100 volumes, BDH Chemicals) and 7% sucrose in 0.05 M Tris-HCl (pH 7.4) at 25°C for 60 mins. The cells were post-fixed and dehydrated in graded ethanol solutions, mounted and examined for staining. Clones in which greater than 95% of the cells were stained were considered to be peroxidase positive. Cytochemical controls for the peroxidase activity were performed by incubation without hydrogen peroxide, and cell type controls were conducted with the "fibroblast-like cells" from the first liver suspension plating.

3.2.6 Microsomal haem oxygenase assay

The assay of MHO activity is described by Tenhunen et al. (1968) and preparation of the cell extract described in section 4.2.2. All solutions were prepared with double glass distilled water. To a reaction tube were added 5 µl 17 mM haemin (Type I, Sigma), 5 µl 140 mM NADP, 5 µl 400 mM glucose-6-phosphate, 5 µl 660 mM magnesium chloride, 450 µl 90 mM phosphate buffer (pH 7.4) and 30 µl cell extract. Incubation was undertaken for 45 mins. at 37°C. In a control tube NADP and glucose-6-phosphate were replaced by buffer. At the end of incubation the bilirubin formed was determined by the fluorescence method described by Roth (1967). A 100 µl aliquot of incubation mixture was mixed with 50 µl 6% BSA in PBS/A and 600 µl 85% phosphoric acid. After 2 mins., 2 ml of water was added and fluorescence determined (ex. 435 nm/em. 500 nm). A blank was prepared by first mixing incubation mixture and water, then adding phosphoric acid. Standard tubes were established as for the control tubes but contained known concentrations of bilirubin (Sigma). Enzyme activity was expressed,as moles bilirubin formed/mg. protein/min. Since assay of a microsomal fraction isolated by high speed centrifugation had negligible effect on enzyme activity, all assays were performed on crude cell extracts.

3.2.7 Cell suspension properties and culture kinetics

Cell number was estimated with a Neubauer haemocytometer. Exclusion of 0.5% trypan blue (BDH Chemicals) in PBS/A containing 3% BSA was used as an indication of cell viability. Due to phagocytic activity, it was important to estimate viability within a few minutes. The proportion of unstained cells relative to total stained and unstained cells provided an estimate of cell viability.

Plating efficiency of cells in the first subculturing was the proportion of cells forming colonies when a known number of cells (approximately 100) were seeded into a 30 mm petri dish. Subsequent plating efficiency estimates were the proportion of cells forming colonies when 200 cells were seeded into a 50 mm petri dish. The petri dishes were incubated at 37°C in a humidified incubator for 8 days, stained with Giemsa and colonies counted.

Initial cell population doubling time was based on the time required to double the number of cells in a colony. Subsequent estimation of cell population doubling time was based on the time taken for the cell population to double in number when in the exponential phase of growth. Cell population doubling times during the initial stages of culture were not based on cell lines used for other studies. Parallel cultures were used to obtain estimates of population doubling time. Estimates of the length of the lag period after seeding and plating efficiency were necessary before cumulative cell population doublings could be calculated.

3.2.8 Karyology

Karyotype preparation followed the G banding method described by Slack et al. (1976)

3.3 Results

3.3.1 Isolation of liver cell suspensions

The cell isolation technique was characterized by studying liver cell suspensions from three animals of the same sex and age as those which were used for establishing cell lines. All values are quoted $\pm$ standard deviation (SD). The yield of material after the primary dissociation with collagenase was calculated from the amount of cellular protein in the suspension and total protein in the whole liver. The livers contained 335.7 $\pm$ 25.5 mg of protein and the isolated cells accounted for 217.7 $\pm$ 26.1 mg of protein. Thus, 65% of liver protein was recovered in the cell suspension. The total number of cells isolated from a liver was $171 \times 10^6 \pm 17 \times 10^6$. In the suspension, 92% of the cells were single and the remainder were accountedfor by aggregates of up to five cells. Trypan blue exclusion, and thus viability was estimated to be 90.1 $\pm$ 3.5%.

After centrifugation and protease digestion to remove hepatocytes, only 8.8 $\pm$ 1.8 mg of cellular protein was recovered. This reticuloendothelial cell suspension contained $57 \times 10^6 \pm 8 \times 10^6$ cells or 33% of all cells in the total liver cell suspension. Contamination by cells other than those of reticuloendothelial origin was low. The 1% which appeared to be of hepatocyte origin were usually nonviable. Polymorphonuclear cells accounted for 3% of the cell suspension. Viability of the cell suspension was 93.7 $\pm$ 2.5%.

Colloidal carbon was added to the reticuloendothelial cell suspensions and phagocytic activity demonstrated in 23% of the cells. These cells were considered to be Kupffer cells and the yield from each

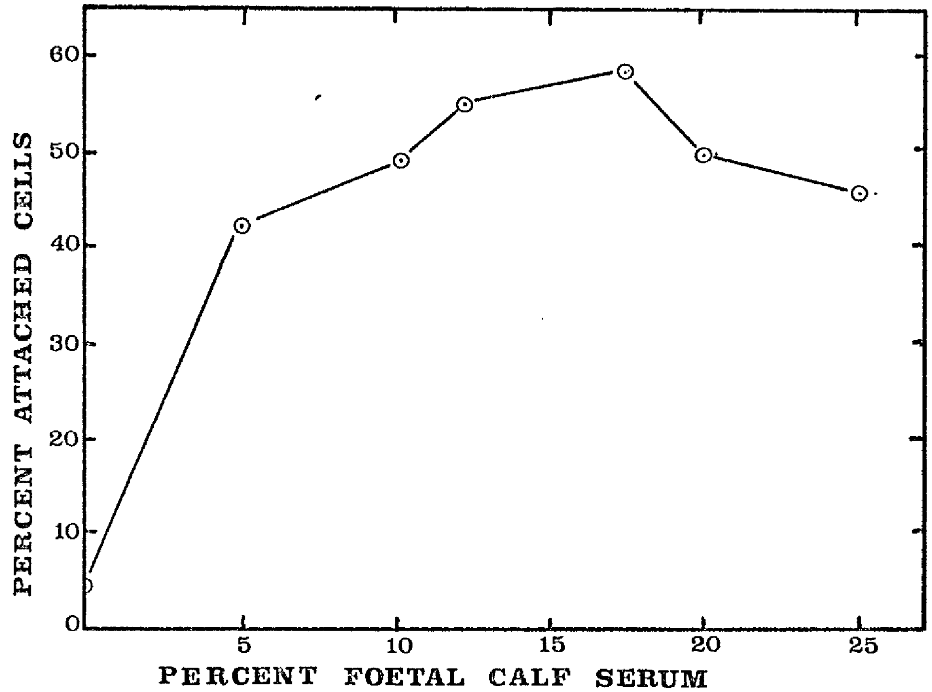

Figure 3.2:- The effect of foetal calf serum on the attachment of freshly isolated adult Kupffer cells to plastic petri dishes.

Attachment was determined 24 hrs. after plating and is shown as the mean of duplicate plates.

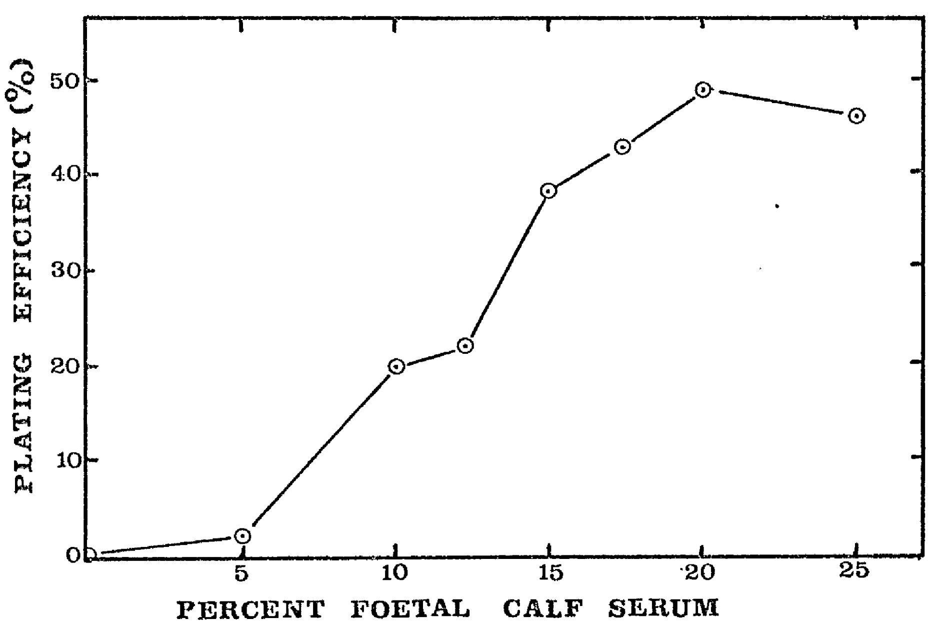

Figure 3.3:- The effect of foetal calf serum on the plating efficiency of adult Kupffer cells after 40 cell population doublings in culture.

Each point is the mean of duplicate plates.

animal was $9.3 \times 10^6 \pm 0.9 \times 10^6$ cells/gm wet weight of liver. The presence of carbon in the Kupffer cell cytoplasm made it impossible to estimate viability by dye exclusion. An estimate of the yield as a proportion of total Kupffer cell population can be obtained from the data of Bissell et al. (1972). Their data reveal that rat liver contains 31×10^6 Kupffer cell/ gm wet weight. On the assumption that Chinese hamster liver contains a similar concentration of Kupffer cells, the isolation technique employed in this study yields approximately 30% of the total Kupffer cell population. Since no data are available for the concentration of Kupffer cells in the Chinese hamster liver, this figure can only be taken as an estimate of yield.

3.3.2 Establishing dissociated Kupffer cells in culture

Cells in the final suspension were plated into plastic petri dishes. Those cells which attached during the short period of the first plating subsequently formed compact colonies with extreme "fibroblast-like" morphology. The cells which phagocytized colloidal carbon attached only during the second plating period. After labelling with colloidal carbon it was apparent that 58% of the freshly isolated, labelled cells adhered to the substrate.

The proportion of foetal calf serum in the culture medium greatly influenced the fraction of labelled cells which attached to the substrate. Figure 3.2 presents the effect of different serum proportions, and demonstrates that maximum attachment of freshly isolated Kupffer cells occurs when the culture medium contains 17% foetal calf serum. Continued culture revealed that approximately 10% of the labelled cells in the final cell suspension formed colonies, this figure providing an estimate of the initial plating efficiency.

The effect of serum proportion on plating efficiency of Kupffer cells after 40 population doublings in culture is presented in Figure 3.3. The cell line used was one of the lines studied in section 3.3.5. The data demonstrate that the optimum proportion of foetal calf serum for attachment of cells when Kupffer cell lines were initiated (17%), was not optimal for plating efficiency of Kupffer cells after an extended period in culture. However, since the most critical stage in establishing a cell line appears to be the initial attachment and first few divisions of the precursor cells, it was considered that optimal conditions at this stage had priority over less critical later stages. Since one of the pre-requisites of this study was a culture environment kept as constant as possible, the foetal calf serum proportion of 17% was used for all studies.

When phagocytized carbon had been diluted by a few cell divisions, it was possible to detect peroxidase activity in the progeny of cells which demonstrated phagocytic activity. In a study of 58 colonies which developed from the reticuloendothelial cell suspension, 38 demonstrated phagocytic activity. In a sample of 64 colonies from the same cell suspension, 43 colonies demonstrated peroxidase. It thus appears as if approximately 2/3 of colonies which develop from the reticuloendothelial cell fraction are derived from Kupffer cells and the remaining 1/3 are probably related to endothelial cells. Nearly all cell lines initiated from clones which demonstrated peroxidase and phagocytic activities also possessed MHO activity (see section 3.3.3). Thus, dividing cells were obtained in culture and these possessed all three Kupffer cell functions. It is concluded that the cells which adhere to the substrate and subsequently form colonies from the second plating period after preparation of the liver reticuloendothelial cell suspension are primarily Kupffer cells and that these colonies can be readily identified from those which are derived from other cell types.

3.3.3 Isolation of cloned cell lines

After attachment to coverslip fragments, the progress of the cells was carefully observed. Only a single attached cell was allowed to remain on the fragment. In this way it was certain that a colony had developed from a single cell. Liver reticuloendothelial cell suspensions from three sibling female Chinese hamsters (nos. 6, 7 and 8 in Figure 2. 1) were plated into petri dishes containing coverslip fragments. Coverslip fragments carrying colonies derived from cells after the second plating period were used to intiate 195 cell lines from selection of 248 colonies. Thus, 79% of colonies yielded viable cell lines which survived at least until the first examination stage which corresponded to approximately 26 population doublings in culture (stage 3 in Figure 3. 1).

At the first examination stage all cell lines were examined for Kupffer cell characteristics. Of the 195 cell lines, 130 demonstrated phagocytic, peroxidase and MHO activities, 2 possessed only peroxidase and MHO activities and 63 did not exhibit any of these characteristics. The 130 cell lines which possessed phagocytic, peroxidase and MHO activities were considered to be derived from Kupffer cells and were retained for subculture and further study.

3.3.4 Cell and culture morphology

In the early stages of culture, the Kupffer cells appeared similar to other cultured macrophages (see Bennett, 1966). The cells readily attached to the substrate and extended cytoplasmic processes and appeared to possess a stellate outline with numerous thin projections and ruffling at the cell periphery. The result of this morphology was a very large, flat cell which measured approximately 20 microns in the smallest diameter (see Figure 3. 4a). Although the cells did not appear to be very mobile their outline was constantly changing. The Kupffer cells were generally pellucid with a pale cytoplasm and nucleus. The nucleus was often eccentric and indented, the nucleolus being clearly visible. The cytoplasm possessed a granular appearance and small vacuoles could be discerned. The pigment which often appeared in freshly isolated Kupffer

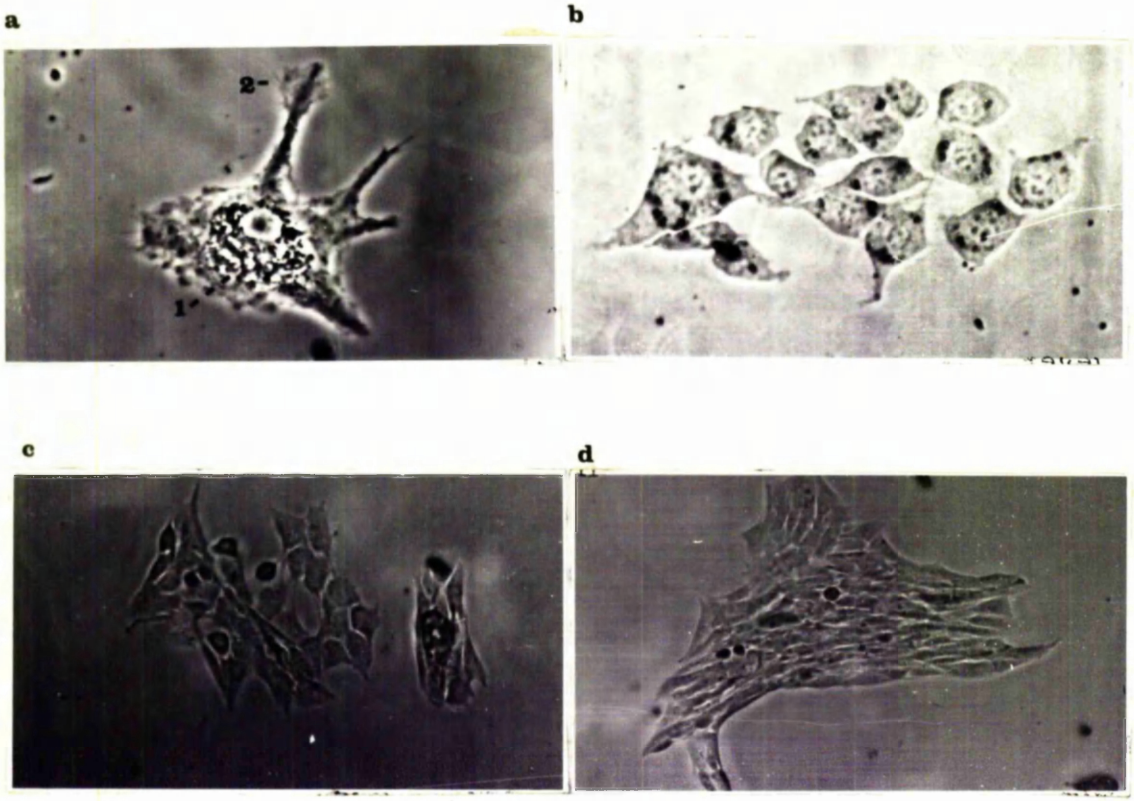

Figure 3. 4:- Chinese hamster Kupffer cell colony morphology at various stages in culture.

(a) Freshly isolated Kupffer cell before any mitoses in culture. Note phagocytized carbon colloid (1) and ruffling periphery (2) (approx. x 1500).

(b) Colony of Kupffer cells after approximately 5 days in culture. Dark Granules are phagocytized carbon (approx. x 750).

(c) Colony of Kupffer cells after approximately 6 days in culture (approx. x 200).

(d) Colony of Kupffer cells seeded after approximately 26 population doublings in culture (approx. x 200).

The culture morphology of primary Kupffer cell lines can be seen in Figure 5. 3 (facing page 108).

cells may have resulted from the ingestion of red blood cells during the isolation procedure. This pigment disappeared after a few hours in culture. During the early phases in culture Kupffer cells appeared connected by numerous cytoplasmic processes, some very thin and others of considerable thickness.

The cell morphology underwent considerable transition during the period of culture (see Figure 3.4a, b, c, d). The ratio of cytoplasmic area to nuclear area was initially greater than six. However, as the culturing period progressed this ratio declined to approximately 2-3. The general transition in morphology was from a cell with constantly changing stellate outline to one which was "fibroblast-like" in appearance. During this period the cytoplasm lost its granular appearance and became relatively clear. After the cell lines had been maintained in culture for 30-40 population doublings, the cells had assumed a "fibroblast-like" appearance (see Figure 5.3). The transition to a "fibroblast-like" morphology generally commenced within the first few cell divisions in culture. These cells extended fewer cytoplasmic processes and became elongated. At later stages in culture, cell morphology was partially dependent on cell density. As cell density increased, the Kupffer cells became elongated, cytoplasmic processes at the sides were withdrawn, and the cells adopted a classical fibroblast morphology. The size of the cultured Kupffer cells also underwent considerable change. While the smallest diameter of freshly isolated and attached cells was generally greater than 20 microns, this was reduced to less than 5 microns after 40 population doublings in culture.

The culture morphology was a reflection of cell morphology. Colonies of cells in the first few divisions in culture were dispersed in appearance and the loosely associated cells had a polygonal appearance (see Figure 3.4 b). As the cells were maintained in culture for further population doublings, colony morphology changed to that of fusiform fibroblasts in parallel array forming a dense reticulum (see Figure 3.4d and Figure 5.3).

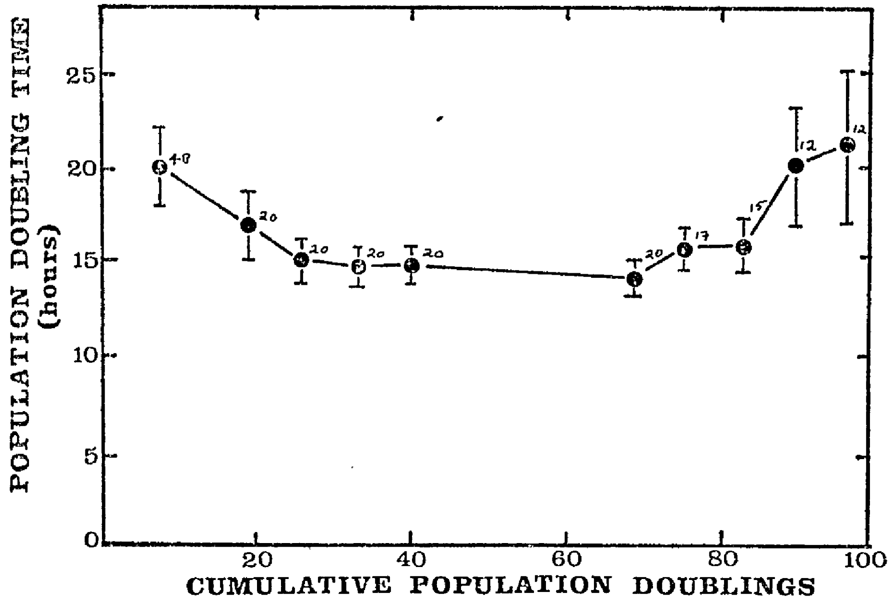

Figure 3.5:- The relationship between population doubling time and cumulative population doublings in culture for adult Kupffer cell lines.

Figures indicate sample size, values are means ± SD.

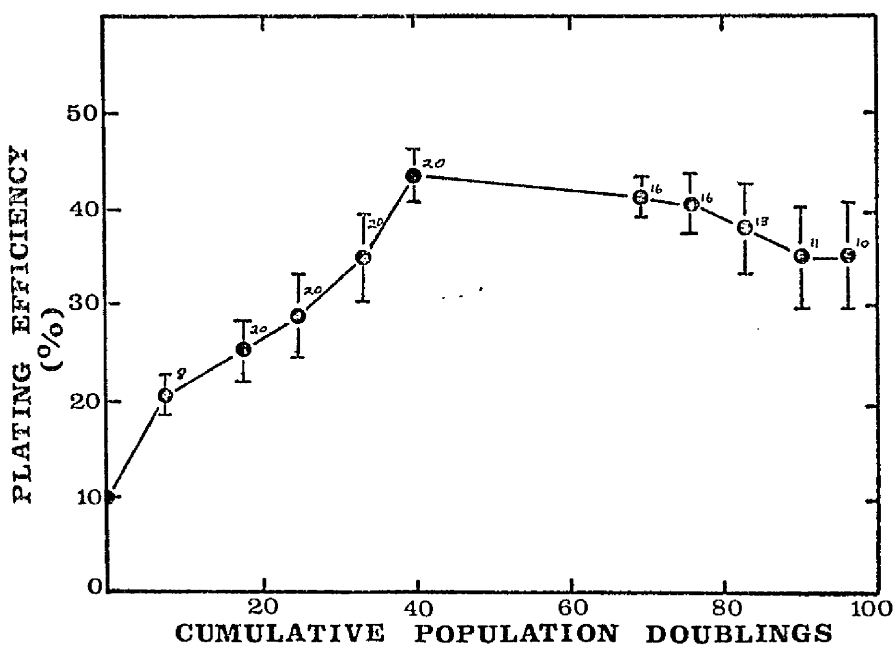

Figure 3.6:- The relationship between plating efficiency and cumulative population doublings in culture for adult Kupffer cell lines.

Figures indicate sample size, values are means ± SD. The initial value is based on the approximation presented in section 3.3.2.

At all stages, and even beyond 90 population doublings in culture, the Kupffer cells exhibited strong density dependent inhibition of division. When the culture had attained a cell density of approximately 8.9×10^4 cells/cm^2, cell division ceased. Although at saturation density and non-proliferating, this confluent monolayer remained metabolically active for a long time. After the second subculture, one particular Kupffer cell line was maintained at confluence for seven months with only one change of medium after three months. Upon subculturing, the cells were capable of proliferation, plating efficiency was reduced, but they still possessed Kupffer cell functions.

3.3.5 Culture kinetics of Kupffer cell lines

Cell population doubling time was estimated on a number of occasions during culture of Kupffer cell lines established in parallel to the 130 cell lines which are the subject of this study. The population doubling time of the initial cell colonies was determined for 48 colonies obtained from the same cell suspension which was used to initiate the 130 cell lines. These colonies demonstrated phagocytic activity and were thus presumed to be of Kupffer cell origin. The colonies were of approximately 60-80 cells in size and doubled in number every 20.3 ± 2.0 hours. The population doubling times of cell lines derived from 20 of these colonies were determined after the first subculture - the stage when cells were dissociated from the colonies and seeded into plastic culture wells. Aliquots of 5,000 from these cell lines demonstrated a population doubling time of 17.1 ± 1.3 hours during the exponential phase of growth. The studies were continued and the relationship between cell population doubling time and cumulative population doublings in culture in shown on Figure 3.5. The cumulative population doubling times were estimated from the number of cells plated and recovered, and the plating efficiency estimates described below. The subculturing routine of these 20 cell lines was the same as that described in Figure 3.1. At the point equivalent to examination stage I, small aliquots of cells were removed and used for the determination of phagocytic, peroxidase and MHO activities. All of the cell lines demonstrated these activities and it was concluded that they were of Kupffer cell origin. Thus, Kupffer cells in culture undergo a change in population doubling time. The doubling time is minimal between approximately 40 and 70 cumulative doublings in culture. After this stage, both the doubling time and its variation increases.

Estimations of plating efficiency were based on colonies selected from the original 48 used for culture kinetics studies. The plating efficiency of the first subculturing was based on 8 colonies which were trypsinized, the cells counted and seeded into 30 mm plastic petri dishes. At this stage the plating efficiency was 20.2 $\pm$ 1.9%. The remaining colonies were subcultured according to the routine described in Figure 3.1 and the plating efficiency determined on aliquots of cells obtained at each subculturing. Small aliquots were removed at the point equivalent to examination stage I, and the presence of phagocytic, peroxidase and MHO activities confirmed.

With knowledge of the plating efficiency at any given stage in the culturing of these cell lines and the number of cells plated and recovered, it was possible to estimate cumulative population doublings in culture. In Figure 3.6 appears the relationship between plating efficiency and cumulative population doublings. The plating efficiency of Kupffer cells steadily increases to a maximum after approximately 40 population doublings in culture. After this point there is a decline in plating efficiency and a concomitant increase in its variation.

Irrespective of cell line age, the lag period after subculturing before mitotic activity commenced was approximately 3 hours. Knowledge of cumulative population doublings in culture, lag period and population doubling time yielded estimates of cumulative time in culture which were in close agreement with chronological age in culture (see Figure 3.1).

Table 3.1:- The persistence of Kupffer cell functions in Kupffer cell lines maintained in culture.

At the stage of 55 population doublings in culture, 130 cell lines were considered and shown to possess all three Kupffer cell functions. Thereafter the sample size was reduced to 15 cell lines, 7 of these expiring between 83 and 97 population doublings in culture. + indicates presence of function, - indicates absence of function.

69 population doublings

	Cell line														
Activity	002	021	032	043	055	066	072	095	103	105	112	118	123	027	088
Peroxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MHO	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Phagocytic	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-

83 population doublings

	Cell line														
Activity	002	032	043	055	066	095	103	021	105	118	123	027	072	088	112
Peroxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MHO	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Phagocytic	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-

97 population doublings

	Cell line							
Activity	055	021	095	066	072	088	112	123
Peroxidase	+	+	+	+	+	+	+	+
MHO	+	+	+	-	-	-	-	-
Phagocytic	+	-	-	-	-	-	-	-

3.3.6 Persistence of Kupffer cell functions in culture

After approximately 55 population doublings in culture, all 130 primary Kupffer cell lines had survived and were re-examined for the presence of Kupffer cell functions. Phagocytic and peroxidase activities were present in all cell lines. Although reduced below the first value, MHO activity was also present in all 130 cell lines after 55 population doublings (MHO activity is described in greater detail in section 4).

A sample of 15 of these cell lines was selected for continued culture and Kupffer cell functions examined at the 69, 83 and 97 cell population doubling stages in culture. The survival of these cell lines and persistence of Kupffer cell functions are summarized in Table 3.1. After 83 population doublings, all cell lines demonstrated peroxidase activity; however, intensity of staining was reduced in those cell lines which did not possess MHO activity. A more detailed description of the persistence of MHO and peroxidase specific activities can be found in section 4.

Although no attempt was made to quantify phagocytic activity, it was apparent that both phagocytic capacity and rate were diminished when compared with cells at an earlier stage in culture. After 97 population doublings, only one cell line of the original 15 possessed all three functions. By this stage, only 8 of the 15 cell lines had survived and were capable of proliferation in culture. At all stages, those cell lines in which MHO activity could not be detected, also did not exhibit phagocytic activity. The sample size is too small for an assessment of whether those cell lines which loose Kupffer cell functions first have a greater chance of survival to at least the stage of 97 population doublings.

3.3.7 Karyology of cultured adult Kupffer cells

The karyotypes of a number of primary adult Kupffer cell

Table 3.2:- The effect of the number of population doublings in culture on the proportion of diploid cells in adult Kupffer cell lines.

Cell line	Percent diploid cells: Population doublings 30	50	90
002	92	88	*
004	88	-	-
010	86	-	-
013	92	-	-
017	94	-	-
021	80	82	60
027	86	82	*
029	84	-	-
032	86	78	*
043	94	92	*
055	96	90	82
061	94	-	-
066	84	80	66
072	88	92	68
088	80	74	58
092	94	-	-
095	94	92	88
099	92	-	-
103	88	84	*
105	94	88	*
112	78	84	68
118	84	80	*
123	92	82	60
127	90	-	-
130	90	-	-
Mean proportion of diploid cells (± SD)	88.8 ± 5.1	84.5 ± 5.6	68.7 ± 10.8

* Cell line expired before this stage

'able 3.3:- The effect of the number of population doublings in culture on the dis-:ibution of chromosome number in adult Kupffer cell lines.

'opulation doublings	Number of cell lines	Chromosome number: Proportion of cells (%) 15-17	18,19	20	21	22	23-25	43	44	n
30	25	0.3	0.5	2.0	3.3	88.8	3.3	0.6	1.3	1250
50	15	0.7	1.1	1.9	5.6	84.5	2.3	0.9	3.1	750
90	8	1.0	2.0	7.8	13.5	68.7	3.2	1.3	2.5	400

= number of cells examined for all cell lines at each stage lines were examined on three separate occasions. The first examination was performed on cells obtained from 25 cell lines at the stage of 26 population doublings. These cells were subcultured and grown until in the exponential growth phase and karyotypes were thus examined after approximately 30 population doublings. The second examination was performed on cells from 15 of the above cell lines after approximately 50 population doublings and the final examination performed on cells from the eight surviving cell lines after approximately 90 population doublings. A description of the karyotypes for each cell line was based on observation of 50 metaphase cells.

In Table 3.2 appears the proportion of diploid cells in each cell line after the three periods in culture. The diploid number of chromosomes in the Chinese hamster is 2n = 22 (see Kakati and Sinha, 1972). After 30 population doublings the proportion of diploid cells was 88.8 $\pm$ 5.1%. The proportion of diploid cells was slightly reduced to 84.5 $\pm$ 5.6% after 50 doublings and by the time 90 doublings had been achieved, only 68.7 $\pm$ 10.8% of cells were diploid. At least 75% of cells must be diploid before a cell line can be regarded as diploid (Federoff, 1967). Thus, all cell lines examined remained diploid for at least 50 population doublings. After this stage a number of cell lines expired and karyotypic variability was apparent in the survivors. Only 2 of the 8 cell lines examined after 90 population doublings were diploid. The variation in karyotype is also reflected in the large standard deviation of the proportion of diploid cells after this time in culture.

There was a tendency for the proportion of aneuploid cells to increase after extended periods of culture. Table 3.3 presents the effect of the number of population doublings on the distribution of chromosome numbers in all cells scored. The data for all cell lines have been pooled. With the exception of the two diploid cell lines after 90 population doublings, the cell lines possessed a similar proportion of aneuploid cells at any given stage. The proportion of cells

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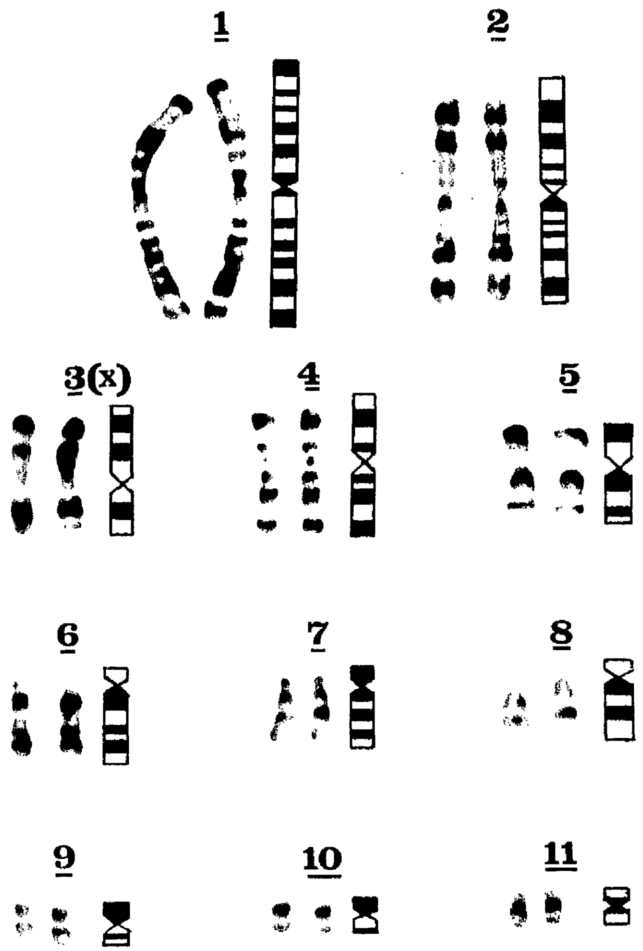


Figure 3.7:- The banded karyogram of a cultured diploid Chinese hamster Kupffer cell.

Presented with each chromosome pair is a schematic representation of the major bands apparent after treatment with trypsin and Giemsa. When the relative sizes and banding patterns are taken into account, chromosomes of normal morphology can be unequivocally identified.

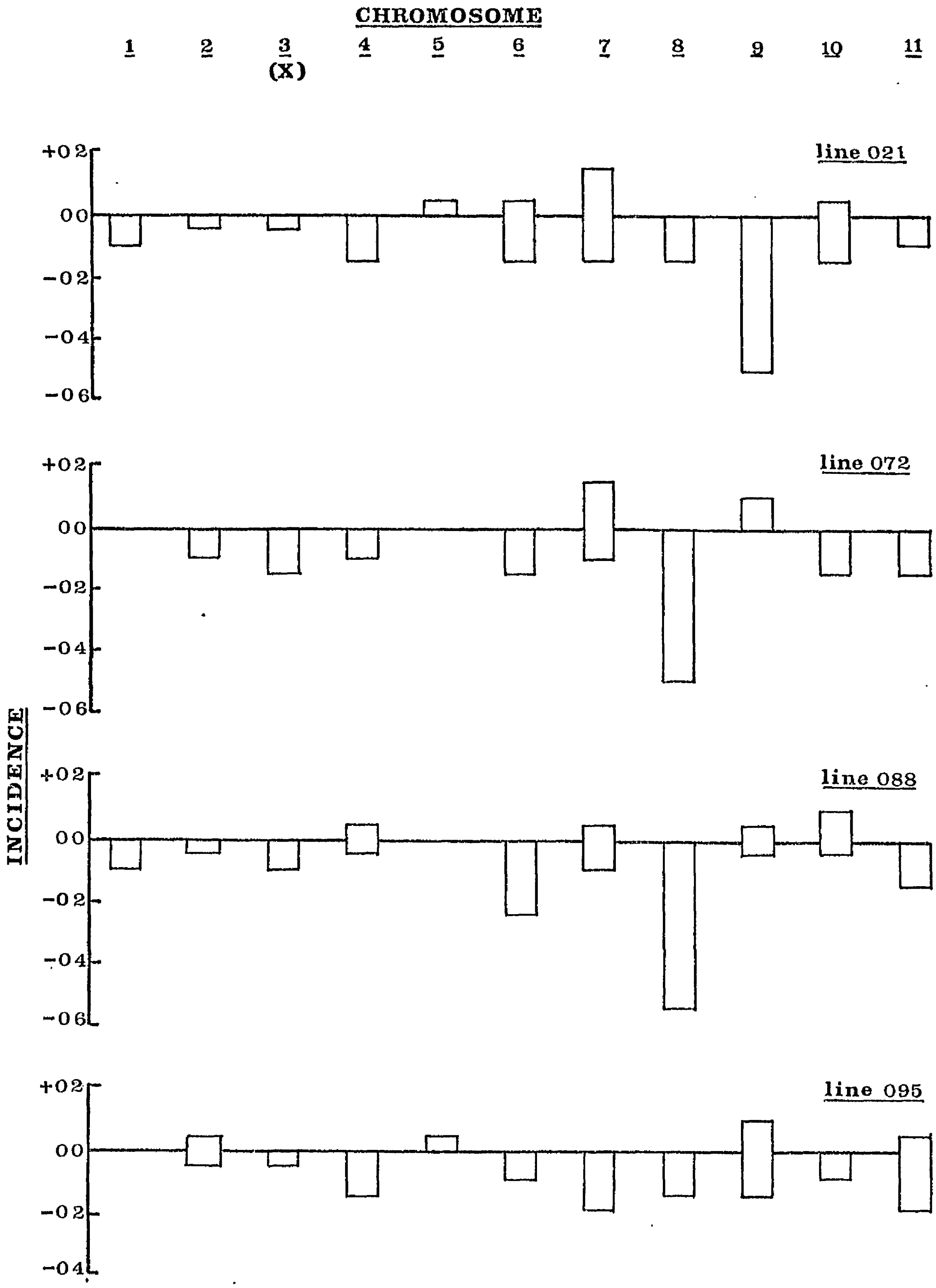


Figure 3. 8:- Incidence of individual chromosomes in aneuploid cells from four primary adult Kupffer cell lines after 90 population doublings in culture.

See text for explanation; (+) indicátes gain of chromosome while (-) indicates loss of chromosome.

with 20 or 21 chromosomes rose from 5% after 30 to over 20% after 90 population doublings. In contrast, the proportion of cells containing 23-25 chromosomes remained relatively constant, and there was only a slight increase in the proportion of cells containing 43 or 44 chromosomes.

Analysis of chromosome banding patterns in diploid cells of cell lines revealed that all chromosomes from the normal complement could be identified and they were of normal morphology when compared with Kakati and Sinha's (1972) description of the Chinese hamster karyotype. Examination of 10 diploid cells from each cell line at each stage revealed an identical banding pattern. This pattern is shown in Figure 3.7. At each stage the diploid cells appeared to contain a full complement of chromosome material and no gross rearrangements had occurred in these cells. Even after 90 population doublings the normal, diploid karyotype was modal.

The incidence of individual chromosomes in aneuploid cells from four cell lines after 90 population doublings is shown in Figure 3.8. The incidence of individual chromosomes is presented by the method used by Levan (1972). The zero line represents the number of chromosomes in the normal diploid cell. The scale is graduated in units of the number of chromosomes gained or lost: a value of + 0.1 (-0.1) means that every tenth aneuploid cell has the gain (loss) of one chromosome of the type concerned. The incidence is based on examination of 20 aneuploid cells from each cell line. The data reveal that there exist differences in chromosome incidence both between and within cell lines after 90 population doublings. The degree of diploidy of the four cell lines has been presented in Table 3.2. The smaller metacentric and acrocentric chromosomes are involved in aneuploid change to a greater degree than the large metacentric chromosomes. Loss of individual chromosomes is more frequent than gain. When these changes in chromosome incidence occur, they invariably involve single rather than multiple gains or losses. In all cell lines alteration in the incidence of chromosomes 1 and 5 was a comparatively rare event. In two cell lines each, both chromosome 1 and 5 did not deviate from the diploid incidence. In contrast were the incidences of some of the acrocentric chromosomes and the small sub-metacentric chromosomes. Chromosome 7 demonstrated both gains and losses in the three heteroploid cell lines (021, 072, 088), while the incidence of chromosomes 6, 8 and 9 was reduced. In each of the heteroploid cell lines there occurred the emergence of a relatively common aneuploid cell demonstrating loss of chromosome 8 in lines 072 and 088, and chromosome 9 in line 021. In contrast was the incidence of the chromosomes in the infrequent aneuploid cells from the diploid cell line 095. In this case no particular pattern of gain or loss had emerged. Thus, while the heteroploid cell lines demonstrated a non-random incidence of chromosomes in aneuploid cells, the pattern of karyotypic change in aneuploid cells from the diploid cell lines appeared random.

3.4 Discussion

In recent years the techniques of cell culture have advanced rapidly. The maintenance of differentiated functions in culture has been reported for a considerable number of cell types (see review, Wigley, 1975). The data presented in this study demonstrate that Kupffer cells can be established in culture. These cells both proliferate and maintain functions associated with their differentiated properties *in vivo*. The initial morphology of these cultured Kupffer cells was similar to the Kupffer cells isolated by Melly *et al.* (1972) and Munthe-Kaas *et al.* (1975).

Previous attempts to culture Kupffer cells for long periods have not been successful. The Kupffer cells cultured by Munthe-Kaas *et al.* (1975) were rapidly overgrown with unidentified "fibroblast-like" cells, whilst Von Kramer and Oftebro (1971) were not able to subculture Kupffer cells. In this study, the removal of unidentified, "fibroblast-like" cells by differential attachment and daily removal of contaminating cell types eliminated the problem of overgrowth. While the Kupffer cells could be subcultured, it was evident that detachment of these cells from the substrate was more difficult than for some commonly used cell lines (e.g. BHK, CHO, L etc.). A combination of several factors would seem to be of importance in successfully culturing Kupffer cells. Firstly, enzyme dissociation of the liver should be performed in as short a time as possible, with only gentle mechanical agitation. Removal of contaminating cell types and daily changes of medium facilitate establishment of Kupffer cells in culture. The proportion of foetal calf serum in the culture medium appears an important factor in determining the initial attachment rate of freshly isolated Kupffer cells.

Since published reports of attempts to isolate Kupffer cells do not provide all the details of yield and cell suspension properties, it is not possible to make close comparisons between these methods and those adopted in this study. A few comparisons suggest that the isolation procedure in this study does not produce a liver reticulo-endothelial cell fraction substantially different from those obtained in other studies. The yield of Kupffer cells in this study of 9.3×10^6 cells/gm wet weight of Chinese hamster liver was not greatly different from the yield of 5.8×10^6 cells/gm wet weight of rat liver obtained by Lentz and Di Luzio (1971). The 58% of isolated Kupffer cells which attach to the surface of a plastic petri dish in this study compares favourably with the attachment rate of 50% reported by Munthe-Kaas et al. (1975) and 60% reported by Bissell et al. (1972). Bissell et al. (1972) obtained 15% of the total number of Kupffer cells in suspension, and provided that the concentration of Kupffer cells in Chinese hamster liver is not greatly different from that in rat liver, the isolation procedure employed in this study resulted in the recovery of approximately 30% of the total Kupffer cell population. Thus, the proportion of total Kupffer cell population isolated in this study is probably greater. If the above estimate of yield is accepted, then, with the techniques employed in this study, approximately 2% of the total Kupffer cell population can attach and divide in culture and subsequently form colonies. Thus, with these techniques, study of Kupffer cells in culture is confined to a small proportion of the total population of these cells.

The Kupffer cells which survived isolation and formed colonies maintained differentiated functions characteristic of the Kupffer cell in vivo. Thus, although there was selection for ability to survive isolation, the culture environment and a capacity for division in this environment, cells which survived could still express these functions. The Kupffer cells which survive in culture and form cell lines demonstrate a considerable capacity for division and can be maintained in exponential growth for at least 70 population doublings. It is with this respect that the cultured Kupffer cell differs most from its in vivo counterpart; there has been a selection for mitotic activity either by the isolation procedure or the culture conditions. It is not possible to assess whether all Kupffer cells in vivo are capable of division once attached in culture or whether the dividing cells in culture represent selection of a section of the Kupffer cell population. It is possible that Kupffer cells may be able to divide in culture only when isolated at a certain stage in the cell cycle. Such a situation is suggested by the synchronous first division of freshly isolated Kupffer cells. The studies of Pariza et al. (1975) demonstrate that liver parenchymal cells must be in the S phase of the cell cycle at the time of isolation if they are to divide in culture.

During the primary cloning of the Kupffer cell lines from the reticuloendothelial cell suspension, it is of interest to note that clones generally have either all three or no Kupffer cell functions. In the

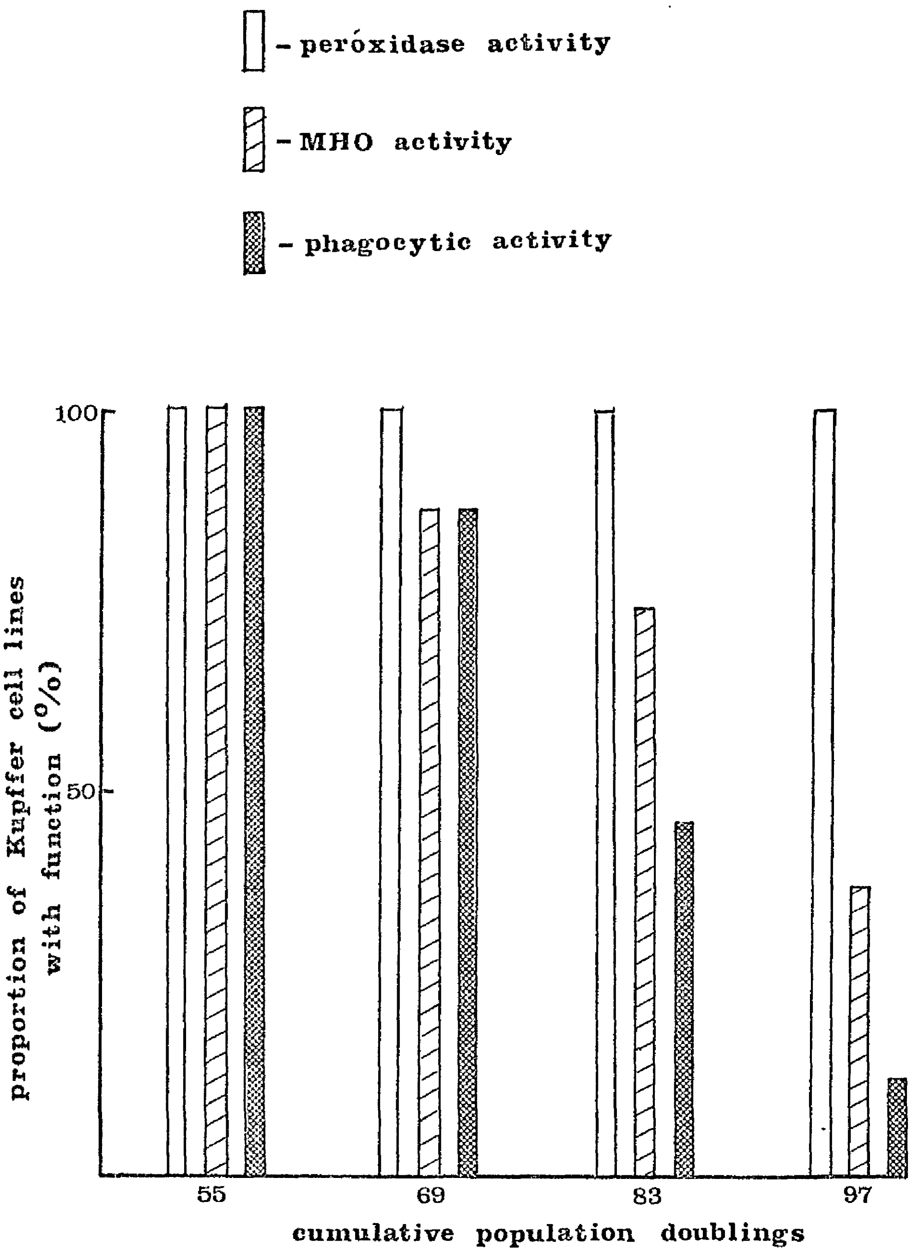


Figure 3.9:- Persistence of differentiated functions in primary adult Kupffer cell lines after an extended period in culture.

sample of 195 liver reticulendothelial cell lines examined after approximately 26 population doublings, only 2 cell lines possessed just peroxidase and MHO activities - all the other cell lines possessed phagocytic, peroxidase and MHO activities or none of these activities. Whether these 2 cell lines represent an early, intermediate loss of Kupffer cell functions, is not clear. The 63 cell lines which did not exhibit the three Kupffer cell functions were probably endothelial cells. Although they may represent Kupffer cells which had lost all three functions by the stage of 26 population doublings, the discontinuous distribution of persistence of these functions makes this an unlikely explanation. The majority of liver reticuloendothelial cell lines retained all three Kupffer cell functions until at least 55 population doublings.

When persistence of Kupffer cell functions is examined, phagocytic activity is the first function to be lost. This observation, based on cells after 80 or more population doublings, suggests that the 2 cell lines obtained after the primary cloning which possess peroxidase and MHO but not phagocytic activity, may represent a relatively rare, early loss of a Kupffer cell function.

The studies on persistence of Kupffer cell functions suggests there is a definite and ordered pattern of loss. Phagocytic activity is the first function to be lost, followed by MHO activity. At no time was phagocytic activity observed in the absence of MHO activity. Peroxidase activity persisted throughout the study period. While a pattern of loss of Kupffer cell differentiated functions is evident, the temporal distribution of this loss demonstrates variation. Some cell lines had lost both peroxidase and MHO activities by the time 69 population doublings had been achieved, while one cell line still possessed both functions after 97 population doublings in culture. There is also variation in the rate of loss of individual functions. Figure 3.9 demonstrates that although there is a pattern in the loss of differentiated functions, the rate of loss of phagocytic activity is greater than that for MHO activity. Thus, it appears possible that individual functions, while part of a pattern, are lost at their own particular rate. The basis for this pattern of loss is not clear. It is conceivable that phagocytic activity is the culmination of a number of structural protein and enzyme realizations and that loss or disruption of any one of these functions will eliminate phagocytic activity. In contrast, peroxidase and MHO activities presumably rely on a smaller number of realizations and are thus less vulnerable to extinction. It is interesting to note that once lost, a Kupffer cell function is not re-expressed at a later stage. Thus, on the basis of the small sample studies, it would appear that the loss of differentiated functions in cultured Kupffer cells is an irreversible process.

The morphological variability and transition of Kupffer cells in culture suggests that the traditional description of cell lines as being either "fibroblast-like" or "epithelial-like" may not be adequate criteria for identification of some cell types. Wigley (1975) has discussed the identification of cultured cells by these morphological criteria. Sandstrom (1965) described considerable morphological transition of various liver cell types under constant culture conditions, and when culture or growth conditions were altered there was an associated change in cell morphology. The cultured Kupffer cells in this study adopted both morphologies depending on age or number of population doublings in culture, stage of the culture cycle and cell density. Cells which had only a short history of culture, or are present at low densities appear to be more "epithelial-like". However, as age increases, or saturation density is approached there is a transition towards a "fibroblast-like" morphology. At any given period, the presence of Kupffer cell functions appeared to be independent of morphology. In general, the transition to a "fibroblast-like" morphology was rapid and occurred in the first few divisions in culture. The transition was always towards a more "fibroblast-like" morphology and reversion to an "epithelial-like" morphology never occurred. No attempt was made to alter morphology or the presence of differentiated functions by environmental stimuli or modifying culture conditions. The apparent uniformity of transition in morphology could be interpreted to indicate an adaptive response to the culture environment.

The fact that Kupffer cell differentiated functions are maintained for a considerable period in culture suggests that expression of these functions is not incompatible with either the culture environment or proliferation. While the cause of their loss remains a mystery, it may be the result of a crisis in development; this crisis may be interpreted as an expression of ageing. A number of phenomena occur at roughly the same stage of the culture history. Population doubling time increases, plating efficiency decreases, the survival rate of the cell lines is reduced and the karyotype undergoes changes away from the diploid state. During the same period, the variation of all these phenomena also increases. These changes are occurring at approximately the same time that Kupffer cell functions are lost. Although causal relationships cannot be established, the co-incidence of these phenomena indicates a crisis or a major alteration in the developmental sequence.

The role of karyotypic change in this process is not clear. The gradual loss of differentiated functions in cultured Kupffer cells appears to be associated with a disruption of the normal diploid karyotype which persisted for at least 60 population doublings. Cell line 055 is an interesting exception to the pattern of disruption generally observed. Unlike the other cell lines, this line retained all three Kupffer cell functions and a diploid karyotype after 97 population doublings. Although the subculturing routine described in Figure 3.1 was not strictly adhered to after 97 population doublings had been achieved, the 8 cell lines were cultured for a further period. It is estimated that cell line 055 expired after approximately 105 population doublings; the other diploid cell line, 095, expired shortly later at 107 population doublings. The only cell lines which survived past the point of 120 population doublings in culture were 072, 088 and 112. All of these cell lines had lost peroxidase and MHO activities by the time they had experienced 83 population doublings. The fact that there was a high frequency of chromosome 8 loss in two of these cell lines (072, 088) may be worth noting. Since detailed karyotypic examination of cell line 112 was not performed, the significance of this observation is not established. However, it would seem that ability to survive long periods in culture was coincident with an increased rate of loss of chromosome 8 in at least 2 out of 3 surviving cell lines. In contrast, the comparative stability of chromosome 1 and 5 incidence in the four cell lines examined in Figure 3.8 suggests that there is less tolerance towards alteration in the incidence of these two chromosomes in the evolution of the karyotype in culture.

In conclusion, this study demonstrates that it is possible to isolate, establish and maintain in culture Chinese hamster Kupffer cells. The Kupffer cells demonstrate stability of a number of properties and functions for at least 60 population doublings in culture. After this time an ageing crisis appears to disrupt the stability and the cell lines exhibit alteration of a number of properties. During this crisis a few cell lines survive and become "established" in culture. The fact that Kupffer cells can be identified and cultured for a considerable period, whilst maintaining a diploid karyotype and stability of growth kinetic parameters makes them potentially valuable material for the study of phenotypic variation in culture. The ability to establish a large number of cell lines from the same individual by primary cloning also provides additional impetus to the study of variation.

SECTION 4

ENZYME ACTIVITIES IN CULTURED ADULT KUPFFER CELLS

4.1 Introduction

The data presented in section 3 demonstrate that it is possible to establish primary, adult Kupffer cell lines in culture. These cell lines express differentiated functions and are viable for at least 70 population doublings. The 130 Kupffer cell lines initiated from three sibling female Chinese hamsters provide suitable material for the study of enzyme activity variation in cultured cells. The cell lines were all closely related and a large number were of genetically identical origin, i.e. they were initiated from the same animal.

In the absence of data on phenotypic variation of specific enzyme activities as a phenomenon, the selection of enzyme activities for study was made with reference to several criteria. The major consideration was accuracy, ease and economy of assay when a large scale screening operation was undertaken. The second requirement was to include diverse enzyme activities related to both differentiated Kupffer cell functions and basal metabolism. The study of several enzyme activities which catalyze similar reactions, utilize common substrates or are involved in related functions may be of interest when considered in the light of co-ordination and relationships of activities. The final criterion of some significance in the selection of enzyme activities for study was that of existing reports of phenotypic variation or mutations affecting enzyme levels. With respect to an intent of this dissertation to examine variation in transformed cells, the selection of enzyme activities for study was also based on a few activities shown to be variable in neoplasms (see section 1.5).

The following enzyme activities were selected for routine screening in the 130 primary adult Kupffer cell lines:-

catalase, EC 1.11.1.6.
arginase, EC 3.5.3.1.

microsomal haem oxygenase (MHO), EC 1.14.99.3.
β-glucuronidase, EC 3.2.1.31.
peroxidase, EC 1.11.1.7.
alcohol dehydrogenase (ADH), EC 1.1.1.1.
lactate dehydrogenase (LDH), EC 1.1.1.27.
isocitrate dehydrogenase (IDH), EC 1.1.1.42.
glucose-6-phosphate dehydrogenase (G6PDH), EC 1.1.1.49.

A general description of the biochemistry of these enzymes can be found in Barman (1969) and Boyer (1970).

Peroxidase and microsomal haem oxygenase activities are related to the differentiated functions of Kupffer cells and have been considered in section 3.1. Catalase is a haem-containing enzyme closely related to peroxidase and both enzymes base their catalytic action on hydrogen peroxide as substrate. The only difference in the chemical reaction catalyzed by these two enzymes is that catalase does not utilize an electron donor. A high β-glucuronidase activity is also a prominent feature of Kupffer cells (Wachstein, 1963) and thus it may be of interest to consider this lysosomal enzyme activity in cultured Kupffer cells.

The dehydrogenase activities, although diverse in the specific reactions catalyzed, are related in-so-far as these reactions are intimately associated with pyrimidine nucleotide co-factors and thus have a potential role in influencing the redox state of the cell. The studies presented in Table 1.2 and those of Potter et al. (1969) and DeLuca and Matheisz (1976) indicate that dehydrogenase activities are capable of considerable variation in similar classes of neoplasm or their derived cell lines.

Catalase and β-glucuronidase have both been the subject of genetic studies on the regulation of enzyme levels. In section 1.4 were cited studies devoted to the recognition of regulatory mutations affecting these two enzyme activities. Although previous studies have been restricted to the mouse, the fact that such regulatory mutations exist suggests that there is the possibility of detecting such alterations when a large survey is conducted.

The final enzyme studied was arginase; an enzyme with high activity in the mammalian liver (Knox, 1972). This enzyme is part of the urea cycle and Tashjian et al. (1975) have demonstrated that its activity is usually lost in cultured cells, whether primary or transformed. However, these workers isolated a clone of hepatoma cells which expressed this enzyme activity as well as those of the other urea cycle enzymes.

It is the purpose of this section to present data on the specific activities of these nine enzymes in the 130 primary adult Kupffer cell lines at the three examination stages described in Figure 3.3 and corresponding to 26, 33 and 40 population doublings in culture. Enzyme activities were studied over an extended period in a smaller sample of cell lines, as were the initial changes in enzyme activity when a Kupffer cell becomes established in culture.

The results of this section will be more fully discussed in section 7.

4.2 Materials and Methods

4.2.1 Subculturing routine

The subculturing routine has been described in section 3.2.3.

4.2.2 Preparation of cell extracts

Whole liver homogenate was prepared from a small piece of tissue prior to enzymatic dispersion of the organ. The piece of liver was hand homogenized in 1 ml of cold PBS/A, the extract centrifuged (10,000 g, 4°C, 10 mins.) and protein concentration adjusted to 1 mg/ml. Cell extracts of hepatocyte and Kupffer cell fractions from freshly dissociated liver and harvested cells were prepared by an identical procedure. Aliquots of the cell suspensions (see section 3.2.3) were transferred to a cold glass homogenizer (1 ml capacity) containing 0.05% (v/v) Triton-X100 (Sigma) and homogenized with 15 thrusts by hand. The homogenate was rapidly frozen in liquid nitrogen and used for both enzyme assays and protein determination. When such extracts were stored at -70°C with a protein concentration of approximately 0.2 mg/ml, no loss of activity was observed for all enzymes during storage for one week. The extract contained the equivalent of 1,100 cells/µl or approximately 0.5 µg cell protein/µl. All enzyme activities were determined within three days of preparing the extract. Adjustment of protein concentration was achieved by addition of BSA (Armour Pharmaceutical).

4.2.3 Routine enzyme assays

Catalase. The assay of catalase activity was based on the fact that in the presence of hydrogen peroxide, heated dichromate in acetic acid is reduced to chromic acetate. Sinha (1972) utilized this reaction in a colourimetric assay of catalase and the method was adopted in this study. To a 10 mm x 75 mm tube were added 675 µl of reaction mixture containing 900mM hydrogen peroxide ("Aristar", BDH Chemicals) and 0.2 mg/ml BSA in 0.01 M phosphate buffer (pH 7.0) and 75 µl of cell extract. After incubation for 5 mins. at 25°C, 1.5 ml of dichromate/acetic acid reagent was added and the mixture heated for 10 mins. at 100°C, allowed to cool and absorbance read in a spectrophotometer at 570 nm. The dichromate/acetic acid reagent was prepared by mixing a 5% (w/v) solution of potassium dichromate with glacial acetic acid (1:3, v/v). In the blank tube, cell extract was added at the end of incubation. A standard curve was obtained by following the above procedure with known amounts of hydrogen peroxide, but without incubation. Catalase activity was expressed as moles of hydrogen peroxide converted/min./mg protein.

Arginase. The assay of arginase activity was based on the fluorometric determination of urea after incubation of the enzyme in the presence of arginine. The conditions of incubation were those described by Geyer and Dabich (1971). The enzyme was activated by pre-incubation of 20 µl of cell extract with 20 µl of 0.05 M manganous sulphate for 5 mins. at 55°C (Schimke, 1962). After pre-incubation and cooling to room temperature, 0.9 ml of a solution containing 0.29 M arginine, 0.01 M maleate, 0.001 M manganous sulphate (pH 9.5) and 0.2 mg/ml BSA was added and incubation proceeded for 30 mins. at 37°C. The concentration of urea in the reaction mixture was determined using the fluorometric method described by McCleskey (1964). To 0.2 ml of reaction mixture was added 2 ml of water. After 15 mins. protein was precipitated with 2 ml 30% trichloracetic acid followed by brief centrifugation. A 2 ml aliquot of supernatant was transferred to a glass stoppered tube containing 2 ml diacetyl monoxime reagent and 0.3 ml conc. sulphuric acid, then heated in boiling water for 15 mins. The diacetyl monoxime reagent was 0.05% (w/v) diacetyl monoxime and 15% NaCl (w/v) in aqueous solution. After cooling, the fluorescence was determined (ex. 380 nm/em. 415 nm) relative to a blank established by the addition of cell extract at the end of the incubation. In the preparation of standards of known concentration, the first incubation was omitted. Arginase activity was expressed as moles of urea liberated /min. /mg protein.

Microsomal haem oxygenase. The assay of this enzyme has been described in section 3.2.6.

β-Glucuronidase. The fluorometric assay described by Verity et al. (1964) and Greenberg (1966) was used to determine β-glucuronidase activity. The assay was based on the fluorescence of 1-naphthol when liberated from 1-naphthyl-β, D-glucuronide by β-glucuronidase. The substrate, 1-naphthyl-β, D-glucuronide was extracted with ethyl ether in 100 mg batches at 40°C for 45 mins.

in order to remove traces of free 1-naphthol. The purified substrate was then dissolved in a few drops of N, N-dimethyl formamide and made up to the appropriate volume with double distilled water.

In 10 mm x 50 mm tubes 20 μl cell extract was added to 240 μl reaction mixture containing 4 mM 1-naphthyl-β, D-glucuronide, 0.15 M KCl and 0.2 mg/ml BSA in 0.1 M sodium acetate buffer (pH 4.5). After 30 mins. at 37°C the tubes were placed on ice and the reaction stopped with 300 μl of 0.5 M NaOH. The fluorescence of this solution was then determined (ex. 345 nm/ em. 455 nm). A blank was prepared by adding extract to a reaction tube at the end of incubation. The activity of β-glucuronidase was expressed as moles 1-naphthol liberated/min./mg protein after comparison with the fluorescence of known concentrations of 1-naphthol in the presence of the other reagents.

Peroxidase. The assay of peroxidase activity was based on the fluorometric method for analysis of low concentrations of hydrogen peroxide described by Keston and Brandt (1965). The method utilizes the fact that a stable, non-fluorescent reagent, diacetyl-dichloro-flourescin (LDADCF), which after activation by alkali, is converted to fluorescent diacetyl-dichloro-fluorescein (DADCF) when treated with peroxidase and small amounts of hydrogen peroxide. The fluorescence in this system is proportional to the hydrogen peroxide concentration for differing enzyme quantities. The method of Keston and Brandt (1965) was modified to assay peroxidase activity when hydrogen peroxide concentration was non-limiting over the time considered.

Stock solutions (10^{-4}M) of LDADCF and DADCF (Eastman-Kodak) in absolute ethanol were stored at 4°C in the dark. Activation of LDADCF for the assay required 1 in 5 dilution with 0.01 M NaOH. The LDADCF was then diluted to 10^{-6} M with buffer (0.025 M phosphate containing 0.04 mg/ml $ZnSO_4 . 7H_20$ and 0.2 mg/ml BSA, pH 7.2). To a cuvette were added 450 µl of reaction mixture containing 9 x 10^{-7}M buffered LDADCF and 5 x 10^{-6}M hydrogen peroxide ("Aristar", BDH Chemicals) and 50 µl cell extract. After rapid mixing and insertion in a recording fluorometer, the reaction was followed for 2 mins. at 25°C (ex. 503 nm/em. 525 nm). Enzyme activity was determined as the initial maximum rate under non-limiting conditions and expressed as moles DADCF produced (and hence moles hydrogen peroxide converted) /min. /mg protein after calibration with known amounts of DADCF when added to assay mixture in the absence of hydrogen peroxide. Blanks for the assay were obtained by substituting distilled water for hydrogen peroxide in the reaction mixture.

Dehydrogenases. The dehydrogenase assays were based on the fluorescence of reduced coenzyme, NADH or NADPH, and are described by Roth (1969).

Alcohol dehydrogenase. The incubation was performed at 25°C in a cuvette to which had been added 750 µl of reaction mixture containing 0.15 mM NAD, 0.2 M ethanol and 0.2 mg/ml BSA in 0.05 M pyrophosphate buffer (pH 8.8) and 10 µl cell extract.

Lactate dehydrogenase. The incubation was performed at 25°C in a cuvette to which had been added 750 µl reaction mixture containing 90 mM lithium lactate, 1.5 mM NAD and 0.2 mg/ml BSA in 0.05 M pyrophosphate buffer (pH 8.8) and 10 µl cell extract.

Isocitrate dehydrogenase. The incubation was performed at 37°C in a tube to which had been added 100 µl of reaction mixture containing 1 mM NADP, 30 mM sodium-DL-isocitrate, 0.04 mM manganous chloride, 20 mM nicotinamide, 0.2 mg/ml BSA and 0.08 M Tris (pH 8.8) and 35 µl of cell extract. After 30 mins. the reaction was stopped by transfer of a 50 µl aliquot to a cuvette containing 700 µl of a mixture of 0.001 M EDTA and 0.05 M K_2HPO_4. The fluorescence of NADPH in the resulting mixture was measured directly at 25°C.

Glucose-6-phosphate dehydrogenase. The incubation was performed at 37°C in a tube to which had been added 20 μl of reaction mixture containing 10 mM glucose-6-phosphate, 3 mM NADP, 7 mM magnesium chloride, 0.2 mg/ml BSA and 0.05 M Tris (pH 8.8) and 20 μl of cell extract. After 30 mins. the reaction was stopped by the addition of 500 μl of 0.01 M NaOH and the fluorescence of NADPH measured at 25°C.

In the case of alcohol dehydrogenase and lactate dehydrogenase, increase in fluorescence was followed with a recording fluorometer. After addition of all solutions to the cuvette, they were rapidly mixed at the same time as the chart drive was started. The slope of the curve between 15 and 135 seconds was taken as a measure of enzyme activity for LDH and until 5 mins. for ADH. For all dehydrogenase assays, blanks were achieved by omission of the substrate. The native fluorescence of NADH and NADPH was measured (ex. 340 nm/em. 460 nm) and compared with secondary standards of quinine sulphate (stock 1 μg/ml in 0.01 N sulphuric acid) which had previously been calibrated with NADH or NADPH. Enzyme activity was expressed as moles of subtrate utilized/min./ mg protein.

4.2.4 Assay of enzyme activities in small numbers of cells.

Reduction of final reaction volume makes it possible to assay a number of enzyme activities in minute quantities of material. Lowry and Passoneau (1972) and Galjaard et al. (1974a, 1974b) describe such an approach to several fluorometric enzyme assays. In this study, reduction of final reaction volume and fluorescence measurement in micro-cuvettes has proved to be a satisfactory method of assaying β-glucuronidase, MHO, ADH, LDH, IDH and G6PDH activities in small samples.

Kupffer cells were cultivated on thin plastic film ("Melinex" polyester type 0, ICI) which lined the bottom of standard plastic petri dishes. After washing with PBS/A containing 0.2 mg/ml BSA, the plastic film was quickly frozen in liquid nitrogen and freeze-dried for 16 hours (-25°C, 0.001 mm Hg). Microdissection of small pieces of plastic film (0.2-1 mm^2) containing 1-200 cells was carried out in an air-conditioned room. Enzyme activities in small numbers of cells in suspension could also be assayed. The suspension of cells in PBS/A containing 0.2 mg/ml BSA was dropped onto plastic film and freeze-dried as above. The cells, which appeared as dark spots, were isolated by hand using a pointed brow hair glued to a glass holder.

Enzyme activity was assayed as soon as the cells had been freeze-dried. All reactions were conducted in small plastic wells (Flow, haemagglutination round bottom well, 200 µl capacity). The reaction mixtures were small volumes of the mixtures described above for the routine assays and fluorescence was measured in a quartz capillary cuvette (10 µl capacity, Baird Atomic) at the above wavelengths.

<u>β-Glucuronidase "micro" assay</u>. The freeze-dried cell material was placed in the reaction well and 5 µl of reaction mixture added. After covering with an oil-drop the material was incubated for 3 hours at 37°C. The reaction was terminated with 5 µl of 0.5 M NaOH and fluorescence measured. It was possible to use this assay to measure activity in as few as 10 cultured adult Kupffer cells.

<u>MHO "micro" assay</u>. After addition of 2 µl of reaction mixture to the sample in a well, the mixture was covered with an oil drop and incubated for 3 hours at 37°C. The reaction was terminated with 1 µl of 6% BSA in PBS/A and 12 µl of 85% phosphoric acid, diluted after 2 mins. with 20 µl of water and fluorescence measured. This assay could be used on 100 or more cultured adult Kupffer cells.

ADH and LDH "micro" assay. The reaction mixture contained twice the concentration of NAD. The sample was dissolved in 10 μl of the appropriate reaction mixture, covered with a drop of oil and incubated at 25°C. After 3 hours the mixture was removed and fluorescence measured. ADH activity could be determined in 10 cells whilst LDH activity could be determined in single cultured adult Kupffer cells.

IDH and G6PDH "micro" assay. The reaction mixture contained twice the concentration of NADP. The sample was dissolved in 2 μl of reaction mixture, covered with an oil drop and incubated for 3 hours at 37°C. After the reaction for IDH had terminated, fluorescence was determined after addition of 20 μl of 0.001 M EDTA containing 0.05 M K_2HPO_4. This assay could be used for determining IDH activity in single cultured adult Kupffer cells. The reaction for G6PDH was terminated by addition of 15 μl of 0.01 M NaOH before fluorescence was measured. G6PDH activity could be determined in as few as 10 cultured adult Kupffer cells.

During incubation, reaction wells for all assays were kept in the dark and occasionally agitated. Blank and standard reactions with known amounts of product were prepared with pieces of plastic film from the same culture but without attached cells. Provided it did not exceed 1 mm^2, the precise size of the plastic fragment did not appear to be critical. The oil droplet added to each reaction well eliminated evaporation. This oil was a mixture of n-hexadecane and liquid paraffin (4:6, v/v) and was cleaned as described by Lowry and Passoneau (1972).

4.2.5 Data analysis

The data analysis was performed with standard statistical techniques (see Sokal and Rohlf, 1969). Analysis of the bulk of the data was too time-consuming to be accomplished manually, so

Table 4.1:- The specific activities of nine enzymes in Chinese hamster liver homogenates and freshly isolated hepatocyte and Kupffer cell extracts

Each value is the mean ± standard deviation of extracts from 5 female animals. t (0.01) = 3.35, 8df. (U = min/mg protein).

Enzyme	Whole liver	Hepatocytes	Kupffer cells	t
Catalase ($\times 10^{-2}$ moles/U)	25.1 ± 1.6	34.6 ± 1.5	17.9 ± 1.2	19.44
Arginase ($\times 10^{-7}$ moles/U)	69.3 ± 4.6	84.2 ± 4.5	28.9 ± 2.2	24.69
MHO ($\times 10^{-11}$ moles/U)	4.4 ± 0.7	2.2 ± 0.7	9.1 ± 0.7	15.59
β-Glucuronidase ($\times 10^{-10}$ moles/U)	42.1 ± 3.8	37.9 ± 3.3	72.3 ± 2.3	19.12
Peroxidase ($\times 10^{-2}$ moles/U)	15.8 ± 1.2	16.9 ± 1.1	9.7 ± 0.8	11.84
ADH ($\times 10^{-7}$ moles/U)	45.5 ± 1.6	44.9 ± 3.7	26.8 ± 1.7	9.94
LDH ($\times 10^{-6}$ moles/U)	60.6 ± 5.5	65.1 ± 5.5	53.5 ± 5.1	3.46
IDH ($\times 10^{-7}$ moles/U)	74.0 ± 1.0	79.0 ± 2.0	30.5 ± 1.9	39.31
G6PDH ($\times 10^{-9}$ moles/U)	95.5 ± 3.4	102.1 ± 2.8	74.9 ± 2.2	17.08

recourse was made to the use of an ICL 1906 digital computer (Nottingham University). The analyses of variance utilized a modification of the BMD programmes (ed. Dixon, 1971). Calculation of correlation co-efficients utilized FORTRAN programmes written in conjunction with Dr. H. Dickinson (Computing Service, University of Glasgow). All data were stored on both punched cards and in a file on magnetic medium, either tape or disc. The data for each enzyme were stored in a separate file.

4.3 Results

4.3.1 Distribution of enzyme activities in Chinese hamster liver

Female Chinese hamsters nos. 6, 7, 8, 12 and 13 (see Figure 2.1) were used for a study of enzyme activity distribution between hepatocytes and Kupffer cells. Whole liver extracts were prepared from the same tissue samples used for the isolation of liver cells. While the enzyme activities are described for dissociated cells, these probably reflect the activities in situ. Dissociation of liver with techniques similar to those used in section 3 does not result in the leakage of enzyme from the dispersed cells (see Berg and Boman, 1972).

The results presented in Table 4.1 demonstrate major differences in the enzyme activities found in hepatocytes and Kupffer cells. There was a significant difference between hepatocytes and Kupffer cells in the specific activity of all nine enzymes. The difference of least significance was for LDH, but even in this case $P < 0.01$. The greatest differences were for arginase and IDH specific activities, both of which were higher in hepatocytes. MHO and β-glucuronidase were the only enzymes whose specific activities were greater in Kupffer cells. However, when enzyme activity/cell is considered, in all cases hepatocytes possess a greater activity than Kupffer cells. The protein content of hepatocytes (41.9×10^{-10} g/cell) was six times greater than that of Kupffer cells (6.7×10^{-10} g/cell). While enzyme activities in whole liver extract were intermediate, they were generally closer to those found in the numerically predominant hepatocytes.

As a result of species, sex and age differences, it is doubtful whether the results of this study can be directly compared with those of other reports. However, some features are worthy of comment. The increased β-glucuronidase activity in Kupffer cells confirms the similar observation of Berg and Boman (1973) and Munthe-Kaas et al. (1975). Similarly, a lower LDH specific activity in Kupffer cells has been reported (Crisp and Pogson, 1972). The distribution of peroxidase specific activity in this study is in contrast with that described by Berkel (1974). This author reported that peroxidase specific activity in approximately 30 times greater in non-parenchymal than in parenchymal cells from rat liver. The data presented in this study indicate that Chinese hamster Kupffer cells contain only 60% of the peroxidase specific activity found in hepatocytes. The reason for this discrepancy is not clear, but the use of different materials suggests that an attempt to explain the difference in distribution would be based on speculation. The data for Kupffer cell catalase specific activity presented in Table 4.1 agree with those reported by Berkel (1974). Kupffer cells possess only one half of catalase specific activity relative to hepatocytes.

It is interesting to note that peroxidase and MHO are found in both Kupffer cells and hepatocytes. These two enzyme activities are associated with the specific functions of Kupffer cells (see section 3). Although MHO specific activity was greater in Kupffer cells, hepatocytes with their greater number and protein content contain most of the liver MHO activity. This observation has also been made by Bissell et al. (1972) who have suggested that

MHO in hepatocytes is related to turnover of the haem of haem-containing proteins in hepatocytes, while the MHO in Kupffer cells is primarily responsible for catabolism of the haemoglobin of ingested red blood cells.

4.3.2 Enzyme activities in adult Kupffer cell lines

Nine enzyme activities were studied in the 130 primary adult Kupffer cell lines initiated in section 3. The subculturing routine and stage when cell extracts were prepared have been described in Figure 3.1. The cell extracts were obtained from all 130 cell lines at stages in culture equivalent to 26, 33 and 40 population doublings. The complete data from this study can be found in appendix 1.1. The data presented in appendix 1.1 are for each enzyme activity and cell line, at each of the three stages. Cell extracts were divided into three for three separate estimates of activity for each enzyme. This section is devoted to presenting the various analyses of the data in appendix 1.1. The cell lines were isolated from three sibling animals (nos. 6, 7 and 8 in Figure 2.1). Lines 001 to 031 were from animal 6, 032 to 077 from animal 7 and 078 to 130 from animal 8. The data were originally analysed on the basis of three separate groups. However, in no case was there a difference in the data between these three groups, and for ease of presentation and analysis, all 130 cell lines are considered together.

In Table 4.2 appear the mean specific enzyme activities in all 130 cell lines at the three stages. All enzymes demonstrated a trend towards less activity as the number of population doublings increased. Without exception, mean enzyme activity after 33 population doublings was less than that after 26 population doublings, but more than that after 40 doublings. Analysis of variance is used below to test the significance of these differences.

Also of interest were the enzyme activities in Kupffer cell lines relative to those found in freshly isolated Kupffer cells (see Table 4.2). The mean activity for all nine enzymes was well below that found in freshly isolated Kupffer cells. The activities in freshly isolated Kupffer cells have been presented in Table 4.1 and three of the five animals used for these estimates provided material from which were initiated the 130 cell lines in question. The degree of decrease in activity exhibited variation between enzymes. For example, after 26 population doublings, arginase activity had demonstrated a reduction to 27% of that found in vivo. In contrast, β-glucuronidase was reduced to 5% of the in vivo activity. In summary, Table 4.2 presents evidence that while all nine enzyme activities were reduced when Kupffer cells were cultured, individual activities were reduced to a different extent. The reduction in activity continued between 26 and 40 population doublings, but at a rate much less than that during the initial reduction to the activity found after 26 population doublings.

Table 4.2 has presented the mean enzyme activities in a sample of 130 cell lines. However, in this dissertation we are interested in phenotypic variation. Therefore, it is necessary to consider each individual observation and describe the distribution of activities from which the means are derived. In Table 4.3 appear distributions of nine enzyme activities in the 130 Kupffer cell lines. These distributions demonstrate that for each enzyme and stage in culture there was a considerable range of activity. At the stage of 26 population doublings the extent of variation ranged from 2-fold for ADH to 7-fold for β-glucuronidase. By inspection, it is apparent that the distribution range of each enzyme activity was reduced as the number of population doublings increased. Concomitant with this reduction in range of activity was a slight decrease in the modal activity. Although no statistical analysis has been conducted, it appears that the distribution of enzyme activities approximates a

Table 4.3:- Distribution of nine enzyme activities in 130 primary, adult Kupffer cell lines after 26, 33 and 40 cumulative population doublings in culture

Catalase (x 10^{-3} moles/min/mg protein)

Doublings	Class activity limit												
	10	15	20	25	30	35	40	45	50	55	60	65	70
26	0	6	9	14	10	14	25	20	8	8	5	7	4
33	1	15	22	16	11	20	20	10	8	4	3	0	0
40	0	31	29	21	14	15	9	6	2	3	0	0	0

Arginase (x 10^{-8} moles/min/mg protein)

Doublings	Class activity limit											
	10	20	30	40	50	60	70	80	90	100	110	120
26	15	0	1	1	27	9	8	8	10	22	19	10
33	15	0	1	18	33	23	15	8	11	5	1	0
40	14	2	13	45	28	15	7	4	0	2	0	0

MHO (x 10^{-12} moles/min/mg protein)

Doublings	Class activity limit										
	8	10	12	14	16	18	20	22	24	26	28
26	1	2	7	21	25	33	20	12	6	2	1
33	4	6	25	27	28	20	11	4	4	1	0
40	4	25	34	25	17	12	7	5	1	0	0

β-Glucuronidase (x 10^{-12} moles/min/mg protein)

Doublings	Class activity limit											
	150	200	250	300	350	400	450	500	550	600	650	700
26	1	2	15	24	21	32	16	11	3	2	2	1
33	3	19	17	30	26	22	7	3	2	1	0	0
40	20	33	21	28	18	2	6	0	2	0	0	0

Peroxidase (x 10 $^{-}$ moles/min/mg protein)

Doublings	Class activity limit											
	50	75	100	125	150	175	200	225	250	275	300	325
26	0	5	18	10	14	24	24	10	12	5	6	2
33	1	22	26	10	24	21	12	10	3	1	0	0
40	5	44	22	24	14	12	6	3	0	0	0	0

ADH (x 10^{-9} moles/min/mg protein)

Doublings	Class activity limit											
	90	100	110	120	130	140	150	160	170	180	190	200
26	0	10	2	15	20	18	19	19	11	11	4	1
33	2	10	14	16	16	25	26	11	8	0	2	0
40	1	10	16	24	29	14	17	13	5	0	1	0

LDH (x 10^{-8} moles/min/mg protein)

Doublings	Class activity limit											
	250	300	350	400	450	500	550	600	650	700	750	800
26	2	10	8	19	17	21	17	19	9	5	2	1
33	2	13	19	14	27	15	19	11	6	1	2	1
40	5	26	16	15	28	17	14	6	3	0	0	0

IDH (x 10^{-9} moles/min/mg/protein)

Doublings	Class activity limit										
	300	350	400	450	500	550	600	650	700	750	800
26	2	4	15	15	12	13	27	16	16	9	1
33	2	5	10	18	17	31	20	12	11	3	1
40	2	6	23	18	20	31	14	13	3	0	0

G6PDH (x 10^{-10} moles/min/mg protein)

Doublings	Class activity limit										
	30	40	50	60	70	80	90	100	110	120	130
26	1	1	9	16	24	25	20	18	14	0	2
33*	1	2	11	23	25	23	17	21	3	1	1
40	1	6	22	18	26	23	18	12	4	0	0

* At this stage there was one cell line with 190 and another with 150 units of G6PDH activity.

normal distribution. However, after 40 population doublings, some distributions were skewed with more enzyme activity class limits above the modal value than below. The development of skewed distributions of enzyme activity suggests that there is a minimal enzyme activity below which the primary adult cell lines may not survive under the particular culture conditions of this study. The lower extremes of the normal distribution could have been eliminated by selection. The lower limit of all enzyme activities was always at least an order of magnitude above the limit of detection. In the case of catalase activity after 40 population doublings, the skewness was extreme, where the lowest class limit was also the modal value.

In no case was there a cell line in the sample of 130 which possessed an enzyme activity near or above the activity present in freshly isolated Kupffer cells. At the limit of the ranges, the activities of enzymes in all cell lines could be regarded as being extreme values of a continuous distribution.

There are two exceptions to these general patterns of distribution. After 26 population doublings arginase activity appeared to exhibit a trimodal distribution. Thereafter, at the stage of 33 and 40 population doublings the distribution was bimodal. This distribution was caused by 15 cell lines which possessed no arginase activity at any of the stages. Thus, a proportion of Kupffer cell lines (approximately 11%) was unique in that arginase activity appeared to be absent. Since the arginase activity did not exhibit a normal distribution in adult Kupffer cell lines, subsequent analyses based on the assumption of a normal distribution may not be reliable. Hence, the cell lines with no arginase activity were omitted from the analysis of variance of arginase activity presented below, but were included in the analysis of correlations with other enzyme activities.

The other exception to the general phenomena described above were the two cell lines which possessed anomalous G6PDH activities after 33 population doublings. Utilizing a t-test, and the null hypothesis that the two variates were from the same statistical population as the other 128 cell lines, the probability of obtaining a value as extreme as 150 or 190 x 10^{-10} moles/min./ mg protein was $P < 0.001$ (127 df). Thus, these two cell lines can be regarded as "anomalous" with respect to G6 PDH activity, i.e. activity was not within the same distribution as for the other Kupffer cell lines. Interestingly, these anomalies had disappeared at the stage of 40 population doublings, and both cell lines possessed G6PDH activities within the range of the other 128 cell lines.

During the period of study, the position of a given cell line in an overall enzyme activity distribution was relatively stable. A cell line which initially possessed an enzyme activity at a given point in the distribution exhibited that activity at the same relative point in the distribution at later stages of the culture history. This observation was verified by considering the ranking of all cell lines for each enzyme activity after 26 and 40 population doublings. A Spearman rank correlation co-efficient was calculated for each enzyme and its significance tested by a t-test (see Siegel, 1956). Under the null hypothesis of no association between enzyme activity rank after 26 and 40 population doublings, $t > 6.5$ for all enzymes; thus with 128 df and a one-tailed test, $P < 0.001$. The null hypothesis is rejected and thus it appears as if, for each enzyme, a cell line maintained its position in the distribution of activities relative to the other cell lines. A cell line with a relatively high (low) activity for a given enzyme after 26 population doublings possessed a relatively high (low) activity for that enzyme after 40 population doublings.

Greater insight into the variation of enzyme activities in the 130 cell lines can be achieved by an analysis of variance of the data presented in appendix 1.1. In Table 4.4 appear analysis of variance tables based on data for each enzyme activity. Since it was proposed to achieve a general analysis, a model II, two-way analysis of variance with replication was conducted so that added variance effects could be detected within the two main effects (variance between cell lines and variance between times). A model II analysis considers the main effects as being random and the aim of the analysis is to estimate the variance of these effects. The cell lines were considered to be a random sample of possible Kupffer cell lines from the three animals. The sampling periods were also considered to be selected at random, since it was not wished in the first instance to establish that time 1 produced values different from those at time 2, but merely to estimate the magnitude of fluctuations in enzyme activity over the period studies. There was no *a priori* reason to believe in a directed trend of enzyme activity over the period of study. The assumptions of such a statistical design have been considered by Eisenhart (1947). The test of significance was a one-tailed test of the null hypothesis that the added variance was equal to zero against the alternative that it was greater than zero. In the calculation of the variance ratio, F_s, and the test of significance, interaction MS/error MS was first tested. If the interaction was significant, the test continued with between cell lines MS/interaction MS and between times MS/interaction MS; when interaction MS was not significant, the interaction SS could be pooled with the error SS.

The analyses presented in Table 4.4 demonstrate that all variance ratios were highly significant, $P < 0.001$. The examination of data for each enzyme activity revealed considerable variation between cell lines, between the three times or stages of assay, and a significant interaction component between cell lines and the stage of assay. Thus the null hypothesis is rejected and we accept instead the alternative of the existence of added variance components both between cell lines and between times of assay. These analyses enforce the previous proposition that there existed considerable variation between cell lines and that enzyme activities were different at each of the stages examined. The direction of change in the mean

Table 4.4:- Analyses of variance for enzyme activities in 130 primary, adult Kupffer cell lines after 26, 33 and 40 cumulative population doublings in culture

df = degrees of freedom, SS = sum of squares, MS = mean squares, F_s = variance ratio. The mean squares were rounded to four figure numbers.

Source of variation	df	SS	MS	F_s
Catalase				
Subgroups	389	204,810	526.5	
between cell lines	129	141,489	1,097	9.90
between times	2	34,728	17,360	157
interaction	258	28,593	110.8	11.3
Error	780	7,661	9.882	
Arginase				
Subgroups	344	678,286	1,972	
between cell lines	114	334,040	2,930	6.21
between times	2	236,674	118,300	251
interaction	228	107,572	471.8	25.0
Error	690	13,057	18.92	
MHO				
Subgroups	389	16,750	43.06	
between cell lines	129	11,776	91.29	9.71
between times	2	2,549	1,274	135
interaction	258	2,425	9.399	16.8
Error	780	438	0.561	
β-Glucuronidase				
Subgroups	389	12,102,914	31,113	
between cell lines	129	8,603,193	66,691	23.7
between times	2	2,774,165	1,387,000	493
interaction	258	725,556	2,812	20.2
Error	780	108,346	138.9	

Source of variation	df	SS	MS	F_s
Peroxidase				
Subgroups	389	2,758,701	7,092	
between cell lines	129	1,910,977	14,810	17.8
between times	2	632,932	316,500	380
interaction	258	214,792	832.5	6.99
Error	380	45,286	119.2	
ADH				
Subgroups	389	580,174	1,491	
between cell lines	129	495,536	3,841	20.0
between times	2	35,079	17,540	91.3
interaction	258	49,559	192.1	44.5
Error	780	3,365	4.314	
LDH				
Subgroups	389	15,605,515	40,120	
between cell lines	129	13,453,983	104,300	22.9
between times	2	974,837	487,400	107
interaction	258	1,176,695	4,561	15.6
Error	780	227,376	291.5	
IDH				
Subgroups	389	12,984,876	33,380	
between cell lines	129	11,300,786	87,600	18.0
between times	2	431,890	215,900	44.5
interaction	258	1,252,199	4,853	35.2
Error	780	107,466	137.8	
G6PDH				
Subgroups	389	474,242	1,219	
between cell lines	129	410,226	3,180	17.1
between times	2	16,146	8,073	43.5
interaction	258	47,870	185.5	19.9
Error	780	7,286	9.341	

Approximate critical values of F:- F, 0.001 (129, 258), F, 0.001 (114, 228) < 1.4; F, 0.001 (2, 258), F, 0.001 (2, 228) < 6.9; F, 0.001 (258, 780), F, 0.001 (228, 690), F, 0.001 (258, 380) < 1.0. Thus all values of F_s were highly significant.

Table 4.5:- Variance components as a proportion of overall variance in a study of nine enzyme activities in 130 primary, adult Kupffer cell lines after 26, 33 and 40 cumulative population doublings in culture

Enzyme	Variance component (%)			
	Between cell lines	Between times	Interaction	Error
Catalase	55.5	22.4	17.1	5.0
Arginase	24.6	50.3	22.3	2.8
MHO	57.4	20.5	18.6	3.5
β-Glucuronidase	60.8	30.4	7.6	1.2
Peroxidase	58.0	30.2	8.9	2.9
ADH	78.5	8.6	12.1	0.8
LDH	79.0	8.8	10.1	2.1
IDH	80.4	4.7	13.7	1.2
G6PDH	79.0	4.8	14.0	2.2

The component 'between times' refers to the variance of enzyme activities determined after 26, 33 and 40 doublings in culture. The variance components are based on the analyses of variance presented in Table 4.3.

enzyme activities presented in Table 4.2 suggests that the variation between times was due to a decrease in activity as culturing proceeded.

In all cases the interaction MS were significantly greater than the error MS. Such an observation suggests a dependence of the effect of one factor on the level of the other factor, and implies that the effects of cell lines and times were not simply additive. Although there was a difference in enzyme activity between times, this difference depended on the cell line considered. With the passing of time in culture an individual enzyme activity did not respond in the same way in all cell lines, although the average response or trend was for a decrease in activity.

The analyses of variance presented in Table 4.4 have established that there existed significant added variance in all sub-groups. Appropriate to a model II analysis it is necessary to express these added variance components as a proportion of the total variance. The contribution of each variance component to total variance is presented in Table 4.5. Variance components were calculated from the expected MS for a model II analysis of variance. Of great importance is the fact that the error component was not greater than 5% for any enzyme activity. This is not an estimate of the variation of the activity determinations, but rather a demonstration that any variation due to enzyme assay or protein determination was small when compared with the major variance components. The extent of variation between cell lines is evident from the variance components. The variation due to time accounted for less of the total variation, while the extent of interaction was variable. Interestingly, the dehydrogenases showed a variance pattern where the interaction contribution was greater than the variance component between times. Inspection of the original data in appendix 1.1 reveals that this was because a number of cell lines maintained a relatively stable dehydrogenase activity after 26 population doublings. The interaction components for β-glucuronidase

Table 4.6:- Product -moment correlation co-efficient (r) matrices for nine activities in 130 primary, adult Kupffer cell lines after 26, 33 and 40 cumulative population doublings in culture

After 26 population doublings

	Cat.	Arg.	MHO	β-Glu.	Perox.	ADH	LDH	IDH
Arg.	0.435							
MHO	0.662	0.243						
β-Glu.	-0.406	-0.174*	-0.207*					
Perox.	0.978	0.421	0.673	-0.387				
ADH	0.613	0.348	0.352	-0.298	0.586			
LDH	0.712	0.314	0.449	-0.258	0.698	0.753		
IDH	0.714	0.364	0.435	-0.359	0.710	0.722	0.836	
G6PDH	0.697	0.384	0.422	-0.269	0.692	0.688	0.771	0.793

After 33 population doublings

	Cat.	Arg.	MHO	β-Glu.	Perox.	ADH	LDH	IDH
Arg.	0.411							
MHO	0.805	0.311						
β-Glu.	-0.406	-0.122*	-0.284					
Perox.	0.987	0.424	0.787	-0.397				
ADH	0.665	0.381	0.555	-0.241	0.686			
LDH	0.830	0.339	0.631	-0.322	0.821	0.740		
IDH	0.785	0.356	0.616	0.363	0.788	0.717	0.812	
G6PDH	0.742	0.330	0.571	-0.247	0.744	0.639	0.786	0.738

After 40 population doublings

	Cat.	Arg.	MHO	β-Glu.	Perox.	ADH	LDH	IDH
Arg.	0.331							
MHO	0.916	0.300						
β-Glu.	-0.269	-0.122*	-0.186*					
Perox.	0.966	0.308	0.888	-0.277				
ADH	0.791	0.343	0.733	-0.289	0.773			
LDH	0.946	0.320	0.847	-0.324	0.920	0.808		
IDH	0.892	0.334	0.799	-0.307	0.864	0.730	0.935	
G6PDH	0.822	0.275	0.747	-0.301	0.802	0.769	0.879	0.850

* -not significant, $P(0.01) < 0.228$, 128 df.

and peroxidase activities were relatively low, suggesting that more cell lines responded in a similar way with respect to these activities. The variance components related to arginase activity had a different distribution to those for the other enzymes. There was a relatively small amount of variation between cell lines, but large variation between times.

The analyses presented above demonstrate that there was significant variation between cell lines and variation existed between stages in culture. The variation was such that when the activities and rates of fall of all enzyme activities were considered, each cell line was unique in its response to the culture environment. This variation can be utilized to study quantitative relationships between the nine enzyme activities. Table 4.6 presents product-moment correlation co-efficient matrices based on the data in appendix 1.1. The most striking feature is the high proportion of significant correlations, only 5 of 108 co-efficients failing to achieve significance. At all stages, catalase, peroxidase and the dehydrogenases appear to possess highly correlated activities, the degree of this correlation generally increasing as culturing proceeds. MHO activity increases its correlation with the above three activities and by the stage of 40 population doublings catalase, peroxidase, the dehydrogenases and MHO form a cluster in the statistical sense.

In contrast are arginase and β-glucuronidase activities. Although correlations between arginase and other enzyme activities are significant, with the exception of those with β-glucuronidase, they are at a lower level than those within the cluster described above. The correlations with arginase are also relatively homogeneous. The correlation co-efficients involving β-glucuronidase demonstrate a different pattern. In all cases they are negative, and the 5 cases of no significant correlation involve this enzyme. Thus without further analysis, three major clusters of enzyme activity can be discerned. These are:- (a) catalase, peroxidase, MHO and the dehydrogenases, (b) arginase and (c) β-glucuronidase.

The presence of at least three enzyme clusters can be verified by homogeneity tests of the relevant groups of correlation co-efficients after, for example, 40 population doublings. The arginase co-efficients are homogeneous (X^2 = 0.512, critical value χ^2_6 (0.05) = 12.59). Similarly the co-efficients with β-glucuronidase are homogeneous (X^2 = 4.78, critical value χ^2_7 (0.05) = 14.07). In the large cluster containing catalase, peroxidase, MHO and the dehydrogenases, the correlation co-efficients are heterogeneous (X^2 = 233.8, critical value χ^2_{20} (0.05) = 31.4). Thus, although obviously different from the other two, this cluster is heterogeneous and could probably be broken down into two or more components. Inspection of Table 4.6 reveals that correlation of enzymes in this cluster is least for pairs with ADH. Catalase and peroxidase are obviously closely associated activities. The co-efficients after 33 and 40 population doublings suggest that MHO is closely associated with these two enzyme activities. However, this must be reconciled with the relatively poor correlations after 26 population doublings. It would appear that the heterogeneous large cluster of enzyme activities could be broken down into three smaller clusters, one containing catalase and peroxidase and in relatively close association LDH, IDH and G6PDH, and the other two are clusters for correlations with ADH and with MHO. These minor clusters are associated with the large cluster to varying degrees.

In summary, examination of inter-relationships between enzyme activities in the 130 cell lines has revealed highly significant correlations and that these correlations can be decomposed into component clusters. The possible biochemical bases for these correlations are considered in section 7.

4.3.3 Enzyme activities in adult Kupffer cell lines during an extended period of culture

The 15 adult Kupffer cell lines utilized for the study in section 3.3.6 were maintained in culture for an extended period and the nine enzyme activities determined at various intervals during this period. The results of this extended study are presented in Figure 4.1. In appendix 2.1 appear the means, standard deviations and co-efficients of variation for each enzyme activity at each time. The first three points in the graphs presented in Figure 4.1 are the three examination stages considered in the previous section. Graphical presentation of data for enzyme activities in the 15 cell lines clearly illustrates the trend towards decreasing enzyme activity during the first three examination stages, i.e. after 26, 33 and 40 population doublings. The second conspicuous feature of the data was the stability of mean enzyme activities between 40 and 69 population doublings. The only exceptions to this are arginase and MHO activities which demonstrateda slight decline in activity during this period. The decline in arginase activity between 40 and 69 population doublings was not significant ($t = 1.22$, critical value $t\ (0.01) = 2.05$, 28 df), while that for MHO was significant ($t = 2.25$, critical value $t\ (0.05) = 2.05$, 28 df). After the stage of 69 population doublings both these enzymes demonstrated a steady decline in activity. With the exception of G6PDH, the other enzyme activities demonstrated stability between 40 and 83 population doublings. The decline in G6PDH activity between 69 and 83 population doublings was significant ($t = 2.66$, critical value $t\ (0.05) = 2.05$, 28 df).

Apart from β-glucuronidase, all enzyme activities appeared to decrease between 83 and 97 population doublings. The apparent increase in β-glucuronidase activity during this period was not significant ($t = 1.57$, critical value $t\ (0.05) = 2.08$, 21 df). In the case of catalase, peroxidase, ADH and LDH the decrease in activity

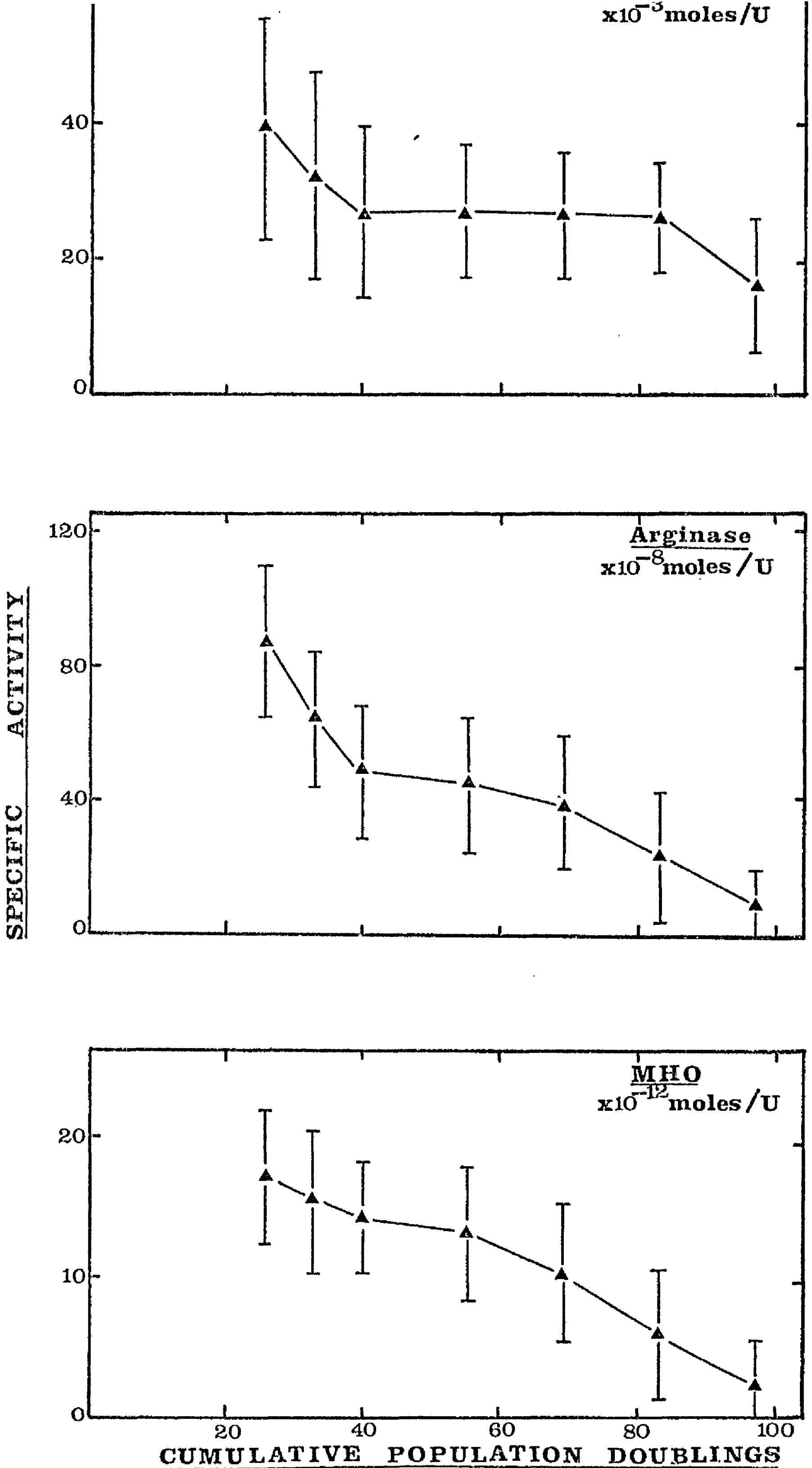


Figure 4.1:- Long term study of nine enzyme activities in 15 primary adult Kupffer cell lines.

The sample size at the stage of 97 population doublings was reduced to 8. Values are means $\pm$ SD (U = min./mg protein).

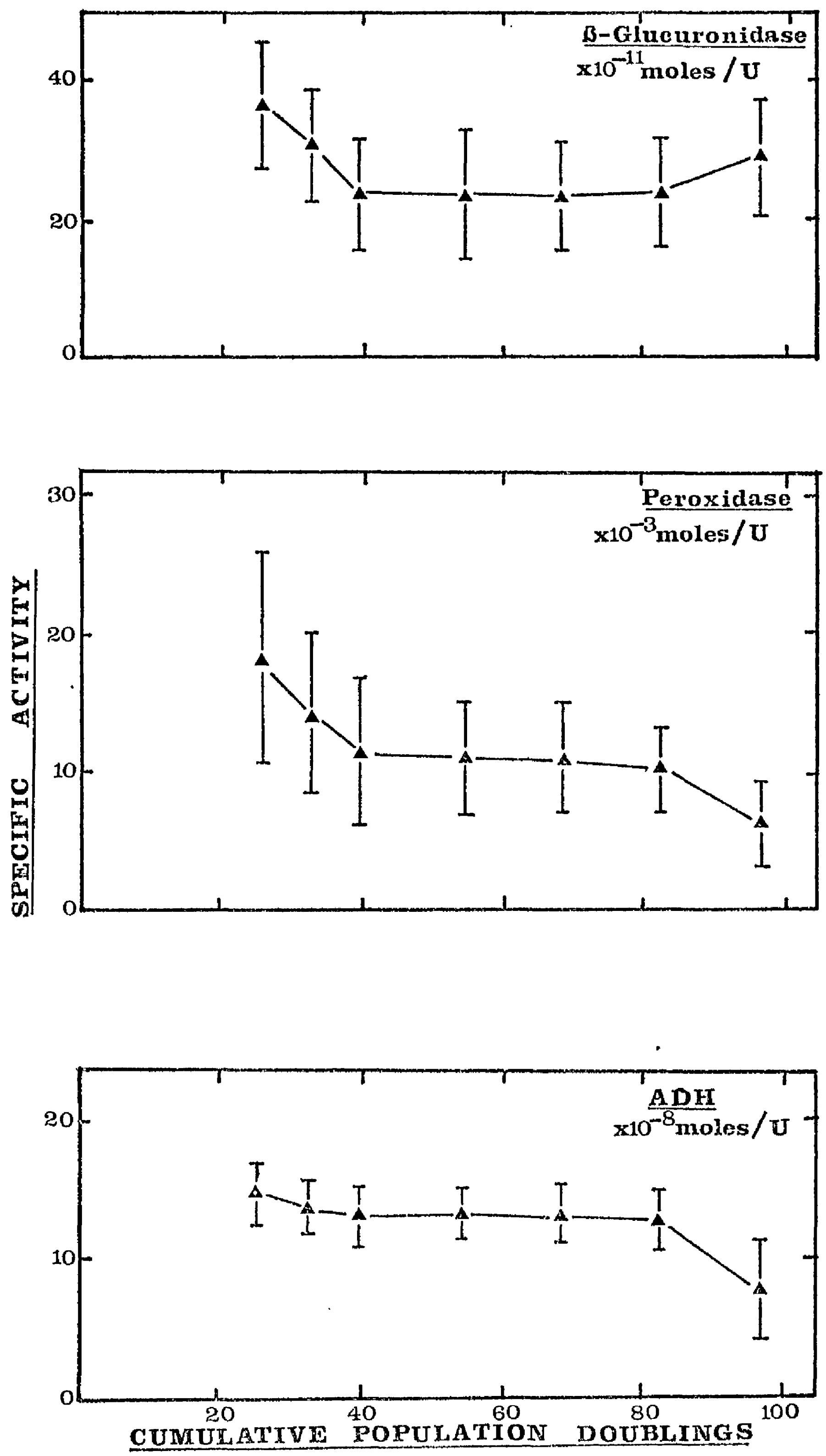


Figure 4.1 (continued)

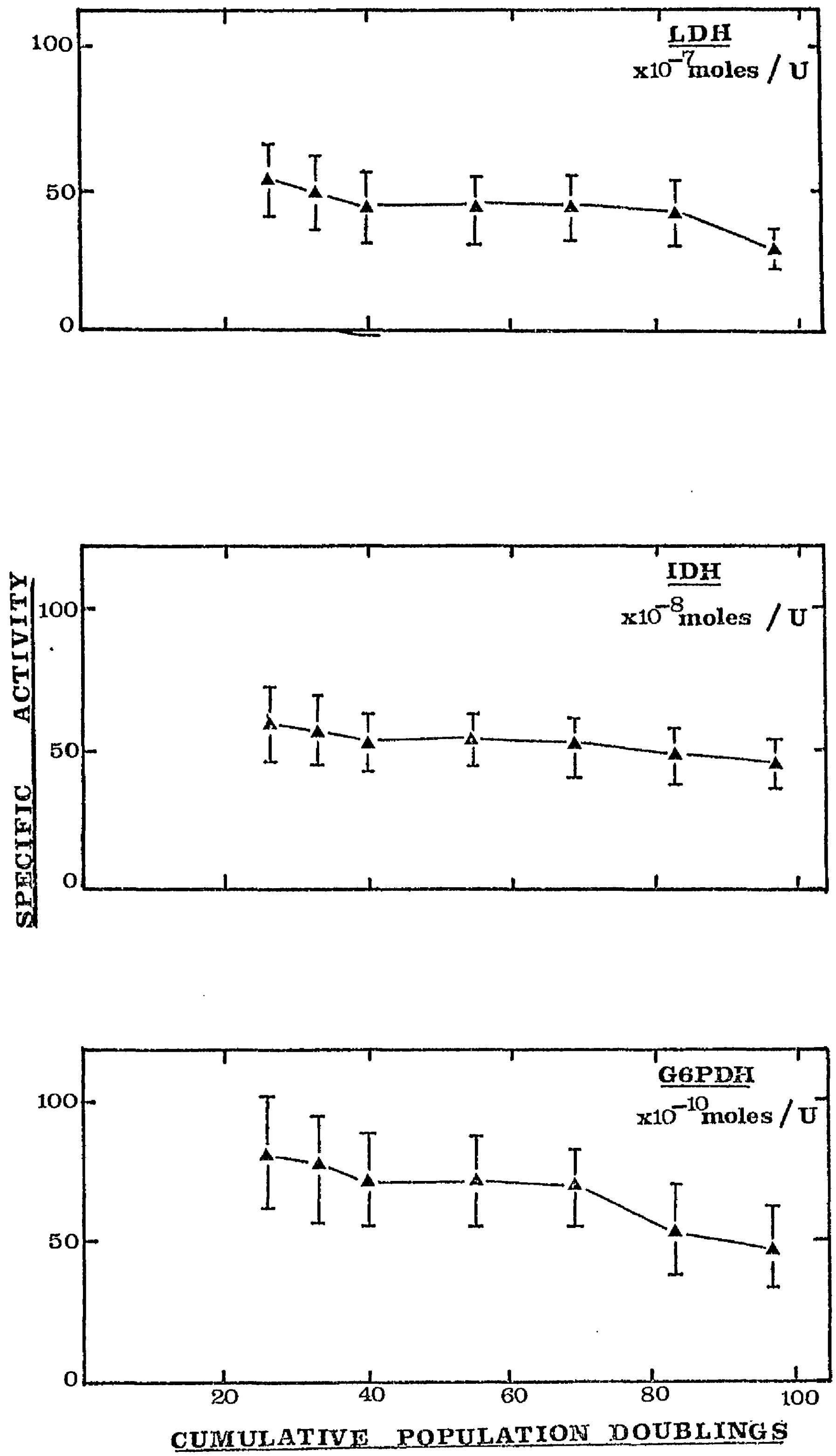


Figure 4.1 (continued)

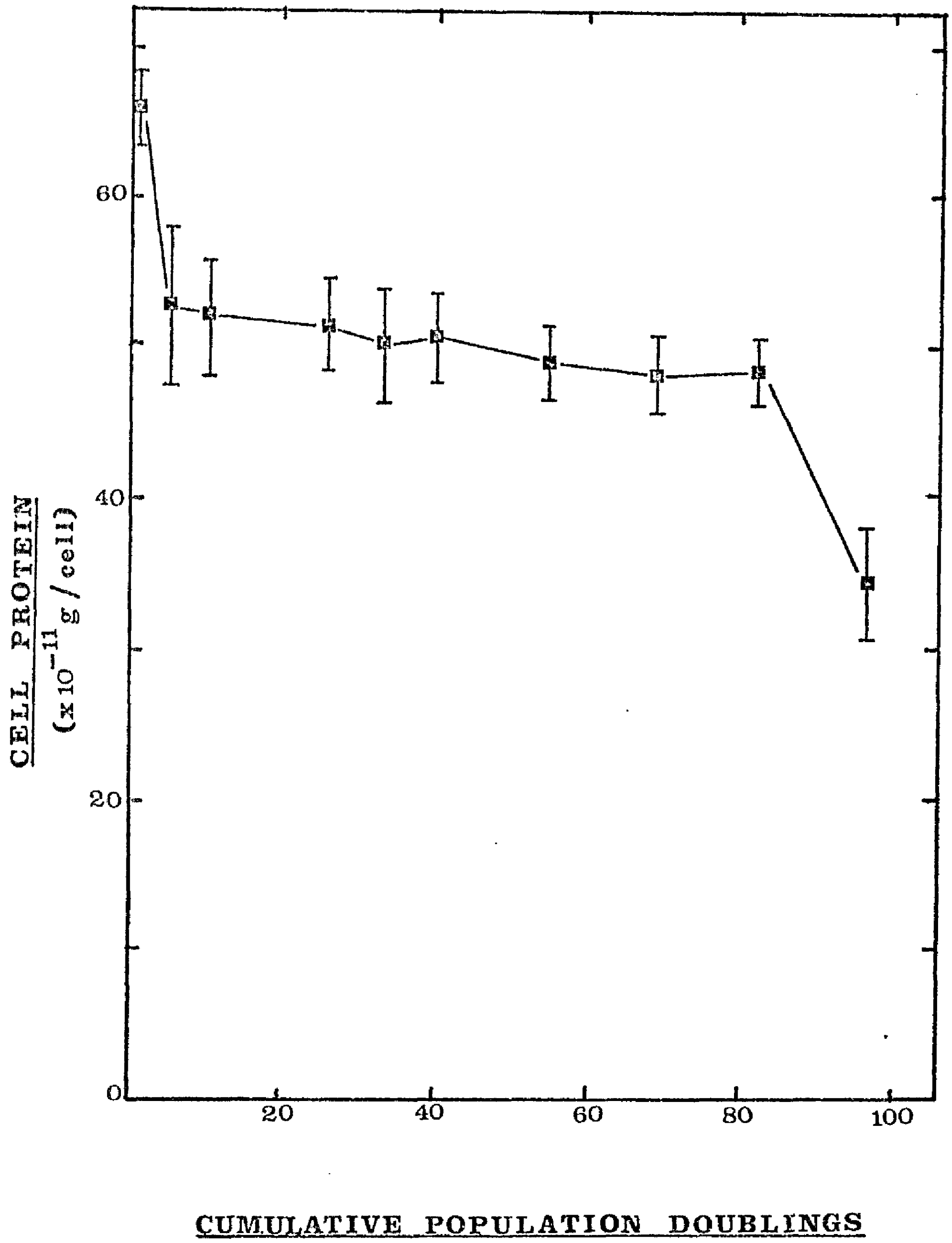


Figure 4.2:- Adult Kupffer cell total protein content as a function of the number of cumulative population doublings in culture.

See text for a description of samples. Values are means ± SD.

was a sudden departure from a value which had been stable for 43 population doublings.

The variation of each enzyme activity between the 15 cell lines underwent considerable changes during the culture period. Inspection of appendix 2.1 suggests 4 patterns of change in co-efficient of variation. The values remain relatively stable during the entire study period for β-glucuronidase, LDH, IDH and G6PDH activities. The co-efficient of variation for ADH activity was constant until 83 population doublings but then demonstrated a 3-fold increase by the time 97 population doublings were achieved. The other two patterns of change were the steady increase in co-efficient of variation for arginase and MHO activity during the entire study period, and the slight decrease which preceeded an apparent increase for variation of catalase and peroxidase activities. Thus, when compared with earlier stages, 5 of the 9 enzyme activities demonstrated considerable variation between the 15 cell lines after 83 population doublings and between the surviving 8 cell lines after 97 population doublings.

The enzyme activities in this and the previous section are presented in the form of specific activities. In order to interpret these values in terms of enzyme activity/cell it is necessary to consider the protein content of the Kupffer cells during the culture period. A decrease in protein content during the culture period is demonstrated in Figure 4.2. The estimations of protein content at 26 population doublings and beyond were based on aliquots of counted cell suspensions from five cell lines. The cells were obtained at the culture density when enzymes were assayed, i.e. at 525 cells/mm^2. The estimation for cells after 5 population doublings was based on 32 colonies each containing approximately 30 cells, and after 10 population doublings in 14 colonies of approximately 1,000 cells each. Estimation of protein in cell colonies was achieved by proportional reduction of all volumes in the protein assay and was based on freeze-dried colonies attached to "Melinex" substrate

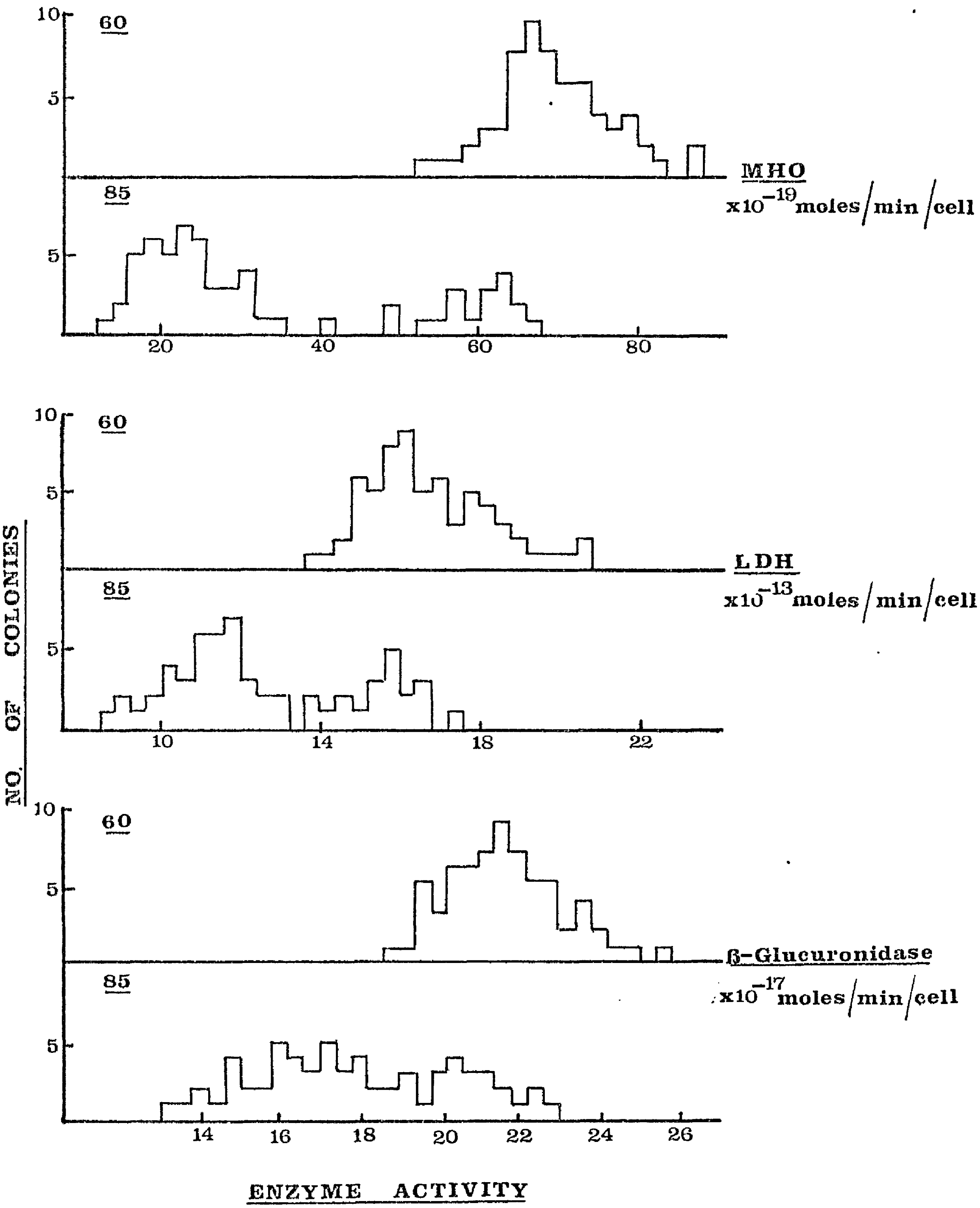


Figure 4.3:- Distribution of three enzyme activities in primary adult Kupffer cell colonies from cell line 095 after 60 and 85 population doublings in culture.

At the stage of enzyme assay colonies were of 60-80 cells in size.

(see section 4.2.4). The estimation at time 0 was based on 5 freshly isolated Kupffer cell suspensions.

There was a rapid decline in the protein content during the first five population doublings in culture. However, between the period of 5 and 83 population doublings there was no further decline in cell protein content ($t = 1.73$, critical value $t\ (0.05) = 2.31$, 8 df). A dramatic reduction in cell protein content occurred between 83 and 97 population doublings ($t = 7.77$, critical value $t\ (0.05) = 2.31$, 8 df). Thus, during the entire study period of the previous section, and until 83 population doublings in this study, cell protein content remained constant. The changes in enzyme specific activity during this period thus reflected the changes in enzyme activity/cell. After 83 population doublings, the decrease in cell protein content implies that the enzyme activity/cell has decreased more than is indicated by the change in specific activity.

The studies presented above suggest that there was considerable variation in enzyme activity between cell lines of similar origin, that this variation tended to increase as the culture period was extended and that for some enzymes there was a decline in activity during the later stages of culture. It is of interest to consider whether these changes within a cell line were the result of a gradual, but co-ordinated change in enzyme activity in all cells in the cell line population, or whether the change in activity was the result of the emergence of a sub-population of cells possessing a different enzyme activity. In order to investigate this point, the distribution of three enzyme activities between individual cells in cell line <u>095</u> after 60 and 85 population doublings were compared. The results of this study are shown in Figure 4.3. At each stage, cells from cell line <u>095</u> were seeded at low density onto "Melinex" substrate and enzyme activities determined by "micro-assay" on colonies of 60-80 cells in size. The enzyme activities were expressed per cell. The enzyme activity of cells in each colony was assumed

Table 4.7:- The calculation of Spearman rank correlation coefficients to test a possible association between the degree of diploidy and enzyme activity in 8 adult Kupffer cell lines after 97 population doublings

The calculation of r_s included correction for tied values:-

$$r_s = \frac{\Sigma x^2 + \Sigma y^2 - \Sigma d^2}{2 \sqrt{x^2 \; y^2}}$$

$$\Sigma x^2 = \frac{n^3 - n}{12} - \Sigma\left[\frac{t_x^3 - t_x}{12}\right]$$

$$\Sigma y^2 = \frac{n^3 - n}{12} - \Sigma\left[\frac{t_y^3 - t_y}{12}\right]$$

d = difference between paired observations at a given rank.

n = number of observation pairs.

t = number of observations tied at a given rank.

For a two-tailed test when n = 8, P (0.10) = 0.643, P (0.02) = 0.833 (see Siegel, 1956). Thus no r_s achieves significance.

Cell line	Ascending Rank									
	Diploidy	Catalase	Arginase	MHO	β-Glucuronidase	Peroxidase	ADH	LDH	IDH	G6PDH
088	1	2.5	1.25	1.2	5	5	7	1	1	1
021	2.5	7	1.25	6	4	2	5	5	7	3.25
123	2.5	8	7	1.2	1	3	2	6	8	8
066	4	2.5	1.25	1.2	3	4	1	3	5	3.25
072	5.5	5	8	1.2	6	7	8	8	4	7
112	5.5	6	1.25	1.2	2	8	6	2	6	3.25
055	7	1	6	7	7	6	3	7	2	2
095	8	4	5	8	8	1	4	4	3	3.25
Σd^2		107.5	63.7	58.8	34.0	77.0	90.0	54.0	104.0	86.2
$\underline{r}_s$		-0.282	0.195	0.205	0.597	0.087	-0.067	0.360	-0.233	-0.022

to reflect the enzyme activity of the colony's founding cell. Two distinct patterns of distribution can be discerned in Figure 4.3. Both MHO and LDH activities decreased between 60 and 85 population doublings, but more significant was the emergence of a bimodal distribution of enzyme activity in colonies of cells after 85 population doublings. The distribution of β-glucuronidase activity was not obviously bimodal and probably represented a normal distribution with large variance.

The bimodal distribution of MHO and LDH activities in cells from line 095 suggests that there had been the emergence of a sub-population of cells with low enzyme activity between 60 and 85 population doublings. Such a study was not performed either with other cell lines or other enzyme activities, but it seems reasonable to propose that the reduction in activity of a number of enzymes at the end of the extended culture period was the result of the emergence of cells with low enzyme activity at the expense of those with a higher activity.

As a conclusion to the extended study of enzyme activities in adult Kupffer cells, the enzyme activity data was analyzed with a view to examining a possible association between the degree of diploidy in a cell line and the activity of nine enzymes. The 8 cell lines which survived to the stage of 97 population doublings and whose karyotype has been described in Table 3.2 were used for this analysis. Possible association between enzyme activity and the degree of diploidy was tested by calculation of a Spearman rank correlation co-efficient. The results of this analysis are presented in Table 4.7. When the rank of diploidy and the rank of each enzyme activity were compared for each cell line, no association was observed between these two parameters. Although not significant, β-glucuronidase activity demonstrated the greatest association with diploidy. This analysis suggests that, at least in these 8 cell lines, enzyme activity was independent of the degree of diploidy. The degree of diploidy in these cell lines varied between 58% and 88% (see Table 3.2).

Table 4.8:- Enzyme activities in adult Kupffer cells during the early stages of culture

Values are means ± SD. Figures in parentheses indicate the co-efficient of variation (%) and sample size, i.e. no. of cells, colonies or cell lines. $d_{\frac{1}{2}}$ = number of divisions required to halve the enzyme activity during the initial stages of culture.

	Enzyme activity (moles/min/cell)					
	β-Glucuronidase ($\times 10^{-15}$)	MHO ($\times 10^{-17}$)	ADH ($\times 10^{-12}$)	LDH ($\times 10^{-11}$)	IDH ($\times 10^{-12}$)	G6PDH ($\times 10^{-14}$)
Kupffer cell homogenate	4.74 ±0.15	5.99 ±0.46	1.76 ±0.11	3.52 ±0.34	2.01 ±0.12	4.93 ±0.14
Single, freshly isolated Kupffer cells	5.13 ±0.21 (4.1; 45)	-	1.85 ±0.12 (6.5; 32)	3.70 ±0.34 (9.2; 25)	2.18 ±0.15 (6.9; 20)	5.34 ±0.14 (2.6; 35)
Kupffer cell colonies:- 2- 5 cells	-	-	-	0.867±0.093 (10.7; 25)	0.703±0.061 (8.7; 25)	-
10- 20 cells	0.446±0.054 (12.1; 34)	-	0.109±0.019 (17.4; 25)	-	-	0.413±0.064 (15.5; 25)
50- 65 cells	0.201±0.049 (24.4; 28)	1.364±0.184 (13.5; 16)	0.068±0.019 (27.9; 15)	0.234±0.046 (19.7; 25)	0.245±0.049 (20.0; 15)	-
150-170 cells	0.213±0.056 (26.3; 20)	1.264±0.254 (20.1; 12)	0.074±0.013 (20.1; 7)	0.218±0.054 (24.8; 15)	0.291±0.064 (22.0; 10)	0.376±0.098 (26.1; 20)
Kupffer cell lines after 26 doublings in culture	0.187±0.052 (27.8;130)	0.885±0.208 (23.5;130)	0.072±0.012 (16.7;130)	0.245±0.063 (25.7;130)	0.279±0.059 (21.2;130)	0.396±0.109 (27.5;130)
Approx. $d_{\frac{1}{2}}$	1.2	2.9	1.1	1.2	1.3	1.1

4.3.4 The rate of the initial decline in enzyme activity

The earliest stage of culture for which enzyme activities have so far been described is after 26 population doublings. The data presented in Table 4.2 indicate that by this stage all enzyme activities had decreased to a fraction of those in freshly isolated Kupffer cells. It is not clear whether this decrease in enzyme activity was a rapid process or whether it was the result of a steady decline during the first 26 population doublings. The enzyme activities presented in Table 4.8 were determined in an attempt to describe the rate of decline in enzyme activity during the first few divisions in culture. Kupffer cells for this study were isolated from animal no. 10 (see Figure 2.1), identified by phagocytic capacity and enzyme activities determined by "micro-assay" (see section 4.2.4). Preliminary studies established that ingestion of colloidal carbon had no effect on any of the enzyme activities. The spaces in Table 4.8 were those stages for which it was not possible to accurately determine enzyme activity. The data demonstrate general agreement between enzyme activity/cell in a Kupffer cell homogenate and the activity as determined in single cells by "micro-assay". In all cases the "micro-assay" resulted in an enzyme activity marginally greater than that determined for the homogenate.

The observations made in this study support the general belief (Davidson, 1964; Terzi, 1974) that enzyme activities rapidly decline when a cell enters the culture environment. The six enzyme activities for which it was possible to assay in small quantities of material demonstrated a rapid decline when the cells were isolated in culture. The initial decline rate was expressed as the number of divisions required to halve the enzyme activity ($d\frac{1}{2}$) and calculated between activities in freshly isolated Kupffer cells and cells from colonies containing 50-65 cells. The rate of initial decline in β-glucuronidase, ADH, LDH, IDH and G6PDH activities was not greatly different from that expected on the basis of simple dilution due to division. Such a situation would imply that there was a sudden reduction in synthesis or activation of these enzymes when the cells were removed from the in vivo environment and allowed to proliferate in culture.

The decline in MHO activity during this period ($d_{\frac{1}{2}} = 2.9$) was much slower than expected from dilution ($d_{\frac{1}{2}} = 1$), and thus followed different kinetics to those for the other enzymes. An explanation for this difference could lie in the inducible nature of MHO (Tenhunen et al., 1970). Red blood cells persisting after the dissociation stage and haem-containing cell debris could result in the induction of this enzyme activity.

Also in Table 4.8 appear estimates of the co-efficient of variation of the enzyme activities. The variation in enzyme activity between freshly isolated Kupffer cells is relatively small, with a co-efficient of variation of less than 10% for the enzymes considered. The data clearly illustrate a progressive increase in variation as proliferation continues. Kupffer cells from one animal were used for this study and were thus presumed to be genetically identical. Thus, enzyme activities in colonies derived from single, identical Kupffer cells rapidly diverged under a presumed constant environment until the stage of approximately 8 cell divisions.

With the exception of MHO activity, enzyme activities in cells from colonies of 150-170 cells were not greatly different from those in cells after 26 population doublings. The extent of variation was also similar to that described in section 4.3.2. Thus, after about the first 8 divisions the enzyme activities and patterns of variation were relatively stable when compared with the initial divisions.

4.3.5 The culture cycle and error due to cell density estimation

The description of variation in enzyme activities and at various times during the culture history presented in section 4.3.2

has demonstrated that most of the variation was not due to error in the assay of enzyme activity or protein. However, the studies have been based on the assumption that the variation was not due to error in the estimation of cell density (525 cells/mm^2) and thus a constant point in the culture cycle at the stage of assay. Pan and Krooth (1968) cite numerous examples of enzymes whose activity changes during the culture cycle. Cell line 095 was used for a study of protein and enzyme activity variation during the culture cycle, and to examine the contribution of error in the estimation of cell density to the observed enzyme activity variations for this cell line. The studies were conducted on the cell line during the stable phase in culture after 40 population doublings.

In Figure 4.4 appears a growth curve for cell line 095. After subculture there was an initial decline in cell number, followed by near exponential growth between days 1 and 5. After day 6, the cells had achieved a saturation density of approximately 8.9×10^4 cells/cm^2. During the culture cycle there was no major change in the protein content/cell (see Figure 4.4). During the period of exponential growth there may have been a slight reduction in cell protein content and a marginal increase at confluence.

In Figure 4.5 appear estimates of seven enzyme specific activities during the culture cycle of cell line 095. Brief inspection reveals considerable variation in some enzyme activities during the culture cycle. The fact that protein content/cell was relatively stable during the culture cycle suggests that the changes in enzyme specific activities presented in Figure 4.5 also reflect the changes in enzyme activity/cell. The variation in enzyme activity during the culture cycle confirms the need for an assay to be performed at a precise cell density.

Cell line 095 was used to determine the significance of error due to cell density estimates. Seven subcultures of line 095 were established according to the routine described in Figure 3.1

Figure 4.4:- Increase in the number of cells in a 50 mm petri dish and relative protein content/cell during the culture cycle of primary adult Kupffer cell line 095.

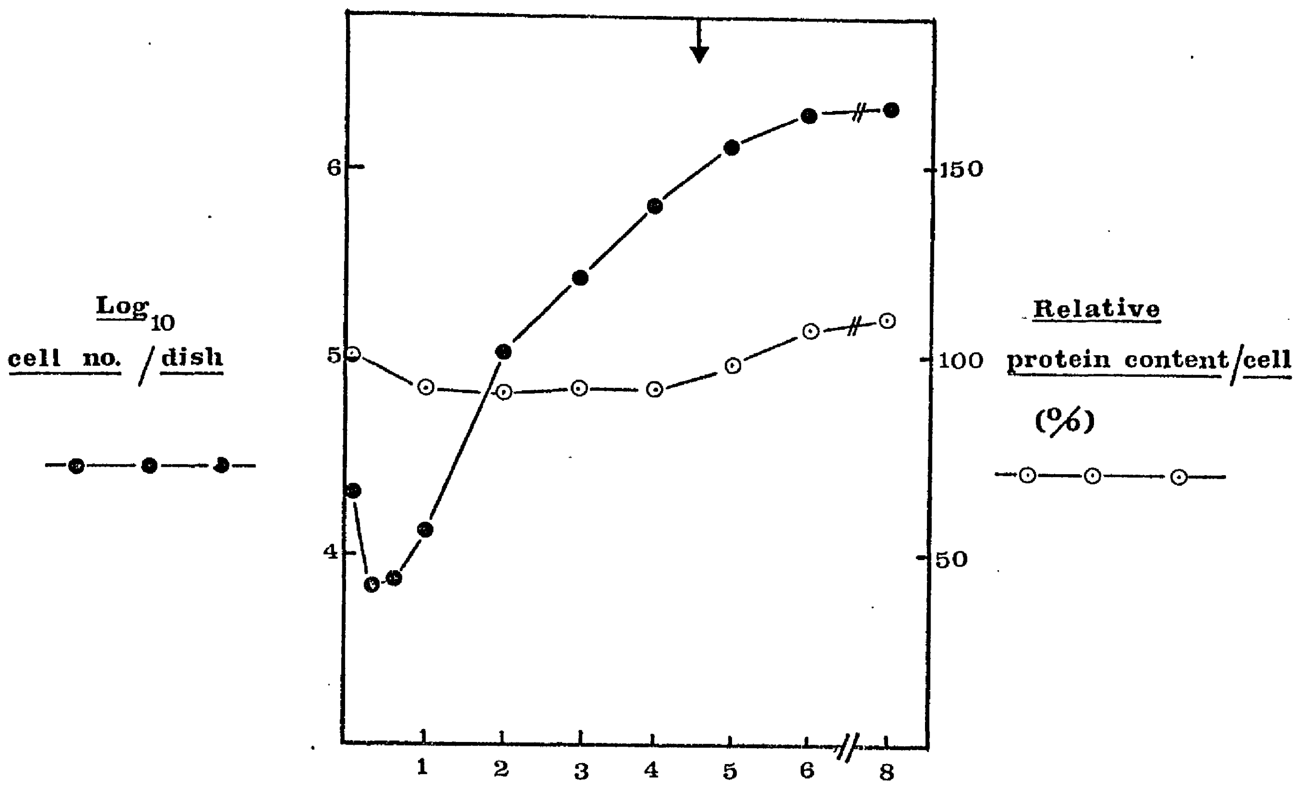
Values are means of triplicate observations. Arrow indicates stage in culture cycle corresponding to 525 cells/mm^2.

Figure 4.5:-

(a) Catalase (Cat.), arginase (Arg.) and β-glucuronidase (β-Gluc.) relative specific activities during the culture cycle of primary adult Kupffer cell line 095.

(b) G6PDH, IDH, ADH and LDH relative specific activities during the culture cycle of primary adult Kupffer cell line 095.

All values are means of triplicate observations. Arrow indicates stage in culture cycle corresponding to 525 cells/mm^2.


Log10
cell no. / dish
Relative
protein content / cell
(%)
6
5
4
150
100
50
1
2
3
4
5
6
8

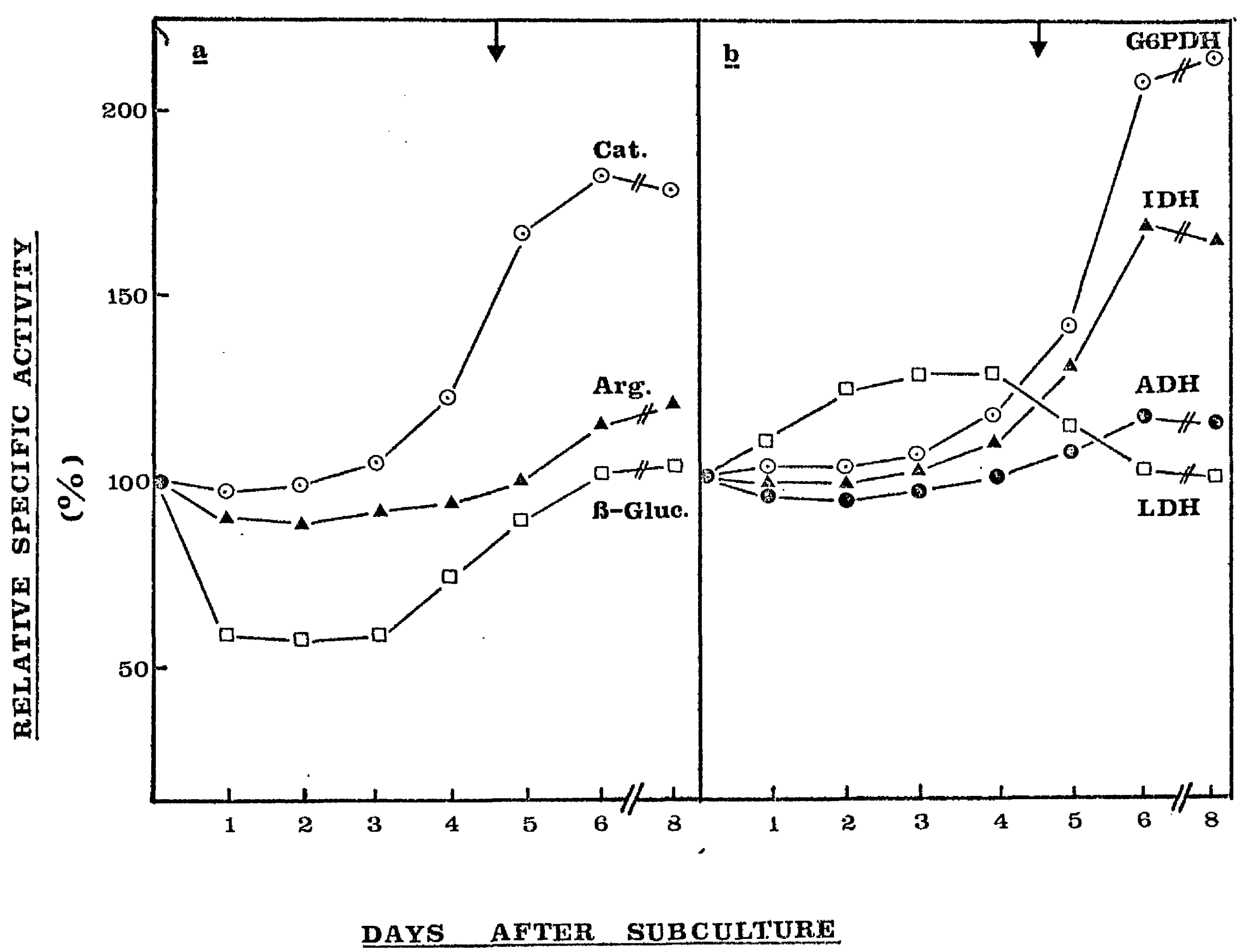

a
b
RELATIVE SPECIFIC ACTIVITY
(%)
200
150
100
50
Cat.
Arg.
ß-Gluc.
G6PDH
IDH
ADH
LDH
1
2
3
4
5
6
8
DAYS AFTER SUBCULTURE

Table 4.9:- A test of the variance contribution of cell density estimates to enzyme activity variance

A single classification, model II analysis of variance was conducted on data from 7 subcultures (among groups) of cell line 095. Five replicate enzyme assays were conducted on one cell extract from each subculture (within groups; error). For a one-tailed test $F_{0.10(6,28)} = 2.00$, $F_{0.05(6,28)} = 2.45$.

Enzyme	Source of variance	df	MS	F_s	Variance component (%)
Catalase	Among groups	6	182.0	1.60	10.7
	Within groups	28	113.9		89.3
Arginase	Among groups	6	58.5	2.07*	17.6
	Within groups	28	28.3		82.4
MHO	Among groups	6	2.33	0.59	7.5
	Within groups	28	3.93		92.5
β-Glucuronidase	Among groups	6	42.7	2.41*	22.0
	Within groups	28	17.7		78.0
Peroxidase	Among groups	6	56.5	2.36*	21.3
	Within groups	28	24.0		78.7
ADH	Among groups	6	9.83	0.50	9.1
	Within groups	28	19.8		90.9
LDH	Among groups	6	19.8	1.21	4.0
	Within groups	28	16.4		96.0
IDH	Among groups	6	43.5	0.73	5.2
	Within groups	28	59.9		94.8
G6PDH	Among groups	6	23.0	1.66	11.6
	Within groups	28	13.9		88.4

* -significant, $P < 0.10$

and five replicate enzyme assays conducted on one cell extract from each subculture at the stage of 525 cells/mm^2. Model II, single classification analyses of variance were conducted on the data and a summary of these analyses appears in Table 4.9. At a level of significance of $P = 0.10$, there was significant variation between subcultures of arginase, β-glucuronidase and peroxidase activities. In contrast was the homogeneity of subcultures with respect to catalase, MHO, ADH, LDH, IDH and G6PDH activities. The significance of the variation in activities of arginase, β-glucuronidase and peroxidase was marginal. Both arginase and β-glucuronidase activities demonstrated less variation during the culture cycle than, for example, catalase and G6PDH activities. However, inspection of Figure 4.5 would suggest that estimations of catalase and G6PDH activities would be the most susceptible to error in cell density estimation. Therefore, it is possible that the observed variations of β-glucuronidase and arginase activities in subcultures of cell line <u>095</u> are the result of a lesser assay variance component and possibly the existence of real, biological, variation between subcultures, i.e. not related to differences in cell density estimates at the time of assay. However, for those enzyme activities which show statistically significant variation between subcultures, the conservative view that this variation was due to error in estimating cell density must be entertained.

The calculation of variance components (see Table 4.9) revealed that in all cases, variation due to the enzyme assays and protein determination accounted for more than 78% of the total variation between subcultures. Conversely, variation between subcultures, presumably due to variation in cell density estimates, accounted for less than 22% of the total variation between subcultures of cell line <u>095</u>.

Although the results of this section consider only one primary, adult Kupffer cell line, it seems reasonable to suggest that the variation in enzyme activities between the Kupffer cell lines was not due to error in cell density estimations or enzyme and protein assays. In section 4.3.2 was presented evidence that enzyme and protein assay was only a small, and insignificant, fraction of the total variation between cell lines. In this section is presented evidence that error due to cell density estimates is even smaller relative to enzyme and protein assay error. Thus, it is concluded that the variationsreported in section 4.3.2 are in fact genuine variations between cell lines and not the result of variations in the techniques employed.

SECTION 5

ENZYME ACTIVITIES IN SV40-TRANSFORMED ADULT AND FOETAL KUPFFER CELL LINES

5.1 Introduction

In recent years it has become apparent that transformation of cultured mammalian cells by oncogenic viruses is an analogous process to the induction of tumours in animals (Tooze, 1973). In the context of this dissertation, the term "transformation" refers to the process whereby normal cells assume a neoplastic potential. Under appropriate conditions these cells form malignant tumours upon injection back to the homologous host. When transformed by oncogenic viruses, cultured cells acquire a set of properties some of which, including increased malignancy are characteristic of tumour cells. Such properties may include high saturation density, reduced serum requirement, ability to grow in semi-solid suspension culture, growth in a less oriented manner, growth on monolayers of normal cells, increased agglutinability by plant lectins, and the emergence of foetal and virus-specific antigens (Benjamin, 1974). Since oncogenic viruses possess limited genetic information, it appears that most of the observed alterations in transformed cells must be pleiotropic or indirect responses of the cell to the virus (Tooze, 1973).

Accompanying the changes associated with neoplastic transformation is an increase in phenotypic variation of the cell cultures. Examples of this variation have been described in sections 1.5 and 1.6. With reference to transformation of cultured mammalian cells by oncogenic viruses, Ponten (1971, p 61) has noted that:-

> "The variability and individuality of the transformed cultures is highly reminiscent of *in vivo* tumours which often have characteristic minor features, which differ even within the same tumour category".

Although a number of different groups of virus have been shown to be oncogenic in cultured cells (see Ponten, 1971), this study is restricted to the use of the oncogenic Papova virus, Simian Virus 40 (SV40). The use of SV40 in studies of transformation of cultured cells has been discussed in several extensive reviews (Defendi, 1966; Black, 1968; Eckhart, 1969; Green, 1970; Ponten, 1971; Tooze, 1973;

Benjamin, 1974; Butel and Estes, 1975) and will not be considered in detail. Infection of Chinese hamster cells with SV40 leads to non-productive infection, i.e. abortive or stable transformation, and no detectable syntheses occur of either viral DNA or viral structural proteins (see Benjamin, 1974). Cells permanently transformed by SV40 contain all the genetic information of the virus as shown by nucleic acid hybridization (Sambrook et al., 1968), release infectious virus upon fusion with permissive cell lines (Watkins and Dulbecco, 1967), and synthesize virus-specific intra-nuclear tumour (T) and surface transplantation antigens (Black et al., 1963; see Ponten, 1971). SV40 transformed cells also possess unrestrained growth potential in culture and demonstrate irregular orientation (Ponten, 1971).

At present, there is no general agreement on which particular property or set of properties of virus-transformed cells underlies their neoplastic growth potential. The fact that presence of T-antigen is positively correlated with transformation in culture (Black, 1966) and tumorigenicity (Diamandopoulos and Enders, 1966) provides a property with pathological significance. The presence of properties such as irregular growth, decreased density dependent inhibition of growth and decreased serum requirement do not necessarily imply tumorigenicity (Benjamin, 1974). Since most, if not all virus-transformed cells contain virus-specific T-antigen, its detection provides an easy method for determining whether or not a cell is transformed by a particular virus. It is not known whether T-antigen is required for transformation or for the maintenance of the transformed state (Tooze, 1973). The work described in this section attaches the most weight to presence of T-antigen as being the criterion of transformation and thus neoplastic potential.

The nature and significance of T-antigen are not understood, and at present it serves only as a marker of transformation. Evidence indicates that T-antigens are virus-coded proteins or specifically modified cellular antigens (Benjamin, 1974). The recent studies of Tenen et al. (1975) utilizing temperature sensitive SV40 mutants suggest that

T-antigen is a product of the SV40 A gene.

In this section it is proposed to examine the variation in enzyme activities between SV40-transformed Kupffer cell lines originating from genetically identical material. Such a study could provide insight into the nature of phenotypic variation, oncogenic virus/mammalian cell interaction, and neoplastic transformation. Since neoplastic transformation results in the emergence of several foetal characteristics (Knox, 1972; see section 1.5), it could be of value to describe variation in foetal cell lines, and examine to what extent foetal characteristics emerge in SV40-transformed adult Kupffer cells.

While the study of primary adult Kupffer cell lines in section 4 revealed considerable heterogeneity between cell lines, no cell lines possessed an enzyme activity near or above the in vivo level. The fact that transformation of cells in vivo or in culture has been reported to result in considerable variation was to be exploited in an attempt to isolate cell lines with an "anomalous" enzyme activity. Cell lines with extreme activities could be of considerable value in future enzyme regulation studies. The possibility of isolating cell lines with "anomalous" enzyme activities may be enhanced by the mutagenic action of SV40 in cultured cells (Marshak et al., 1973).

By way of a diversion, it is interesting to note that Kupffer cell neoplasms are extremely rare in man. MacSween et al. (1973) and Sussman et al. (1974) have reviewed the available data based on less than 100 known cases. The lethality of this malignancy is striking. The paucity of cases probably reflects not only diagnostic difficulties but also a resistance of this cell type to malignant transformation in vivo. The basis for such stability is not understood but may indicate caution in the generalization of phenomena reported in this section to other cell types.

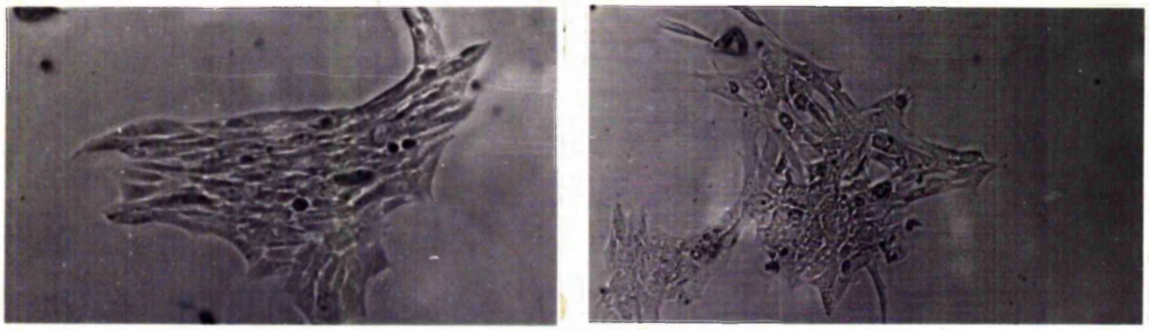
The results of this section are discussed in more detail in section 7.

Figure 5.1:- Examples of normal and SV40-transformed Kupffer cell colony morphology and the subculturing routine for SV40-transformed Kupffer cells.

The phase-contrast photomicrographs are of colonies of cells seeded at the stage of 26 population doublings in culture.

(p. e. - plating efficiency)

untransformed **transformed**



Subculturing routine for SV40-transformed Kupffer cells

Stage		Approximate population doublings since SV40 infection
1.	SV40-infected cells seeded onto glass fragments. Select irregular colonies of approximately 100 cells.	6
2.	Transfer cells to plastic culture wells (p. e. 28%) and grow to approximately 5×10^4 cells	17
3.	Seed 1×10^4 cells into 50 mm petri dish (p. e. 33%) and grow to density of 525 cells/mm^2 (10^6 cells). Prepare cell extracts for examination (I).	26
4.	Seed 4×10^4 cells into 4 oz. glass bottle (p. e. 45%) and grow to density of 525 cells/mm^2 (2.4×10^6 cells). Prepare cell extracts for examination (II).	33
5.	Seed 4×10^4 cells into 4 oz glass bottle (p. e. 45%) and grow to density of 525 cells/mm^2 (2.4×10^6 cells). Prepare cell extract for examination (III).	40

Subsequent subculturing based on an inoculum of 4×10^4 cells into 20 oz glass bottle (p. e. 45%) and grown to a density of 525 cells/mm^2 (6.8×10^6 cells). Results in 8.5 population doublings per subculture.

5.2 Materials and Methods

General culture methods, enzyme assays, data analysis and karyotype preparation have been described in preceeding sections.

Control cell lines:- Tumour-forming SV40-transformed Chinese hamster cell line possessing T-antigen obtained from Flow Ltd. and designated CHSV40-T. Tumour-forming spontaneous-transformed Chinese hamster cell line obtained from Flow Ltd. and designated CHSp-T.

Virus:- The SV40 was a plaque purified, small plaque variant originally obtained from Dr. J. F. Williams (Institute of Virology, University of Glasgow) and had been propagated in BSC-1 cells, an established line of African green monkey kidney cells. The stock titre was 2×10^9 PFU/ml.

5.2.1 Transformation of Kupffer cell lines

After identification (see section 3.2) Kupffer cells were removed from the substrate by trypsin/versene (see section 2.4) and washed in serum-free medium. The cells were resuspended in fresh serum-free medium (4×10^4 cells/ml) and 0.25 ml of cell suspension was incubated with SV40 at a multiplicity of infection of 500 PFU/cell for 3 hours at 37°C with agitation every 20 mins. At the end of incubation the cells were suspended in 5 ml fresh medium (see section 2.3) and centrifuged (200 g, 5 mins, 10°C) before inoculation of plastic petri dishes containing glass coverslip fragments (see section 3.2.2). Inoculation was in the range of 50-1,000 cells/plate. The medium was changed daily, and after approximately 5 days, the cells were inspected and those colonies with irregular, layered morphology selected for further culture as described in section 3.2.2 but with the subculturing routine described in Figure 5.1. Coverslip fragments supporting only one colony were selected. An example of the morphology can be seen in Figure 5.1.

5.2.2 Isolation of foetal cell lines

Foetal Kupffer cells were isolated from a mid-term female Chinese hamster embryo (animal no. 15 in Figure 2.1). The embryonic liver was excised under a dissecting microscope and Kupffer cells isolated by the method described for adult cells (see section 3.2.1) except that the cell suspension was not filtered, and all glassware was coated with "Repelcote" (Hopkins and Williams Ltd.). Cell lines were initiated as for adult Kupffer cells (see section 3.2.2). The sex of the embryo was determined by karyotypic analysis of the resultant cell lines.

A mid-term female foetus (animal no. 17 in Figure 2.1) was used to establish primary foetal Kupffer cell lines which were then transformed by SV40 as described above.

5.2.3 Detection of SV40-T-antigen

Tumour antigen was detected by two methods. The complement-fixation (CF) test was that used by Black et al. (1963) and Diderholm and Wesslen (1965). The indirect immunofluorescence detection of T-antigen was based on the method described by Pope and Rowe (1964). Both these methods utilized commercially available immunological materials.

Materials:-

Anti-SV40-T antiserum (Flow Ltd.). Prepared from tumour-bearing hamsters, non-anti-T antibodies removed, stored at -20°C.

Haemolytic antiserum (Flow Ltd.). Rabbit anti-sheep erythrocyte antiserum stored at a stock dilution of 1/100 at -20°C. Both sera were incubated at 56°C for 30 mins to destroy complement.

Fluorescent conjugate (Flow Ltd.). Rabbit anti-hamster globulin labelled with fluorescein isothiocyanate, stored at -20°C.

Complement (Flow Ltd.). Guinea-pig serum, stored at -20°C.

Sheep erythrocytes (Flow Ltd.). Stored at 4°C.

Veronal buffer diluent. 85 g NaCl and 3.75 g sodium-5, 5-diethyl barbiturate were dissolved in about 1,400 ml distilled water; 5.75 g 5, 5-diethyl barbituric acid were dissolved in about 500 ml hot distilled water. The two solutions were mixed, allowed to cool and 5.0 ml of a stock solution containing 1.0 M $MgCl_2$ and 0.3 M $CaCl_2$ added. The volume was increased to 2 litres with distilled water and the solution stored at 4°C.

Positive control antigen (Flow Ltd.). Tumour-forming, SV40-transformed Chinese hamster cell line possessing T-antigen, designated CHSV40-T.

Negative control antigen (Flow Ltd.). Tumour-forming spontaneous-transformed Chinese hamster cell line without T-antigen, designated CHSp-T.

Complement-fixation test

Haemolytic system:- The haemolytic antibody was titrated for the dilution which resulted in optimal sensitization of sheep erythrocytes. One ml portions of antiserum dilutions (1/400 - 1/6,400) were added to 1 ml portions of a standardized suspension of sheep erythrocytes (10^9 cells/ml) in 10 ml centrifuge tubes with constant agitation. One ml portions of each of these cell suspensions were distributed in a series of 40 ml centrifuge tubes, 5.5 ml of diluent added to each, followed by 2.0 ml of an appropriate complement dilution. The complement dilution was chosen so as to yield approximately 50-70% haemolysis of optimally sensitized cells (1/200 dilution of the guinea-pig serum used in this study). The tubes were incubated at 37°C for 90 mins with occasional agitation. At the end of this period, the tubes were centrifuged (100 g, 5 mins, 4°C) and the degree of lysis estimated by photometric measurement of the supernatant at 541 nm. Tubes yielding complete lysis contained 1 ml of cell suspension and 6.5 ml of the 1/200 dilution of complement. The contribution to optical density made by the complement was subtracted from all readings on a proportional basis.

Table 5.1:- Complement fixation (CF) test control reactions.

In the controls, antiserum and antigen were at half the dilution routinely used in the CF test. + indicates presence and - indicates absence of component.

	Antiserum	Antigen	Complement	Haemolytic system
CF test	+	+	+	+
Controls 1	+	-	-	+
2	-	+	-	+
3	-	-	+	+
4	+	+	-	+
5	+	-	+	+
6	-	+	+	+
7	-	-	-	+

Table 5.2:- Two-dimensional complement fixation (CF) test with CHSV40-T cells as antigen and anti-SV40-T antiserum.

Dilution of antigen refers to dilution of the suspension. 0 means no lysis, 4 means complete lysis.

		Antiserum dilution (reciprocal)							
		10	20	40	80	160	320	640	1280
Antigen dilution (reciprocal)	2	0	0	0	1	4	4	4	4
	4	0	0	0	0	3	3	4	4
	8	0	0	0	0	0	3	3	4
	16	0	0	0	0	0	0	4	4
	32	0	0	0	0	0	0	2	4
	64	0	0	0	0	0	0	3	4
	128	4	4	4	4	4	4	4	4
	256	4	4	4	4	4	4	4	4
	512	4	4	4	4	4	4	4	4

On the basis of the titration of haemolytic antibody, an appropriate dilution was prepared for optimal sensitization (generally a 1/800 dilution of rabbit anti-sheep erythrocyte antiserum), and this dilution was slowly pipetted into an equal volume of standardized cell suspension with agitation. Sensitized cells were prepared only as required. The titration scheme was repeated when different batches of complement, erythrocytes or haemolytic serum were used.

Fixation:- CF tests were performed in round-bottom plastic wells (500 μl capacity, Linbro). The fixation system contained 0.05 ml anti-SV40-T antiserum, 0.05 ml antigen solution and 0.05 ml complement. The mixture was incubated for 24 hours at 4°C, allowed to warm to room temperature and 0.1 ml of the haemolytic system containing 2×10^8 sensitized cells/ml added. The fixation was scored by inspection after incubation at 37°C for 60 mins. The scoring was based on a system of 0 = no lysis, 1 = 25% lysis, 2 = 50% lysis, 3 = 75% lysis and 4 = complete lysis. In Table 5.1 appear the control reactions conducted with every series of CF tests. A test was discarded if any control demonstrated positive fixation.

The complement was a 1/200 dilution of guinea-pig serum and 0.05 ml contained 5 H_{50} units (one H_{50} unit is that amount of complement required for 50% lysis of the above number of cells in the stated reaction volume). The antigen was a 10% (v/v) suspension of the appropriate cells in veronal buffer. The suspension was three times frozen in liquid nitrogen and thawed, and stored at -70°C.

In order to determine the appropriate anti-SV40-T antiserum dilution for routine CF screening of cell lines, a two-dimensional fixation test was conducted. This test is presented in Table 5.2 and examined fixation as a function of both antiserum and antigen dilution. The antigen was prepared from the positive control cell line CHSV40-T. There existed a constant "cut-off" at a dilution of 1/64 which was independent of antiserum dilution to 1/160. The highest dilution of antiserum giving a positive CF reaction varied in direct proportion to

a. b.

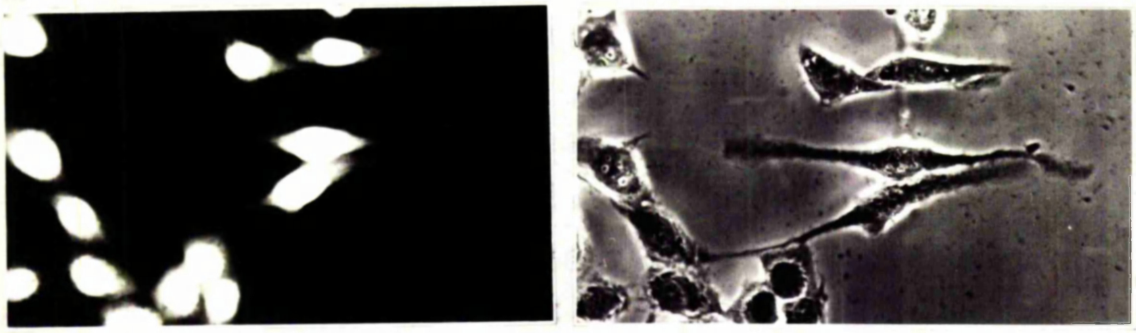


Figure 5. 2:- The localization of SV40-T-antigen in SV40-infected cultured Chinese hamster Kupffer cells by indirect immunofluorescence.

Over-exposure of the photograph of cells under fluorescence illumination (a) clearly illustrates the intra-nuclear location of the T-antigen. Included is the same field of cells under phase-contrast illumination (b). Poor morphology is due to fixation in cold acetone (approx. x 1500).

a. b.

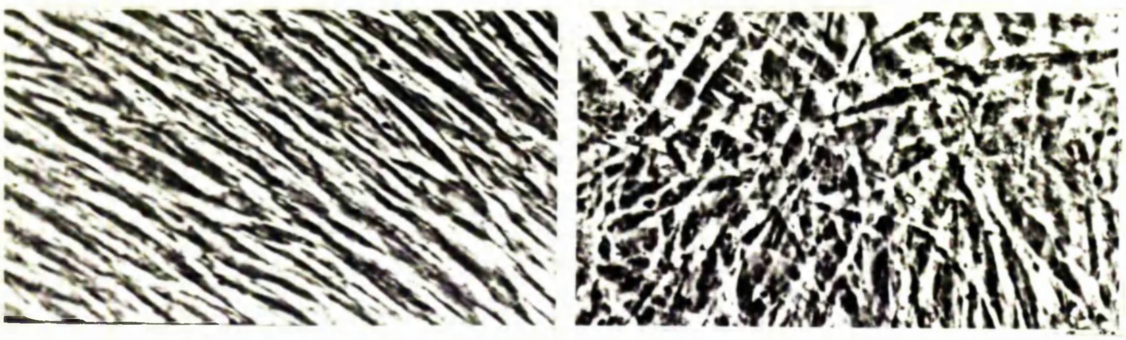


Figure 5. 3:- The morphology of confluent cultures of primary adult (a) and SV40-transformed adult Chinese hamster Kupffer cells (b).

Phase contrast (approx. x 150).

the antigen dilution. Antigen quantities which exceeded the equivalence zone resulted in inhibition of CF although ample antibody was present. On the basis of this test an antiserum dilution of 1/80 was selected as the most appropriate for subsequent tests. In CF tests, CF results were quoted as antigen titres and were the maximum antigen dilution which result in CF (i.e. a score of 0) with a 1/80 dilution of the antiserum. The anti-SV40-T antiserum showed no CF reaction with SV40 as antigen, thus antibodies to viral capsid antigen(s) did not contribute to the CF titre.

Immunofluorescence:- Cells were grown on glass coverslips until approximately 50% confluent, washed twice in cold PBS/A, fixed for 10 mins in cold acetone, air-dried and stored in a nitrogen atmosphere at -70°C. In the indirect immunofluorescence test the cells were incubated with a 1/10 dilution of anti-SV40-T antiserum at 37°C for 30 mins in a humid atmosphere, washed twice in PBS/A for 5 mins and rinsed in distilled water. Fluorescein isothiocyanate-labelled rabbit anti-hamster globulin antiserum was added at a dilution of 1/10 and incubation performed for 30 mins in a humid atmosphere at 37°C. The coverslips were washed as above and mounted in buffered glycerol (10% v/v in PBS/A, pH 8.5) and the cells examined for nuclear fluorescence with a Leitz "Orthoplan" fluorescence microscope (see Figure 5.2). A positive result was recorded when greater than 90% of nuclei were fluorescent. Control coverslips were prepared where no conjugate was added and when conjugate was added without pre-incubation of the cells with anti-SV40-T antiserum. Native fluorescence was also considered in a control.

5.3 Results

In order to facilitate comparison, SV40-transformed and foetal Kupffer cell lines are considered together. The data in this section were derived from 65 SV40-transformed adult Kupffer cell

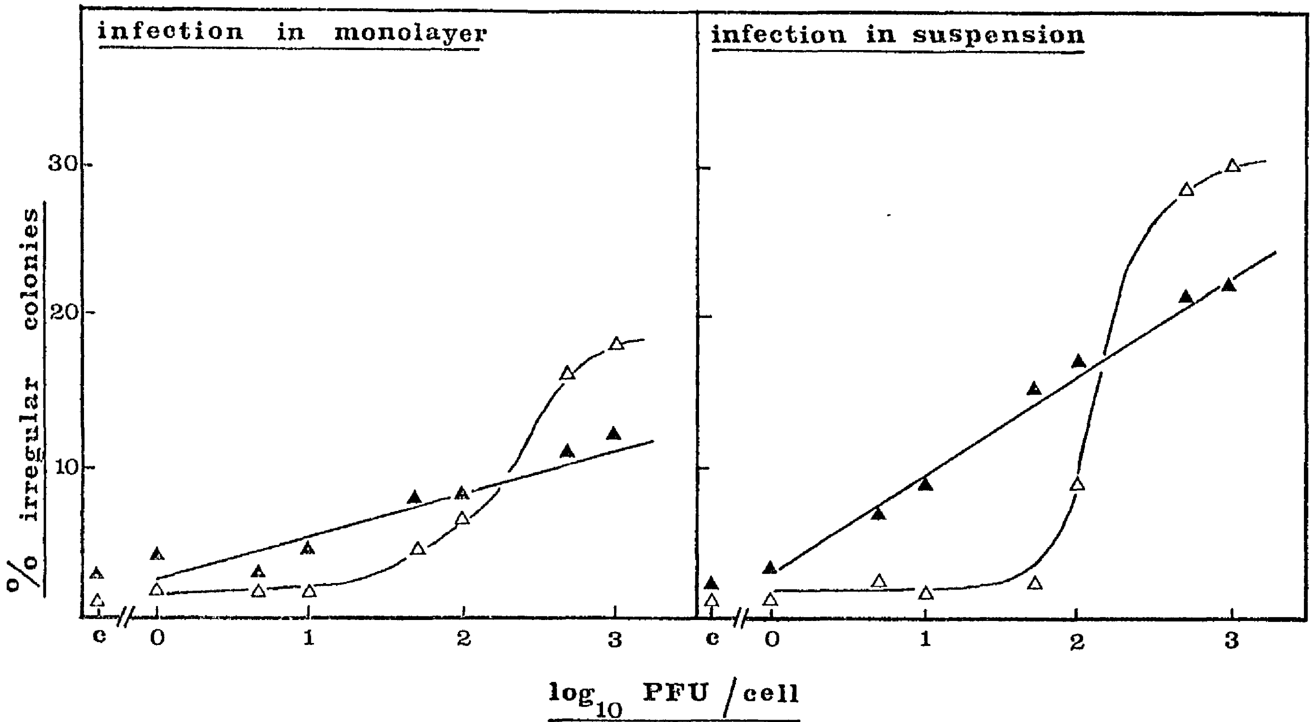


Figure 5.4:- The effect of SV40 infection multiplicity on the frequency of colony morphology transformation in adult Kupffer cells and unidentified "fibroblast-like" cells.

Points are means of duplicate determinations.

C = frequency after mock infection.

—△——△— Kupffer cell line

—▲——▲— unidentified "fibroblast-like" cell line lines, 24 primary foetal Kupffer cell lines and 4 SV40-transformed foetal Kupffer cell lines. The first step in the study was to characterize the SV40-transformation of Kupffer cells.

5.3.1 Multiplicity of SV40 infection and the frequency of transformed colonies

The purpose of this study was to establish the most efficient method of transformation. The criterion for transformation during the initial stages of post-infection culture was irregular colony formation (see Figure 5.1). This criterion has been described by Benjamin (1974). In Figure 5.4 appear the effects of multiplicity of infection on the proportion of irregular colonies for cells infected in monolayer and in suspension. The two cell lines used for this study were a control, unidentified "fibroblast-like", cell line obtained from the initial plating of a freshly isolated Kupffer cell suspension, and an adult Kupffer cell line at the stage of 26 population doublings in culture. The Kupffer cell line demonstrated peroxidase, MHO and phagocytic activity. Colony morphology transformation was more frequent when infection occurred in suspension. Such an observation has been made by Todaro and Green (1966). The other prominent feature of this data was the difference in the kinetics of colony transformation between the two cell types. The "fibroblast-like" cell line demonstrated a steady increase in transformation frequency as the multiplicity of infection increased. In contrast, the Kupffer cell line appeared to exhibit a threshold of infection multiplicity below which there was no increase above the spontaneous transformation frequency. Variation in the transformation frequencies between different cell types from the one species has been reported by Macnab (1972). In this study, the phagocytic activity of the Kupffer cells may have inactivated the virus (see Benacerraf, 1963), and infection may not have occurred until the phagocytic system had been saturated. At high multiplicities of infection the Kupffer cells demonstrated a greater frequency of transformation than the "fibroblast-like" cells. Subsequent transformation studies utilized a multiplicity of infection of 500 PFU/cell. Infection of Kupffer cells with SV40 did not alter the plating efficiency.

A parallel experiment was conducted with the above adult Kupffer cell line where the presence of T-antigen was determined in colonies derived from cells infected with SV40. Immunofluorescence studies demonstrated concordance between irregular colony growth and the presence of T-antigen. The concordance was greater than 90% and the few irregular colonies which did not demonstrate T-antigen may have been spontaneous transformants. Thus, for subsequent experiments, selection of colonies demonstrating irregular growth provided a high probability that the cells had been transformed by SV40.

5.3.2 Establishing SV40-transformed adult and foetal cell lines

Animal no. 9 (female adult, see Figure 2.1) was used to initiate Kupffer cell lines in the manner described in section 3.2. A sample of 65 cell lines possessing peroxidase, MHO and phagocytic activities was isolated. At the stage of 26 population doublings the cells were harvestedand an aliquot infected with SV40. Two colonies with irregular morphology were selected from each cell line and cultured as described in the routine presented in Figure 5.1. At a stage equivalent to approximately 26 population doublings since SV40 infection, small aliquots from each clone were removed, seeded onto glass coverslips and the presence of T-antigen determined by immunofluorescence. Thereafter, only one clone from each cell line was maintained in culture. These clones demonstrated T- antigen and were thus termed "SV40-transformed cell lines".

Animal no. 15 (female foetus, see Figure 2.1) was used to initiate 24 primary foetal Kupffer cell lines. These cell lines were subcultured in the manner described for primary adult cell lines (see Figure 3.1) and possessed peroxidase, MHO and phagocytic activities when screened after 26 population doublings in culture.

Animal no. 16 (female foetus, see Figure 2.1) was used to initiate 4 SV40-transformed foetal cell lines. The method for establishing these cell lines was identical to the procedure used for the SV40-transformed adult cell lines. All 4 foetal cell lines demonstrated peroxidase, MHO and phagocytic activities prior to transformation.

All SV40-transformed cell lines possessed a culture morphology different from that exhibited by primary cell lines (see Figure 5.3). The transformed cells grew in an irregular manner so as to produce a multilayered culture without apparent orientation of growth. This irregular morphology is further evidence of transformation (Benjamin, 1974). The culture morphology of primary foetal cell lines was indistinguishable from that exhibited by primary adult cell lines (see section 3.3.4 and Figure 5.3). In both cases the cells grew in a monolayer with parallel array.

The SV40-transformed Kupffer cell lines appeared to possess a longer lifespan in culture than the primary cell lines. In section 3 it was noted that some of the primary adult Kupffer cell lines possessed a finite lifespan in culture. In a sample of 15 primary adult cell lines 8 survived 97 population doublings in culture. At least 3 of these 8 survived past the stage of 120 population doublings. A sample of 5 SV40-transformed adult Kupffer cell lines was selected for extended culture. All 5 cell lines survived to at least the stage of 200 population doublings since infection with SV40 without any indication of a reduction in viability. Similarly, the 4 SV40-transformed foetal Kupffer cell lines selected for extended culture survived to the stage of 200 population doublings since infection with SV40. The SV40-transformed cell lines selected for continued culture were selected after 90 population doublings since infection with SV40. In

Table 5.3:- Distribution of reciprocal CF titres in SV40-transformed adult and foetal Kupffer cell lines at 40 and 90 population doublings since infection with SV40.

Figures in parentheses indicate the number of cell lines considered.

	Population doublings	Reciprocal CF titre				
		32	64	128	256	512
		no. of cell lines				
SV40-transformed	40	29	19	17	-	-
adult cell lines (65)	90	24	21	19	1	-
SV40-transformed	40	-	-	-	3	1
foetal cell lines(4)	90	-	-	-	2	2

contrast, the 5 primary foetal Kupffer cell lines selected for extended culture had all expired by the stage of approximately 130 population doublings.

Thus, although the sample sizes were not large, the results suggest that SV40-transformed Kupffer cell lines survived a longer period in culture than primary Kupffer cell lines. Although some of the primary adult cell lines had expired before 90 population doublings, primary adult and foetal Kupffer cell lines would generally seem to be limited to 120 population doublings. The 3 primary adult cell lines which survived to this point were not cultured further so it is not possible to assess their potential to survive continued culture. SV40-transformed adult and foetal Kupffer cell lines survived at least 200 population doublings since infection with SV40, and thus survived a considerable period in culture. It was not established whether the SV40-transformed cell lines could survive culturing beyond this stage.

In all SV40-transformed cell lines, the presence of T-antigen was confirmed by CF tests. While T-antigen was present in all cell lines described as positive by immunofluorescence, its titre demonstrated variation between the various SV40 transformed cell lines. The reciprocal CF titre of the negative control cell line, CHSp-T, was consistently <2. The positive control cell line, CHSV40-T demonstrated a reciprocal CF titre of 64. The reciprocal CF titres of SV40-transformed adult Kupffer cell lines varied between 32 and 128. In contrast, was the high reciprocal CF titre for the 4 SV40-transformed foetal cell lines which varied between 256 and 512. The distribution of reciprocal CF titres between SV40-transformed cell lines is presented in Table 5.3. The basis for the variation in CF titre in SV40-transformed adult Kupffer cell lines is not clear. Since immunofluorescence revealed that greater than 90% of the cells were T-antigen positive, the variation does not appear to be due to differences in the proportion of cells possessing T-antigen. Rather, it would appear that the variation was due to quantitative differences

Table 5.4:- Population doubling times of Kupffer cell lines at various stages of culture.

Values are means ± SD, figures in parentheses indicate number of cell lines used for the estimation. Population doublings are post-isolation for primary cell lines and post-SV40 infection for transformed cell lines.

Type of cell line	Population doubling time (hrs):- population doublings 26	40	90
Primary adult	15.3 ± 1.0 (20)	15.0 ± 0.6 (20)	20.2 ± 3.1 (12)
Primary foetal	13.7 ± 0.5 (4)	13.4 ± 0.4 (4)	14.2 ± 0.8 (4)
SV40-transformed adult	13.9 ± 1.0 (4)	13.0 ± 0.8 (4)	13.4 ± 1.1 (4)
SV40-transformed foetal	14.0 (2)	14.3 (2)	13.6 (2)

Table 5.5:- Saturation densities of Kupffer cell lines.

Each value is the mean for two cell lines.

Type of cell line	Saturation density (x 10^4 cells/cm^2)
Primary adult	8.9
Primary foetal	24.5
SV40-transformed adult	57.8
SV40-transformed foetal	46.3

in T-antigen expression between the cell lines. The results presented in Table 5.3 suggest that when compared with SV40-transformed adult Kupffer cells, T-antigen is produced to a greater extent in SV40-transformed foetal Kupffer cells.

5.3.3 Culture kinetics of SV40-transformed adult and foetal cell lines

The plating efficiency of adult Kupffer cells prior to transformation with SV40 was approximately 28%. Upon transformation, the plating efficiency was 45%. Such a value was not greatly different from the maximum for primary adult cell lines (43%, see section 3.3.5) and was evident for both SV40-transformed adult and foetal cell lines. However, in contrast to the primary adult cell lines, the maximum plating efficiency of SV40-transformed cell lines was maintained throughout the culture history, and by the stage of 100 population doublings since SV40 infection, the plating efficiency was 50%. The pattern of change for the plating efficiency of the primary foetal cell lines was indistinguishable from that obtained for the primary adult cell lines (see Figure 3.6).

In Table 5.4 appear the population doubling times of the various types of cell line used in this study. The doubling time for SV40-transformed cell lines, whether of foetal or adult origin, was less than that for primary adult cell lines. Although the differences in doubling time are not great, it would appear that primary foetal cell lines proliferate at a rate similar to that exhibited by the SV40-transformed cell lines. The data also suggested a constancy of population doubling time during the culture period for SV40-transformed and primary foetal cell lines. In contrast is the lengthening of doubling time for primary adult cell lines as the culturing proceeds (see Figure 3.5).

Estimates of plating efficiency and population doubling time make it possible to determine the approximate number of cumulative population doublings the SV40-transformed cell lines had achieved in culture. Throughout section 5, the stage of culture of SV40-transformed cell lines will be with reference to the number of population doublings since infection with SV40. This infection was performed in primary Kupffer cell lines after 26 population doublings in culture.

Subcultures of two cell lines of each type used in these studies were allowed to reach confluence, the stage after which no increase in cell number occurred, and the saturation density determined. Table 5.5 reveals considerable differences in saturation densities between the various types of Kupffer cell line. Transformation of adult Kupffer cells with SV40 resulted in at least a 5-fold increase in saturation density. Such an increase in density is further evidence for the transformation of these cells (Benjamin, 1974). Once the saturation density of the SV40-transformed cell lines was achieved, mitotic activity ceased and a non-proliferating culture was apparent. Although at a greater density than primary adult cells, the SV40-transformed cells still demonstrated a density-dependent inhibition of division. The saturation density of primary foetal cell lines was almost 3-fold greater than that for primary adult cell lines. While these data provide evidence of different saturation densities between various types of Kupffer cell line, it must be stressed that at no time was a cell line used for enzyme activity studies allowed to reach confluence.

5.3.4 Kupffer cell functions in SV40-transformed adult and foetal cell lines.

Prior to transformation by SV40, all adult and foetal Kupffer cell lines possessed peroxidase, MHO and phagocytic activities. The detection of these Kupffer cell properties has been described in section 3.2. By the time the cell lines had achieved 26 population doublings in culture since infection with SV40, none of these activities could be detected. Thus, it would appear that transformation of adult and foetal Kupffer cells by SV40 results in the extinction of properties associated with the in vivo functions of these cells. The next step was to determine at what stage these functions were lost. The earliest stage at which irregular colony morphology could be detected was when the colonies contained more than 60 cells, i.e. over 8 cell divisions since infection of the parent cell.

Adult Kupffer cells at the stage of 26 population doublings in culture were infected with SV40 and the resulting colonies examined for Kupffer cell functions. When 10 colonies possessing irregular morphology and approximately 70 cells in size were examined, peroxidase and phagocytic activities were not detected. Colonies with normal morphology possessed both phagocytic and peroxidase activities. Although it was not possible to examine the presence of both Kupffer cell functions and T-antigen in the one colony, the observations of section 5.3.2 suggest that colonies exhibiting irregular morphology also possessed T-antigen and were thus considered to be transformed. Foetal Kupffer cells at the stage of 26 population doublings in culture were infected with SV40. Ten colonies derived from SV40-infected foetal Kupffer cells which exhibited irregular morphology and approximately 70 cells also failed to demonstrate peroxidase and phagocytic activities. These preliminary observations suggest that SV40-transformation of Kupffer cells, foetal or adult, results in the loss of Kupffer cell functions, and that this loss occurs within the first 8 cell divisions after infection with SV40.

A detailed study of the persistence of Kupffer cell functions in primary foetal cell lines was not conducted. A sample of 5 primary foetal cell lines which exhibited phagocytic, MHO and peroxidase activities after 40 population doublings was cultured for an extended period. After 97 population doublings all of the cell lines had lost phagocytic activity but demonstrated MHO and peroxidase activities,

Table 5.6:- Enzyme activities in SV40-transformed adult, SV40-transformed foetal and primary foetal Kupffer cell lines relative to those in primary adult Kupffer cell lines.

Primary cell line enzyme activities are compared after 40 population doublings in culture; SV40-transformed cell line enzyme activities are those at 40 population doublings after infection with SV40.

Enzyme activity	Kupffer cell lines			
	Primary adult	Primary foetal	SV40-transformed adult	SV40-transformed foetal
Catalase	1.0	0.20	0.07	0.06
Arginase	1.0	0.08	0.00*	0.00*
MHO	1.0	0.29	0.00*	0.00*
β-Glucuronidase	1.0	0.44	1.29	1.26
Peroxidase	1.0	0.46	0.00*	0.00*
ADH	1.0	0.20	0.12	0.10
LDH	1.0	0.53	0.43	0.49
IDH	1.0	0.38	0.17	0.19
G6PDH	1.0	0.71	1.10	0.94
no. of cell lines	130	24	65	4

* Activity not detected.

albeit at reduced levels. These activities will be described in greater detail in section 5.3.6. Thus, two of the Kupffer cell properties persisted in primary foetal cell lines until at least the stage of 97 population doublings. Comparable persistence was also observed in several primary adult Kupffer cell lines (see section 3.3.6).

5.3.5 Enzyme activities in SV40-transformed adult and foetal cell lines

Nine specific enzyme activities in SV40-transformed adult and foetal, and primary foetal cell lines were different from those found in primary adult Kupffer cell lines. The complete data for each enzyme and cell line at the first three assay stages can be found in appendices 1.2 and 1.3. In Table 5.6 appear enzyme activities in the various types of Kupffer cell line relative to those in primary adult Kupffer cell lines. The relative activities were obtained by comparing mean enzyme activities extracted from appendix 1. The activities for primary cell lines were data obtained after 40 cumulative population doublings since initiation of the cell lines. The activities for SV40 transformed cell lines were those obtained 40 population doublings since infection with SV40.

The most prominent feature of Table 5.6 is the absence of arginase, MHO and peroxidase activities in SV40-transformed cell lines, whether of adult or foetal origin. The absence of MHO and peroxidase in SV40-transformed cells has been considered above in section 5.3.4. At no time in the study of the 65 SV40-transformed adult and the 4 SV40-transformed foetal cell lines were these three enzyme activities observed. Thus, it appears that transformation of cultured Kupffer cells by SV40 results in the extinction of arginase, MHO and peroxidase activities. In SV40-transformed cell lines, catalase, ADH, LDH and IDH activities were only a fraction of the activities in primary adult cell lines. In contrast was β-glucuronidase activity. When compared with primary adult cell lines, this enzyme activity was elevated in SV40-transformed cell lines. There was little difference between G6PDH activities in SV40-transformed cell lines and primary adult cell lines.

Another feature of the data presented in Table 5.6 is the similarity of enzyme activities in adult and foetal SV40-transformed cell lines. Since primary adult and primary foetal cell lines differ markedly in their enzyme activities, this similarity would appear to be the result of transformation. Primary foetal cell lines were qualitatively similar to primary adult cell lines but possessed lower activities of all enzymes considered. The primary foetal cell lines all possessed arginase activity and the Kupffer cell activities, MHO and peroxidase. Since primary adult cell line enzyme activities were a fraction of those in freshly isolated Kupffer cells (see section 4.3.2), it follows that primary foetal and SV40-transformed adult and foetal cell lines possessed an even smaller fraction of the <u>in vivo</u> activity.

A more detailed analysis of the data in appendices 1.2 and 1.3 will now be presented. The statistical methods were identical to those described in section 4. Since only 4 SV40-transformed foetal cell lines were considered, the sample size was too small for detailed analysis. The enzyme activities in these four cell lines will be considered in section 5.3.6.

The distribution of enzyme activities in 65 SV40-transformed adult cell lines and 24 primary foetal cell lines is presented in appendices 3 and 4. The distributions for all enzyme activities were continuous and approximate a normal distribution. As in the case of enzyme activities in primary adult cell lines, there was a considerable range in activity. The range in enzyme activities was greatest in the SV40-transformed cell lines, where the differences between cell lines with the lowest and highest activities were 6.6 fold for catalase, 3.4 fold for β-glucuronidase, 23.7 fold for ADH, 4.7 fold for LDH, 2.5 fold for IDH, and 2.3 fold for G6PDH. In contrast were the enzyme activity ranges in primary foetal cell lines. The maximum difference was 3.4 fold for arginase and the least was 1.5 fold for ADH. Thus, it appears that range of activity was greatest for SV40-transformed cell lines.

Inspection of appendices 3 and 4 reveals that there is little difference in distribution of enzyme activities between the three stages of examination. The distributions of enzyme activity in both SV40-transformed adult cell lines and primary foetal cell lines appear to be stable and the differences between the distributions after 26 and 40 population doublings are small. This conclusion is to be verified by the analyses of variance presented below. Although transformation of adult cell lines by SV40 increased the range of enzyme activities, no cell line possessed an activity near or above the activity in freshly isolated Kupffer cells. There was no exception to the absence of enzyme activities; i.e. arginase, MHO and peroxidase activities were absent, or rather, not detected, in all SV40-transformed adult cell lines. The enzyme activities at the limits of the ranges were considered to be values at the extremes of a normal distribution of enzyme activities encompassing the other cell lines. A similar pattern was observed for the primary foetal cell lines. Utilizing a t-test and the null hypothesis that the most extreme values of each distribution were from the same statistical population as the other cell lines, the probability of obtaining such values was $P > 0.05$ in all cases for both SV40-transformed adult cell lines and primary foetal cell lines and all enzymes. No individual enzyme activity could be described as an infrequent quantitative variant and thus no "anomalous" enzyme activity was detected.

As in the case of primary adult cell lines, the rank of each cell line in the enzyme activity distribution during the culture period was considered. The statistical basis for the test was presented in section 4.3.2. A Spearman rank correlation coefficient was calculated for each enzyme activity, and considered rank at 26 and 40 population doublings. SV40-transformed adult cell lines maintained their rank for each enzyme activity ($t > 2.9$ for all enzymes and thus $P < 0.01$ with 63 df). Similarly, the rank of primary foetal cell lines in each enzyme activity distribution was maintained during the period considered ($t > 3.4$ for all enzymes and thus $P < 0.01$ with 22 df). Thus, during the period of culture between 26 and 40 population doublings, SV40-transformed adult cell lines and primary foetal cell lines maintained their relative position in each enzyme activity distribution. Such a situation was also noted for primary adult cell lines (see section 4.3.2).

The analysis of variance design presented in section 4.3.2 was used to examine in more detail enzyme activities in SV40-transformed adult cell lines and primary foetal cell lines. The analyses were based on the data presented in appendices 1.2 and 1.3. In Table 5.7 appears a summary of analyses of enzyme activities in SV40-transformed adult cell lines. The variation between cell lines was highly significant for all six enzyme activities considered. Variation between times was not apparent for three enzyme activities, notably catalase, β-glucuronidase and ADH. There was no change in these activities over the period between 26 and 40 population doublings since infection with SV40. In contrast, LDH, IDH and G6PDH demonstrated significant differences in activity between the times considered. There was a significant interaction component for all enzyme activities except IDH. When compared with variance in primary adult cell lines (see Table 4.4), interaction and variance between times was less in SV40-transformed adult cell lines. However, the degree of variation between cell lines is greatest for the SV40-transformed cell lines. In no case was variance due to error found to be significant.

Table 5.7:- Analyses of variance for enzyme activities in 65 SV40-transformed adult Kupffer cell lines after 26, 33 and 40 population doublings since infection with SV40.

df = degrees of freedom, SS = sum of squares, MS = mean squares, F_s = variance ratio, n. s. = not significant. The MS were rounded to four-figure numbers.

Source of variation	df	SS	MS	F_s
Catalase				
Subgroups	194	1,925,423	9,925	
between cell lines	64	1,628,490	25,460	11.33
between times	2	9,405	4,703	2.09 n.s.
interaction	128	287,528	2,246	11.22
Error	390	78,111	200.3	
β-Glucuronidase				
Subgroups	194	3,573,935	18,420	
between cell lines	64	3,458,696	54,040	64.25
between times	2	7,579	3,789	4.51 n.s.
interaction	128	107,660	841.1	54.94
Error	390	5,972	15.31	
ADH				
Subgroups	194	2,878,598	14,840	
between cell lines	64	2,806,720	43,860	81.86
between times	2	3,301	1,651	3.08 n.s.
interaction	128	68,577	535.8	9.26
Error	390	22,552	57.83	
LDH				
Subgroups	194	1,620,385	8,353	
between cell lines	64	1,598,282	24,970	107.13
between times	2	3,314	1,657	11.29
interaction	128	18,789	146.8	11.08
Error	390	5,168	13.25	
IDH				
Subgroups	194	128,370	661.7	
between cell lines	64	125,492	1,961	102.93 (117.34)
between times	2	440	220	11.55 (13.17)
interaction	128	2,438	19.05	1.19 n.s.
Error	390	6.219	15.95	

Source of variation	df	SS	MS	F_s
G6PDH				
Subgroups	194	81,529	420.3	
between cell lines	64	75,188	1,175	26.45
between times	2	657	328.5	7.40
interaction	128	5,684	44.41	5.85
Error	390	2,960	7.590	

Approximate critical values of F:- F, 0.01 (128, 390) < 1.3; F, 0.01 (2, 128) < 4.8; F, 0.01 (64, 128) < 1.7. When not significant interaction was pooled with the error. The values of F_s in such a situation are presented in parentheses with F, 0.01 (2, 518) < 4.6 and F, 0.01 (64, 518) < 1.5.

Table 5.8:- Analyses of variance for enzyme activities in 24 primary foetal Kupffer cell lines after 26, 33 and 40 cumulative population doublings in culture.

df = degrees of freedom, SS = sum of squares, MS = mean squares, F_s = variance ratio, n.s. = not significant. The mean squares were rounded to four-figure numbers.

Source of variation	df	SS	MS	F_s
Catalase				
Subgroups	71	1,083,944	15,267	
between cell lines	23	1,062,058	46,176	448.14
between times	2	17,146	8,573	83.20
interaction	46	4,740	103.0	2.75
Error	144	5,400	37.50	
Arginase				
Subgroups	71	15,673	220.7	
between cell lines	23	11,547	502.0	6.39
between times	2	513	255.5	3.27 n.s.
interaction	46	3,613	78.54	2.30
Error	144	4,920	34.17	
MHO				
Subgroups	71	13,983	196.9	
between cell lines	23	12,565	546.3	28.26 (37.47)
between times	2	529	264.5	13.68 (18.14)
interaction	46	889	19.33	1.48 n.s.
Error	144	1,882	13.07	
β-Glucuronidase				
Subgroups	71	125,432	1,767	
between cell lines	23	116,650	5,072	27.99
between times	2	447	223.5	1.23 n.s.
interaction	46	8,335	181.2	4.24
Error	144	6,149	42.70	
Peroxidase				
Subgroups	71	2,859,594	40,280	
between cell lines	23	2,660,588	115,700	46.72
between times	2	85,099	42,550	17.18
interaction	46	113,907	2,476	5.72
Error	144	62,350	433.0	

Source of variation	df	SS	MS	F_s
ADH				
Subgroups	71	82,745	1,165	
between cell lines	23	62,669	2,725	6.91
between times	2	1,925	962.5	2.44 n.s.
interaction	46	18,151	394.6	5.50
Error	144	10,323	71.69	
LDH				
Subgroups	71	247,750	3,489	
between cell lines	23	225,989	9,826	25.12
between times	2	3,788	1,894	4.85 n.s.
interaction	46	17,973	390.7	5.23
Error	144	10,759	74.72	
IDH				
Subgroups	71	143,351	2,019	
between cell lines	23	131,078	5,699	24.35
between times	2	1,505	752.5	3.21 n.s.
interaction	46	10,768	234.1	3.44
Error	144	9,809	68.12	
G6PDH				
Subgroups	71	12,102	170.5	
between cell lines	23	10,383	451.4	13.42 (16.28)
between times	2	172	86.00	2.56 n.s. (3.10)
interaction	46	1,547	33.63	1.30 n.s.
Error	144	3,721	25.84	

Approximate critical values of F:- $F, 0.01 (46, 144) < 1.5$; $F, 0.01 (2, 46) < 5.1$; $F, 0.01 (23, 46) < 2.2$. When not significant, interaction was pooled with the error. The values of F_s in such cases are presented in parentheses with $F, 0.01 (2, 190) < 4.6$ and $F, 0.01 (23, 190) < 1.8$.

Table 5.9:- Variance components as a proportion of overall variance in a study of six enzyme activities in 65 SV40-transformed adult Kupffer cell lines after 26, 33 and 40 population doublings since infection with SV40.

The component "between times" refers to the variance of enzyme activities determined after 26, 33 and 40 population doublings since infection with SV40. The variance components are based on the analyses of variance presented in Table 5.7.

Enzyme	Variance component (%)			
	Between cell lines	Between times	Interaction	Error
Catalase	74.2	0.4	19.6	5.8
β-Glucuronidase	95.1	0.2	4.4	0.3
ADH	95.6	0.1	3.2	1.1
LDH	97.6	0.3	1.6	0.5
IDH	92.4	0.4	--- 7.2*	----
G6PDH	85.5	1.0	8.3	5.2

* Since interaction was not significant in this case, it was pooled with the error (see Table 5.7).

Table 5.10:- Variance components as a proportion of overall variance in a study of nine enzyme activities in 24 primary foetal Kupffer cell lines after 26, 33 and 40 cumulative population doublings in culture.

The component "between times" refers to the variance of enzyme activities determined after 26, 33 and 40 population doublings in culture. The variance components are based on the analyses of variance presented in Table 5.8.

Enzyme	Variance component (%)			
	Between cell lines	Between times	Interaction	Error
Catalase	96.7	2.2	0.4	0.7
Arginase	47.8	2.5	15.0	34.7
MHO	76.6	4.5	---- 18.9*	----
β-Glucuronidase	85.9	0.1	7.3	6.7
Peroxidase	88.3	3.9	4.8	3.0
ADH	58.0	1.8	24.1	16.1
LDH	83.9	1.7	8.4	6.0
IDH	82.3	1.0	7.5	9.2
G6PDH	62.3	1.1	------ 36.6*	----

* In these cases, interaction was not significant and was pooled with the error (see Table 5.8).

Analyses of variance of nine enzyme activities in the primary foetal cell lines are presented in Table 5.8. These analyses reveal significant variation between cell lines for all enzyme activities. In contrast to primary adult cell lines (see Table 4.4), primary foetal cell lines do not demonstrate variation in six enzyme activities during the period from 26 to 40 population doublings since initiation of the cell lines. The only enzyme activities which demonstrated significant variation during this period were catalase, MHO and peroxidase. It may be of significance that two of these enzymes, MHO and peroxidase, are responsible for some Kupffer cell functions. The interaction component was not significant for MHO and G6PDH activities. As for analyses of enzyme activities in primary adult cell lines and SV40-transformed adult cell lines, the variance due to error was not significant for any enzyme activity.

The added variance components for enzyme activities in SV40-transformed adult cell lines are presented in Table 5.9. These calculations support the above conclusions. The greatest variance component for all enzyme activities was between cell lines. The contribution of error to the total variance was less than 6%. The added variance component between times was not greater than 1% of the total variance. Similarly, for all enzyme activities except catalase, interaction made only a small contribution to the total variance.

Calculation of added variance components as a proportion of overall variance in primary foetal cell lines reveals that most of the added variance was accounted for between cell lines (see Table 5.10). A possible exception was arginase, where less than 50% of the variance was between cell lines. The proportion of variance between times was small for all enzyme activities. Interaction was a variable component ranging from 0.4% for catalase to 24.1% for ADH. Similarly, the error demonstrated variation between enzymes. Those enzymes with a large interaction component also possessed a large error component. The error contribution to arginase

Table 5.11:- Product-moment correlation coefficient (r) matrices for six enzyme activities in 65 SV40-transformed adult Kupffer cell lines after 26, 33 and 40 population doublings since infection with SV40.

After 26 population doublings

β-Glu.	0.235*				
ADH	0.199*	0.806			
LDH	0.103*	0.864	0.887		
IDH	0.153*	0.870	0.887	0.838	
G6PDH	0.106*	0.814	0.811	0.911	0.911
	Cat.	β-Glu.	ADH	LDH	IDH

After 33 population doublings

β-Glu.	0.148*				
ADH	0.086*	0.811			
LDH	0.059*	0.897	0.847		
IDH	0.090*	0.898	0.726	0.824	
G6PDH	-0.001*	0.767	0.753	0.882	0.876
	Cat.	β-Glu.	ADH	LDH	IDH

After 40 population doublings

β-Glu.	0.062*				
ADH	0.136*	0.784			
LDH	0.199*	0.843	0.643		
IDH	0.043*	0.835	0.729	0.983	
G6PDH	-0.059*	0.673	0.731	0.822	0.795
	Cat.	β-Glu.	ADH	LDH	IDH

* not significant, critical value $P(0.01) > 0.320$, 63 df.

Table 5.12:- Product-moment correlation coefficient (r) matrices for nine enzyme activities in 24 primary foetal Kupffer cell lines after 26, 33 and 40 cumulative population doublings in culture.

After 26 population doublings

	Cat.	Arg.	MHO	β-Glu.	Perox.	ADH	LDH	IDH
Arg.	0.194*							
MHO	0.975	0.163*						
β-Glu.	-0.833	0.145*	-0.848					
Perox.	0.990	0.179*	0.957	-0.823				
ADH	0.896	0.296*	0.833	-0.728	0.900			
LDH	0.930	-0.002*	0.932	-0.887	0.923	0.768		
IDH	0.838	-0.099*	0.844	-0.801	0.842	0.636	0.953	
G6PDH	0.881	0.027*	0.891	-0.832	0.883	0.803	0.908	0.890

After 33 population doublings

	Cat.	Arg.	MHO	β-Glu.	Perox.	ADH	LDH	IDH
Arg.	0.076*							
MHO	0.941	0.114*						
β-Glu.	-0.780	0.095*	-0.704					
Perox.	0.992	0.057*	0.932	-0.771				
ADH	0.710	0.267*	0.576	-0.646	0.689			
LDH	0.941	0.142*	0.880	-0.683	0.933	0.685		
IDH	0.903	0.033*	0.847	-0.659	0.908	0.590	0.933	
G6PDH	0.899	0.091*	0.826	-0.655	0.893	0.677	0.935	0.868

After 40 population doublings

	Cat.	Arg.	MHO	β-Glu.	Perox.	ADH	LDH	IDH
Arg.	0.152*							
MHO	0.900	0.210*						
β-Glu.	-0.801	0.162*	-0.801					
Perox.	0.918	-0.017*	0.793	-0.688				
ADH	0.881	0.025*	0.863	-0.765	0.819			
LDH	0.926	-0.114*	0.872	-0.778	0.875	0.832		
IDH	0.933	-0.134*	0.885	-0.792	0.907	0.845	0.980	
G6PDH	0.817	-0.065*	0.712	-0.649	0.749	0.739	0.903	0.915

*not significant, critical value P(0.01) = 0.515, 22 df.

variation accounted for 34.7% of the total variation. Such a situation does not reflect inaccuracy of assay but rather the smaller contribution of variance between cell lines. A similar argument can be presented to explain the apparently large contribution of the error component to ADH variation.

In summary, the analyses of variance reveal that there existed considerable variation of several enzyme activities between both SV40-transformed adult cell lines and primary foetal cell lines. Changes in enzyme activity during the culture period in these two types of cell line were less than those evident for primary adult cell lines (see section 4.3.2). In general, the degree of interaction was less in SV40-transformed adult cell lines and primary foetal cell lines than in primary adult cell lines.

The examination of quantitative relationships between enzyme activities was conducted in a manner similar to that used in section 4.3.2. Table 5.11 presents product-moment correlation coefficient matrices for enzyme activities in SV40-transformed adult cell lines. The most striking contrast to correlation coefficients presented for enzyme activities in primary adult cell lines (see Table 4.6) was the fact that catalase activity failed to achieve a significant correlation with any other enzyme activity. The other major feature was the significant, positive, correlation of β-glucuronidase activity with the other enzyme activities. In primary adult cell lines this correlation was at a lower level and negative (see Table 4.6). There appeared to be no difference in correlation coefficients at the three stages of assay. The degree of correlation of dehydrogenase activity may have declined slightly during the culture period.

Correlation coefficients between enzyme activities in primary foetal cell lines are presented in Table 5.12. While correlation coefficients involving arginase were not significant, all other coefficients were significant. As for primary adult cell lines, correlations with β-glucuronidase were negative. Catalase, MHO, peroxidase and the dehydrogenases demonstrated a high degree of activity correlation. The general patterns of correlation were maintained at all three stages examined.

The significance of correlations between enzyme activities in primary adult, SV40-transformed and primary foetal cell lines will be discussed in greater detail in section 7.

5.3.6 An extended study of enzyme activities in SV40-transformed adult and foetal cell lines

The results of an extended study of enzyme activities in SV40-transformed adult and foetal cell lines can be found in appendix 2. Since no consistent trends in the enzyme activities were observed during the extended culture period, the data will not be presented graphically. In each class of cell line considered in this section, the protein content/cell remained constant during the study period.

The six enzyme activities studied in 15 SV40-transformed adult Kupffer cell lines exhibited a marked stability between 26 and 90 population doublings since infection with SV40 (see appendix 2.2). The differences between each mean enzyme activity at 26 and 90 population doublings were compared utilizing a t-test. In all cases $P > 0.1$ and thus there existed no significant difference for all enzymes. The coefficient of variation was relatively stable for all enzymes during the culture period. Although small, changes in coefficient of variation were consistently an increase as the culturing proceeded.

The activities of nine enzymes in 5 primary foetal Kupffer cell lines during an extended period of culture are presented in appendix 2.3. Comparison of mean activities after 26 and 97 population doublings utilizing a t-test revealed stability during the period. There was no significant difference between the mean activities at these stages for eight enzymes ($P > 0.1$). Only MHO demonstrated a significant difference ($P = 0.05$) and inspection of the data reveals that during the culture period there was a constant decrease in MHO activity and a concomitant increase in its coefficient of variation. The other enzyme activities demonstrated a gradual increase in coefficient of variation as culturing proceeded. Thus, while mean

Table 5.13:- Enzyme activities in adult Kupffer cells during the early stages of culture after transformation by SV40.

Cells from a primary adult Kupffer cell line at the stage of 26 population doublings were transformed by SV40 and enzyme activities determined in resulting colonies with irregular morphology containing 60-80 and 240-260 cells. Mock infection of cells from the same cell line yielded untransformed, control, cells. The enzyme activities in such cells were determined in colonies of 60-80 cells. Values are means ± SD, n = number of colonies.

	Enzyme activity (moles/min/cell)			
	β-Glucuronidase ($\times 10^{-16}$)	LDH ($\times 10^{-13}$)	IDH ($\times 10^{-14}$)	G6PDH ($\times 10^{-15}$)
Untransformed cell colonies (n = 15).	2.06±0.35	26.3±4.7	28.3 ±4.5	3.96±0.87
SV40-transformed cell colonies				
60-80 cells (n=15)	2.84±0.40	10.6±5.3	5.08±3.64	4.40±0.93
240-260 cells (n=10)	2.88±0.47	11.0±5.6	4.59±3.96	4.43±0.90
SV40-transformed adult Kupffer cell lines 26 population doublings since infection with SV40 (65 cell lines)	2.70±0.42	11.4±5.9	4.86±4.03	4.35±0.91

activities remained constant in the 5 cell lines, the variation increased. The increase in variation of catalase, LDH and IDH, although slight, followed a consistent trend.

In appendix 2.4 are presented the results of a study of six enzyme activities in SV40-transformed foetal Kupffer cell lines during an extended period of culture. There was no significant difference between mean activity after 26 and 90 population doublings since infection with SV40 for all enzymes ($P > 0.2$). No consistent trends were apparent for changes in the coefficients of variation.

In summary, the results of studies of enzyme activities in SV40-transformed adult and foetal cell lines presented in this section reveal stability of activity during an extended period of culture. Such observations are in contrast to those made in section 4.3.3 where enzyme activities in primary adult cell lines were shown to change during a comparable culture period.

As a conclusion to this extended study, the initial changes in four enzyme activities after transformation of Kupffer cells by SV40 were examined. In section 5.3.1 it was evident that the earliest stage at which SV40-transformed cells could be recognised was approximately 8 cell divisions after infection with SV40. Such cells were found in colonies of irregular morphology and expressed T-antigen. β-Glucuronidase, LDH, IDH and G6PDH activities were determined in irregular colonies of 60-80 and 240-260 cells derived from adult Kupffer cells infected with SV40. The one primary adult Kupffer cell line at the stage of 26 population doublings was used for this study. The enzyme activities were determined using the "micro-assays" described in section 4.2.4. The results presented in Table 5.13 indicate that the changes in the enzyme activities evident after 26 population doublings occur within approximately the first 8 cell divisions after infection and transformation by SV40. The difference between enzyme activity in colonies of un-transformed cells and 60-80 cell colonies derived from an SV40 infected cell was significant for, β-glucuronidase, LDH and IDH ($P < 0.01$ using a t-test). The difference for G6PDH was not significant ($P > 0.1$ using a t-test). Thus, the biochemical alterations which are evident in SV40-transformed Kupffer cell lines occurred within the first few divisions after infection and transformation by SV40.

5.3.7 Karyology of SV40-transformed adult and foetal Kupffer cells

The karyotypes of 10 SV40-transformed adult and 2 SV40-transformed foetal Kupffer cell lines were examined 30, 80 and 94 population doublings after infection with SV40. Three primary foetal Kupffer cell lines were examined 30 and 94 population doublings since isolation. A description of the karyotypes for each cell line was based on observation of 50 metaphase cells.

In Table 5.14 appear the proportions of diploid cells in each cell line. All classes of cell line demonstrated a decline in the proportion of diploid cells as culturing proceeded. At each stage the mean proportion of diploid cells in the primary foetal cell lines was greater than that in both classes of SV40-transformed cell line. SV40-transformed adult cell lines exhibited heterogeneity in the proportion of diploid cells. After 30 population doublings since infection with SV40, 7 of the 10 SV40-transformed adult cell lines were diploid. Only one of these cell lines remained diploid by the time 94 population doublings had been accomplished. Both SV40-transformed foetal cell lines were diploid 30 population doublings after infection with SV40, although by 94 population doublings they had become heteroploid. At least 75% of cells must be diploid before a cell can be described as diploid (Federoff, 1967).

The distribution of chromosome numbers in the three classes of cell line are presented in Table 5.15. At the stage of 30 population doublings, the distribution of chromosome number was similar

Table 5.14:- The effect of the number of population doublings in culture on the proportion of diploid cells in SV40-transformed adult and foetal Kupffer cell lines.

The number of population doublings quoted for SV40-transformed cell lines are since infection with SV40 which occurred in primary cell lines at the stage of 26 population doublings. $\bar{x}$ = mean proportion of diploid cell ± SD.

Cell lines	Proportion of diploid cells (%): Population doublings		
	30	80	94
SV40-transformed adult			
404	68	56	52
409	74	66	64
415	90	76	70
419	84	76	66
424	70	58	54
426	76	60	52
431	80	62	60
435	86	64	56
438	88	72	76
446	92	78	60
	$\bar{x}$ = 80.8 ± 8.5	66.8 ± 8.1	61.0 ± 8.0
Primary foetal			
602	90		72
607	84		64
612	82		66
	$\bar{x}$ = 85.3 ± 4.2		67.3 ± 4.2
SV40-transformed foetal			
502	78	64	56
504	84	76	62
	$\bar{x}$ = 81	70	59

Table 5.15:- The effect of the number of population doublings in culture on the distribution of chromosome number in SV40-transformed adult and foetal Kupffer cell lines.

The number of population doublings quoted for SV40-transformed cell lines are since infection with SV40 which occurred in primary cell lines at the stage of 26 population doublings. 50 cells were scored for each cell line. Figures in parentheses indicate the number of cell lines considered. Deviations from 100% for total % are due to rounding error.

Cell lines	Population doublings	Proportion of cells (%): Chromosone number							
		15-17	18,19	20	21	22	23-26	36-43	44
SV40-transformed adult (10)	30	1.4	1.6	1.8	4.8	80.8	5.4	2.2	2.0
	80	2.0	4.0	5.2	7.6	66.8	8.2	3.8	2.4
	94	1.8	3.8	3.4	9.0	61.0	12.6	5.8	2.6
Primary foetal (3)	30	0.7	0.7	1.3	5.3	85.3	3.3	2.0	1.3
	94	3.3	4.0	6.7	10.0	67.3	4.0	2.7	2.0
SV40-transformed foetal (2)	30	2	1	2	3	81	6	3	1
	80	3	4	5	3	70	9	5	1
	94	2	5	3	7	59	14	8	2

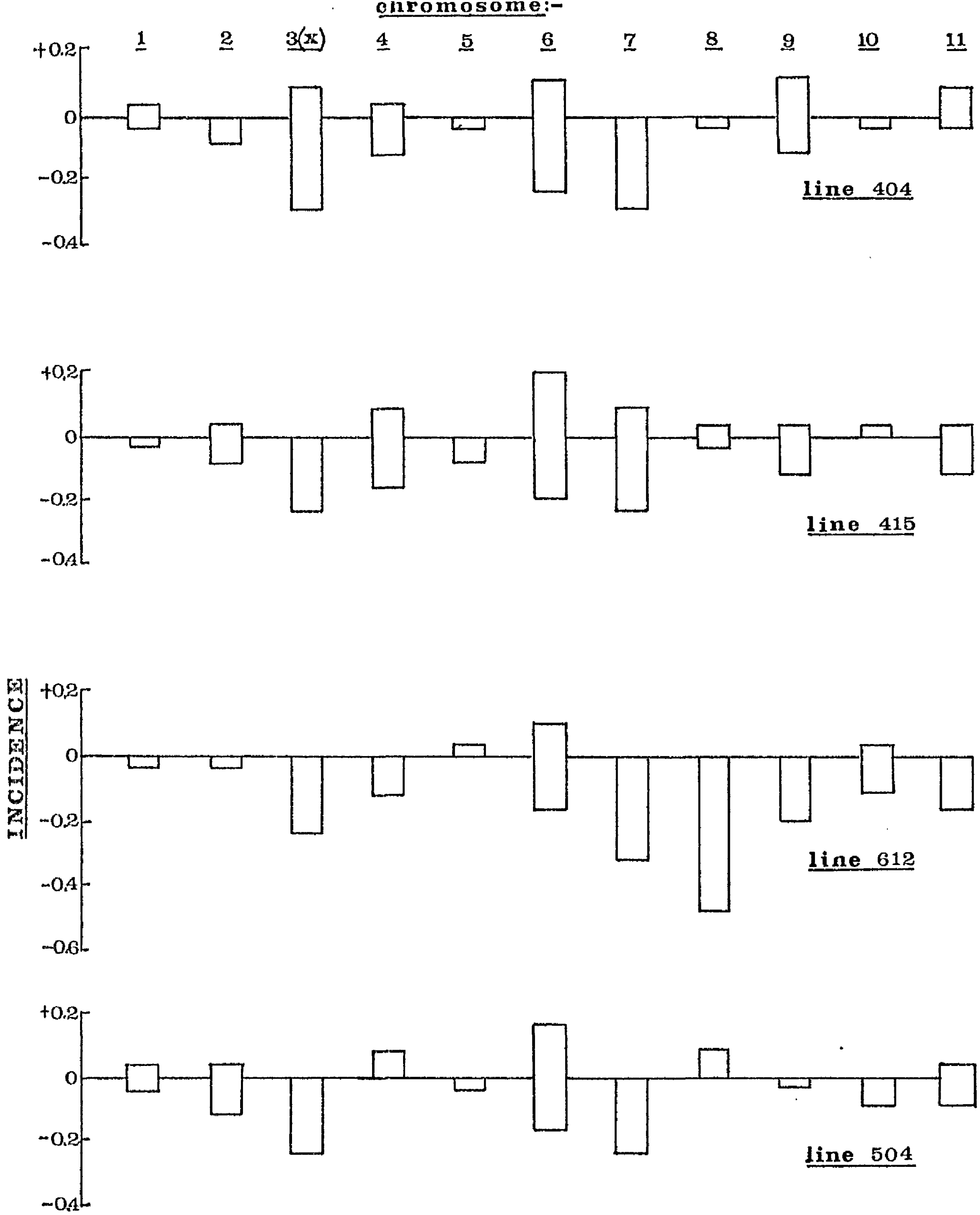


Figure 5. 5:- Incidence of individual chromosomes in aneuploid cells from SV40-transformed adult (404, 415), SV40-transformed foetal (504) and primary foetal (612) Kupffer cell lines.

The zero line represents the number of chromosomes in the normal diploid cell. The scale is graduated in units of the number of chromosomes gained or lost - a value of + 0. 1 (-0. 1) means that every tenth aneuploid cell has the gain (loss) of one chromosome of the type concerned. The karyotypes of SV40-transformed cell lines were considered 80 population doublings after infection whilst those for the primary cell lines were after 94 population doublings in culture.

in all three classes of cell line. Accompanying the decrease in diploid cell proportion as culturing continued, SV40-transformed cell lines possessed an increasing proportion of aneuploid, tetraploid and sub-tetraploid cells. After 94 population doublings, more than 8% of cells were tetraploid or sub-tetraploid. The increase in aneuploid cell proportion involved chromosome numbers both greater and less than the diploid number. The major change in chromosome distribution in primary foetal cell lines as culturing proceeded was an increase in the proportion of aneuploid cells with less than the diploid number of chromosomes. A slight increase in the proportion of tetraploid or sub-tetraploid cells was also apparent.

Figure 5.5 presents the incidence of individual chromosomes in aneuploid cells. In this study 25 aneuploid cells from each cell line were examined and the presence or absence of individual chromosomes recorded. The method of presentation has been described on page 64. The Kupffer cell lines selected for this study were 404 and 415 (SV40-transformed adult), 504 (SV40-transformed foetal) and 612 (primary foetal). The degree of diploidy of these four cell lines has been considered in Table 5.14. The incidence of individual chromosomes demonstrated heterogeneity within each of the cell lines. As was the case for primary adult cell lines (section 3.3.7), the incidence of some chromosomes was more variable than others. In all four cell lines, chromosomes 1 and 5 deviated only slightly from the diploid incidence. In contrast, chromosomes 3, 6 and 7 demonstrated considerable variation of incidence.

The major difference between SV40-transformed and primary Kupffer cell lines was in the incidence of chromosome 8. The incidence of chromosome 8 was less variable in the SV40-transformed cell lines than in primary adult cell lines (see Figure 3.8). This situation also occurred with the foetal cell lines. Chromosome 8 incidence was variable in the primary foetal cell lines but relatively stable in the SV40-transformed foetal cell lines. The number of cell lines examined in each category was too small for comment to be passed on the significance of differences in the incidence of other chromosomes.

The banding pattern of 10 diploid cells from each of the four cell lines considered in Figure 5.5 were identical to those exhibited by diploid primary adult Kupffer cell lines (see section 3.3.7). Structural re-arrangements were rarely encountered and no attempt was made to identify the re-arranged material. Re-arrangements were observed in less than 5% of metaphase cells from SV40-transformed cell lines. The incidence of re-arrangements was even less in primary foetal cell lines. The most common class of re-arrangment appeared to involve unequal exchange between the large and medium metacentric chromosomes.

At this point the main observations made in section 5 will be briefly summarised. The results demonstrated that it was possible to transform cultured adult and foetal Chinese hamster Kupffer cells by SV40. The transformed Kupffer cells possessed many of the properties associated with transformed cell lines. Transformation of Kupffer cells by SV40 resulted in a rapid change in enzyme activity to a level which was stable upon subsequent culturing. Most enzyme activities decreased and Kupffer cell functions were extinguished after transformation. The activity of G6PDH remained uneffected, whilst that of β-glucuronidase was increased after transformation. Accompanying the change in enzyme activities after SV40-transformation was an increase in variation between cell lines which, prior to transformation, were isolated from genetically identical material. Although transformation resulted in greater variation of enzyme activities, no cell line was isolated with an "anomalous" enzyme activity. All of the extreme enzyme activities were values within the limits of a continuous, normal distribution of activities.

SV40-transformation of Kupffer cells also resulted in alteration of the enzyme activity inter-relationships present in primary Kupffer cell lines. Most SV40-transformed Kupffer cell lines remained diploid for a considerable period in culture, and at all stages in all SV40-transformed cell lines examined, the diploid chromosome number was the modal value.

Primary foetal Kupffer cell lines generally possessed properties intermediate between those of primary adult Kupffer cell lines and SV40-transformed adult cell lines. Enzyme activities and their variations were similar in SV40-transformed foetal Kupffer cells and SV40-transformed adult Kupffer cells.

SECTION 6

A STUDY OF LACTATE DEHYDROGENASE ISOENZYMES IN PRIMARY AND SV40-TRANSFORMED CHINESE HAMSTER KUPFFER CELL LINES

6.1 Introduction

In previous sections appeared studies on total activity for a number of enzymes. Total activity is often a combined measure of the catalytic activity of different molecules, and hence an enzyme activity may be the result of expression of two or more enzyme structural genes. The enzymes which exist as different molecular forms and catalyze the same reactions in a given species and tissue are termed isoenzymes and have been of considerable value in studies of molecular control, ontogeny and tissue differentiation. Masters and Holmes (1972) have reviewed the use of isoenzymes in such studies. More general accounts of enzymes which possess isoenzymes can be found in Shaw (1969), Wilkinson (1970) and Schapira (1973).

Our knowledge of isoenzymes is most detailed in relation to those of lactate dehydrogenase. It is the purpose of this study to briefly consider lactate dehydrogenase (LDH) isoenzymes in various Kupffer cell lines. LDH isoenzymes exist as tetramers and represent the possibilities of hybridization between two different types of polypeptide, a and b which are the respective products of the A and B genes. The isoenzymes are LDH-5 ($\underline{a}_4$), LDH-4 ($\underline{a}_3\underline{b}$), LDH-3 ($\underline{a}_2\underline{b}_2$), LDH-2 ($\underline{ab}_3$) and LDH-1 ($\underline{b}_4$). The numbering of the isoenzymes is in order of their relative electrophoretic mobilities, with LDH-1 being assigned to the form of greatest anodic mobility (Webb, 1964).

LDH is ubiquitous in vertebrate cells and its isoenzyme distribution in some mammalian tissues has been summarised by Wilkinson (1970, p. 136). Adult tissues differ markedly in their patterns of isoenzyme activity. During the development of an animal species to its adult stage, the isoenzyme pattern of a particular tissue is also significantly altered. These changes occur during both foetal and neo-natal development and probably reflect the changing metabolic roles of the individual tissues. The shifts in isoenzyme pattern during development are due to real cellular changes and not changes in cell population structure (Markert and Moller, 1959). There also appears to be a relationship between the rate of mitosis in a tissue and the prevalence of LDH-5 (Papaconstantinou, 1967).

LDH isoenzymes have also been studied in cultured cells. Prolonged culture tends to result in a loss of LDH-1 and a corresponding increase in LDH-5 (Philip and Vesell, 1962; Vesell et al., 1962; Nitowsky and Soderman, 1964; Childs and Legator, 1965). Blanco et al. (1967) studied the effects of continued culture on the isoenzyme patterns of differentiated mammary tissue cells from a lactating goat. There was a gradual change in isoenzyme pattern from predominantly LDH-1 to LDH-5. This change was also accompanied by the loss of the ability to synthesize milk components. Although LDH-5 appears to be the predominant isoenzyme in established cell lines, both Ruddle et al. (1970) and Nichols and Ruddle (1973) have described cell lines with appreciable LDH-1 activity. Yasin and Goldenberg (1966) have demonstrated the stability of isoenzyme patterns in a number of cell lines during long-term culture. LDH-5 was the predominant isoenzyme in all cell lines studied. Clonal variation in LDH isoenzyme pattern has also been described in neuroblastoma cells by Tholay et al. (1974). In this case, most clones exhibited only LDH-5 but occasional clones also possessed LDH-4 activity. The small number of studies and doubtful or unknown origin of a number of cell lines makes it impossible to comment on whether the original isoenzyme pattern bears any relationship to the ultimate pattern after establishment of cells in culture.

Uniform LDH isoenzyme patterns have been demonstrated in tumour tissue (see Wilkinson, 1970; Criss, 1971; Schapira, 1973). While the level of LDH activity in malignant tissues tends to be higher than corresponding normal tissues (Meister, 1950), these tissues contain mainly LDH-3, LDH-4 and LDH-5 irrespective of the tissue from which the tumour arose (Poznanska-Linde et al., 1966). Sekiya et al. (1973) demonstrated that the tumourigenicity of cultured rat uterine adeno-carcinoma cells was correlated with an increased proportion of LDH-5.

The purpose of this study was to describe both the changes in LDH patterns and quantitative relationships between the LDH subunits which take place when Chinese hamster Kupffer cells are cultured. Data are presented which describe isoenzyme proportions in primary adult and foetal Kupffer cell lines. The isoenzyme proportions of SV40-transformed adult and foetal Kupffer cell lines are also described. The isoenzyme proportions were related to the activity of LDH b subunits as a proportion of the total number of active LDH subunits, and hence provided estimates of the relative contributions of the LDH A and LDH B gene products to total enzyme activity.

6.2 Materials and Methods

The assay of total LDH activity has been described in section 4.2.3.

6.2.1 Preparation of cell extracts

Cells in culture were harvested in the usual manner, washed in PBS/A, centrifuged (1,000 g, 4°C, 15 mins.) and homogenized in PBS/A (2×10^5 cells/100 μl). The protein concentration in the extract was approximately 1 μg/μl. Extracts of freshly isolated hepatocytes and Kupffer cells (see section 3.2) were prepared in a similar manner and protein concentration was adjusted to the above value with PBS/A. All homogenization was performed by hand in glass homogenizers (1 ml and 0.1 ml capacities). Any subsequent dilution of extracts for electrophoresis was accomplished with PBS/A containing 1 μg/μl BSA (Armour Pharmaceutical).

6.2.2 Electrophoresis

Electrophoresis was based on the method described by Meera Khan (1971). Cellulose acetate strips ("Celagram" 78 mm x 150 mm, Shandon) were briefly soaked in citrate-phosphate buffer containing 1% BSA, blotted dry, pre-run for 10 mins. (200V, 2 mA), and then 10 μl samples applied. After electrophoresis in citrate-phosphate buffer for 90 mins. at room temperature (200V, 2 mA), the strip was impregnated with staining mixture and incubated for 20 mins. at 37°C in a humid atmosphere.

Buffer:- Citrate-phosphate, pH 7.0 (Na_2HPO_4, 0.01 M; citrate, 1.54×10^{-3} M).

Staining mixture:- 1.0 ml Tris-HCl (1.0 M) containing Na_2 EDTA (0.004 M), pH 8.6; 1.0 ml lithium-L-lactate (0.4 M); 0.4 ml NAD (10 mg/ml); 0.4 ml nitro-blue tetrazolium (2 mg/ml, Sigma); 0.4 ml phenazine methosulphate (0.4 mg/ml, Sigma).

Stained strips were fixed with 10% (v/v in distilled water) acetic acid for 2 mins. and dried between glass plates at 60°C. When completely dry, the strips were cleared by impregnation with Whitmore Oil (Gurr) and scanned with a Joyce dual-beam micro-densitometer.

Controls were established from duplicate strips with substitution of buffer for substrate in the staining mixture.

6.2.3 Chromatography

LDH isoenzymes were separated by the technique of ion-exchange chromatography (Fritz et al., 1970). Approximately 5 g of DEAE-Sephadex A50 (coarse, 40-120 μ) were allowed to swell overnight in distilled water and then washed consecutively with 250 ml 0.5 N NaOH, 500 ml distilled water, 250 ml 0.5 N HCl and 500 ml distilled water. The material was simultaneously fined and equilibrated with 2 litres 0.02 M Tris buffer (pH 7.4), then packed into columns, generally 1 x 10 cm. Cell extracts were prepared by homogenizing material in cold 0.02 M Tris (pH 7.4) and after centrifuging (10,000 g, 4°C, 20 mins.) the supernatant was applied to the column, generally

0.5 ml containing 1 mg protein. LDH-5 was not absorbed to the column at pH 7.4, and was recovered by elution with buffer. Elution of the other isoenzymes was accomplished with $1\frac{1}{2}$ column volumes of the following salt concentrations all contained in 0.02 M Tris (pH 7.4):- 0.1 M NaCl (LDH-4), 0.14 M NaCl (LDH-3), 0.18 M NaCl (LDH-2) and 0.22 M NaCl (LDH-1). Fractions were collected and assayed for enzyme activity as described in section 4.2.3.

6.2.4 Evaluation of isoenzyme proportions

Peaks on densitometer tracings of stained cellulose acetate strips were cut out and weighed. Isoenzyme proportions were expressed as percentages of total peak area after taking account of the control contribution to peak area. A similar method was used for activity proportion data of isoenzymes separated by chromatography. Isoenzyme proportions were expressed as percentages of total LDH activity estimated from pooling all fractions.

6.2.5 Estimation of isoenzyme proportions from substrate inhibition studies.

This estimation procedure was based on the observation by Stambaugh and Post (1966) that while LDH-1 is inhibited by 250 mM lactate, LDH-5 maintains its activity. When compared with a standard curve, the ratio of extract activity in low and high lactate concentrations provides an estimate of a and b subunit proportions.

Colonies of cells grown on "Melinex" polyester film (ICI) were excised for assay (see section 4.2.4). Cell number was determined and the "Melinex" fragment placed into a 5 x 60 mm glass tube and 10 μl of hypotonic lysis buffer (1/10 aqueous dilution of PBS/A containing 0.05% Triton X-100 (Sigma), pH 7.0) placed on the colony.

The cells were lysed by rapid freezing in liquid nitrogen and immediate thawing at 37°C. The tubes were then put on ice and the drop of liquid briefly agitated by an air draught from a micro-pipette. Enzyme activity was determined using the method described by Lowry and Passonneau (1972). To 10 µl of reaction mixture were added 2 µl of this cell extract. The reaction mixture contained either 50 mM or 250 mM lithium-L-lactate and 1 mM NAD in 50 mM pyrophosphate buffer (pH 8.8) containing 0.1 µg BSA/µl. Also included were blanks and standards (1 µl of 0.05 - 1 mM NADH). The tubes were sealed with "Parafilm" and incubated at 37°C for 60 mins. After incubation, excess NAD was destroyed by addition of 10 µl of alkaline phosphate buffer (0.25 M Na_2PO_4, 0.25 M K_2HPO_4; pH 12.0) and incubation for 15 mins. at 60°C. Fluorescence was developed by the addition of 100 µl of 6 N NaOH containing 0.03% hydrogen peroxide and incubation at 60°C for 15 mins. The tubes were allowed to cool and aliquots removed for fluorescence determination at ex. 340 nm/em. 460 nm. The ratio of enzyme activities in low:high lactate was compared with a standard curve.

The standard curve was obtained by determining low:high lactate activity ratios for the five LDH isoenzymes. The isoenzymes were partially purified by elution after electrophoresis. The unstained isoenzymes were localized by comparison with stained strips cut from the edges of the cellulose acetate strip. The bands of isoenzyme were excised and placed in 1 ml of 50 mM pyrophosphate buffer containing 1% BSA. After agitation the liquid was placed in small dialysis bags and surrounded with Sephadex-G15 until reduced to approximately 0.25 ml. The activities of partially purified LDH isoenzymes were then assayed in low and high lactate concentrations and an activity ratio determined.

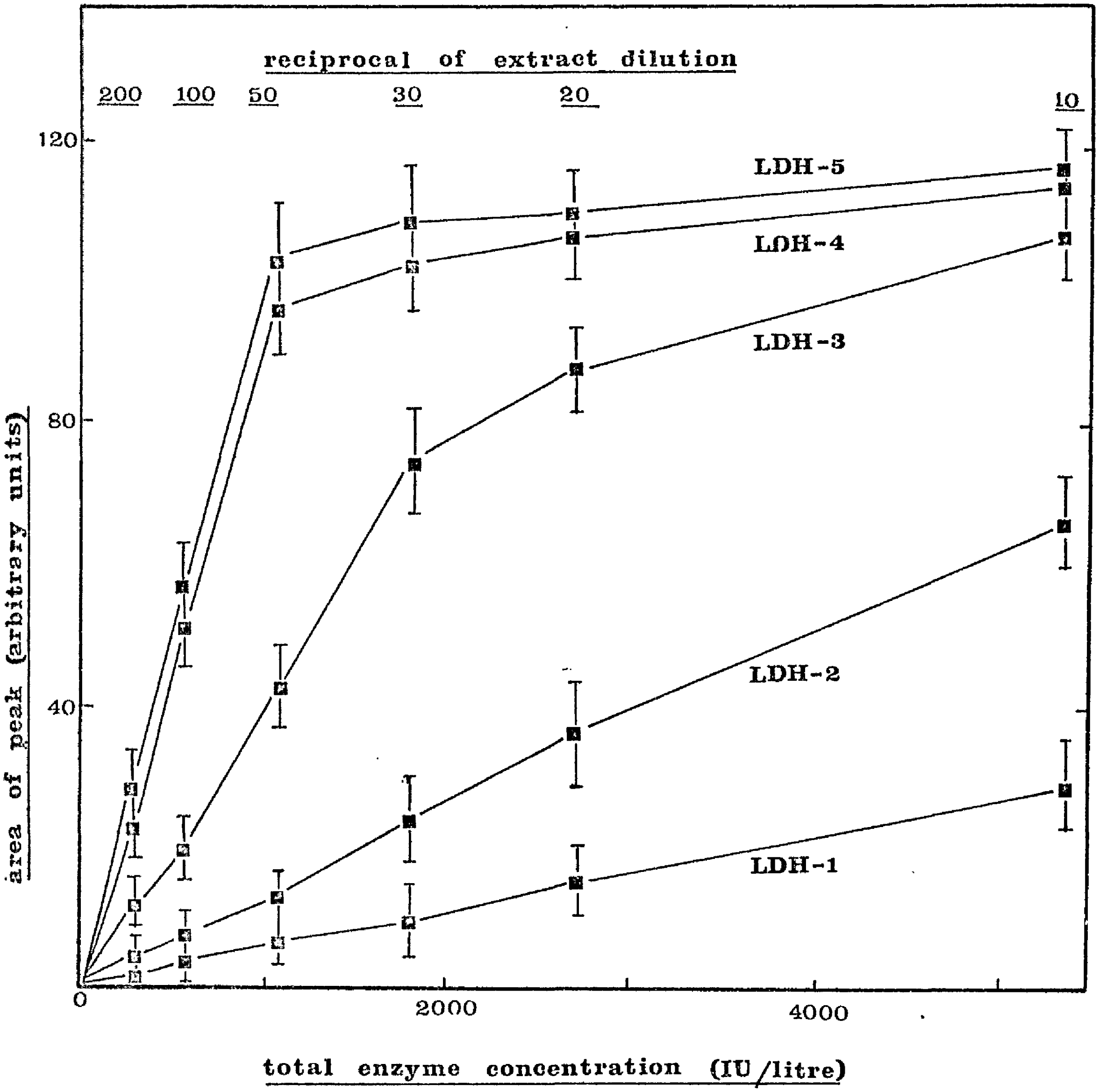


Figure 6.1:- The relationship between the area of lactate dehydrogenase isoenzyme peaks after densitometric scanning of stained electrophoretograms and total enzyme concentration in freshly isolated adult Kupffer cell extracts.

Values are means ± SD of four estimations on cells isolated from two animals. (1 IU = 1 μmole/mg. protein/min.).

6.3 Results

Densitometric scanning of stained electrophoretograms makes it possible to establish the quantitative relationships between LDH isoenzymes. Latner and Turner (1967) described such an assay and demonstrated that the intensity of staining is directly proportional to the activity present in each band, provided that the isoenzyme concentration does not exceed 400 IU/litre (1 IU, International Unit, is the amount of enzyme which converts 1 μmole of substrate/min. at 25°C). In this study of the quantitative relationships between LDH isoenzymes it was necessary to determine the range of extract dilution which resulted in a linear relationship between scanned band peak area and isoenzyme activity. Since the shape of the zones of activity of the cellulose acetate strips were different for each isoenzyme, peak area was found to be a better parameter than peak height.

A freshly isolated adult Kupffer cell extract (protein concentration of 1 μg/μl) possessed a total LDH activity of 54 x 10^3 IU/litre. This extract was diluted with PBS/A containing 1 μg/μl BSA. The relationship between scanned peak area and enzyme concentration is presented in Figure 6.1. Peak area of the most active isoenzymes LDH-4 and LDH-5 is directly proportional to enzyme concentration at dilutions of less than 1/50 of the Kupffer cell extract, i.e. below approximately 10^3 IU of total enzyme activity/litre. In the same extract, the peak areas for LDH-2 and LDH-1 were directly proportional to enzyme concentration at dilutions of 1/10 and greater. The limit of minimum dilution is in agreement with the 400 IU/litre for any single band observed by Latner and Turner (1967). The studies described below indicate that the maximum activity for any LDH isoenzyme in freshly isolated Kupffer cells is for LDH-5, which accounts for 40% of the total activity. Thus routine 1/100 dilution of 1 μg protein/μl extracts resulted in peak area being proportional to enzyme concentrations for all freshly isolated cells. Since total LDH activity in all

Table 6.1:- Total lactate dehydrogenase (LDH) activities in various tissue and cell extracts

Values are means ± SD. Figures in parentheses indicate the number of independent extracts considered. LDH activities in SV40-transformed cell lines were determined 40 population doublings after infection with SV40. The activities in primary foetal cell lines were determined after 69 population doublings in culture.

Extract	Total LDH activity (μmoles/U*)	Activity relative to that in freshly isolated Kupffer cells
Hepatocytes (4)	67.3 ± 7.4	1.26
Adult Kupffer cells (5)	53.5 ± 5.1	1.00
Foetal Kupffer cells (3)	30.9 ± 4.8	0.58
Kupffer cell lines:-		
Primary adult 26 doublings (15)	5.06 ± 1.15	0.09
33 doublings (15)	4.71 ± 1.11	0.09
69 doublings (15)	4.29 ± 1.03	0.08
SV40-transformed adult (10)	1.80 ± 0.50	0.03
Primary foetal (5)	2.01 ± 0.55	0.04
SV40-transformed foetal (4)	1.97 ± 0.38	0.04

* U = min/mg protein

Table 6.2:- Lactate dehydrogenase (LDH) proportions in Chinese hamster tissues and Kupffer cells in culture as determined by electrophoresis and microdensitometry

The figures in parentheses indicate the number of animals or cell lines used for the determination. Values are means ± SD. Isoenzyme proportions in SV40-transformed cell lines were determined 40 population doublings after infection with SV40. The proportions in primary foetal cell lines were determined after 69 population doublings in culture.

	LDH isoenzyme proportion (% of total activity)				
Isoenzyme	1	2	3	4	5
Extract:-					
Hepatocytes (4)	-	-	0.9±1.0	3.8±2.1	95.3±3.0
Adult Kupffer cells (5)	1.7±0.8	4.7±2.0	15.8±2.5	37.7±3.8	40.1±4.3
Foetal Kupffer cells (3)	15.1±0.6	20.2±0.8	16.5±1.8	21.5±1.7	26.7±3.4
Kupffer cell lines:-					
Primary adult (15)					
26 doublings	-	-	2.5±0.8	7.9±2.6	89.6±3.0
33 doublings	-	-	1.8±1.3	5.6±1.8	92.6±3.1
69 doublings	-	-	1.9±1.2	4.8±2.3	93.3±3.4
SV40-transformed adult (10)	-	-	-	-	100.0
Primary foetal (5)	-	-	-	-	100.0
SV40-transformed foetal (4)	-	-	-	-	100.0

cultured Kupffer cell extracts was less than 10% of that in freshly isolated Kupffer cells (see Table 6. 1), dilutions of only 1/10 for a 1 μg protein/μl extract were used for electrophoresis.

In Table 6. 1 appear the total LDH activities present in all cell extracts used for the study of isoenzymes. Also included are estimates of total LDH activity relative to those found in freshly isolated Kupffer cells. The enzyme activities found in cultured Kupffer cells have been described in greater detail in sections 4 and 5. The two predominant cell types in liver apparently possess different LDH activities. The activity of LDH in hepatocytes is greater than that found in Kupffer cells. In addition, foetal Kupffer cells possess only 58% of the LDH activity in adult Kupffer cells.

Greater heterogeneity of LDH activities becomes apparent when individual isoenzymes are considered. Table 6. 2 shows the relative proportion each isoenzyme contributes toward total LDH activity. LDH-1 is the most anodal isoenzyme when a cell extract is subjected to electrophoresis. The other isoenzymes migrate at slower rates, LDH-5 being the slowest. Adult Kupffer cells exhibit a considerably greater proportion of faster migrating isoenzymes than hepatocytes. The proportions of faster migrating isoenzymes in foetal Kupffer cells are greater than those found in adult Kupffer cells. Adult Kupffer cells in culture do not exhibit the LDH-1 and LDH-2 isoenzymes present in freshly isolated cells. However, in contrast to cultured SV40-transformed adult, primary foetal and SV40-transformed foetal cells, a small proportion of total activity can be found in the forms of LDH-3 and LDH-4 in cultured primary adult cells. In cultured SV40-transformed adult, primary foetal and SV40-transformed foetal cells, only LDH-5 could be detected after electrophoretic separation. Thus, SV40-transformed adult, primary foetal and SV40-transformed foetal Kupffer cells in culture appear to possess a common LDH isoenzyme pattern which is different to that found in primary adult Kupffer cell lines. As the culture period continues, primary adult Kupffer cell lines demonstrate an increasing proportion of LDH-5.

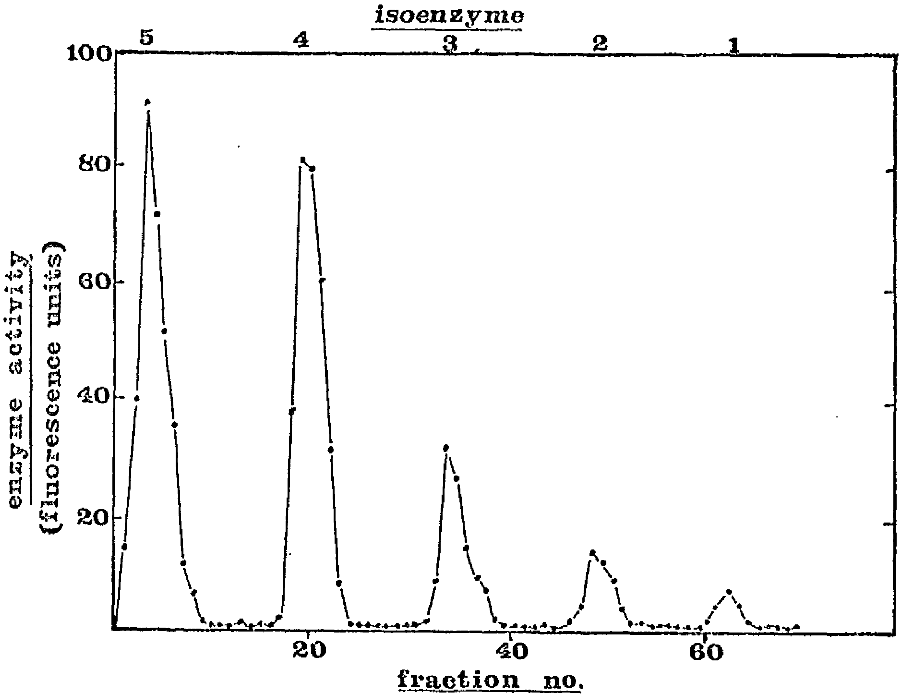


Figure 6.2:- The separation of freshly isolated adult Kupffer cell lactate dehydrogenase isoenzymes by ion-exchange chromatography on DEAE-Sephadex A50 and elution with NaCl solutions.

Table 6.3:- Lactate dehydrogenase (LDH) isoenzyme proportions in cultured Kupffer cells as determined by ion-exchange chromatography.

The figures in parentheses indicate the number of animals or cell lines used for the determination. Values are means ± SD.

Isoenzyme	LDH isoenzyme proportion (% of total activity)				
	1	2	3	4	5
Adult Kupffer cells (4)	0.8±0.4	4.1±0.8	9.5±1.3	38.1± 0.4	47.5±2.6
Kupffer cell lines:-					
Primary adult (6)	-	-	0.9±0.3	1.8±0.9	97.3±1.2
SV40-transformed adult (6)	-	-	-	-	100.0
Primary foetal (5)	-	-	-	0.8±0.4	99.2±0.4
SV40-transformed foetal (4)	-	-	-	-	100.0

Since ion-exchange chromatography is a more sensitive method of separating LDH isoenzymes (Fritz et al., 1970), it was likely that the presence or absence of LDH-3 and LDH-4 in SV40-transformed adult, primary foetal and SV40-transformed foetal Kupffer cell lines could be verified. Possible low activities of these isoenzymes may have been beyond the limits of detection by densitometric scanning. Figure 6.2 shows a typical NaCl elution profile of LDH isoenzymes from freshly isolated adult Kupffer cells. Table 6.3 presents the LDH isoenzyme proportions in various Kupffer cell lines after separation by chromatography. It is evident from the isoenzyme proportions in freshly isolated adult Kupffer cells and primary adult Kupffer cell lines that chromatography yielded results comparable to those obtained after scanning of stained electrophoretograms. All five LDH isoenzymes were present in freshly isolated Kupffer cells. The observation that primary adult Kupffer cell lines possess LDH-3, LDH-4 and LDH-5 confirms the findings presented in Table 6.2. The cell extracts used for chromatography were the same as those used for electrophoretic isoenzyme separation. The extracts for primary adult and primary foetal cell lines were those from cells after 69 population doublings in culture. Cells from the SV40-transformed adult and SV40-transformed foetal cell lines had undergone 40 population doublings since infection with SV40 before the extracts were prepared.

Chromatographic separation reveals a difference in isoenzyme pattern not detected after electrophoresis (see Table 6.3). Transformation of adult Kupffer cells in culture with SV40 results in the loss of all isoenzymes except LDH-5. Ion-exchange chromatography demonstrates that primary foetal Kupffer cell lines possess a small proportion of activity in the form of LDH-4. After transformation of primary foetal Kupffer cell lines by SV40, only LDH-5 can be detected.

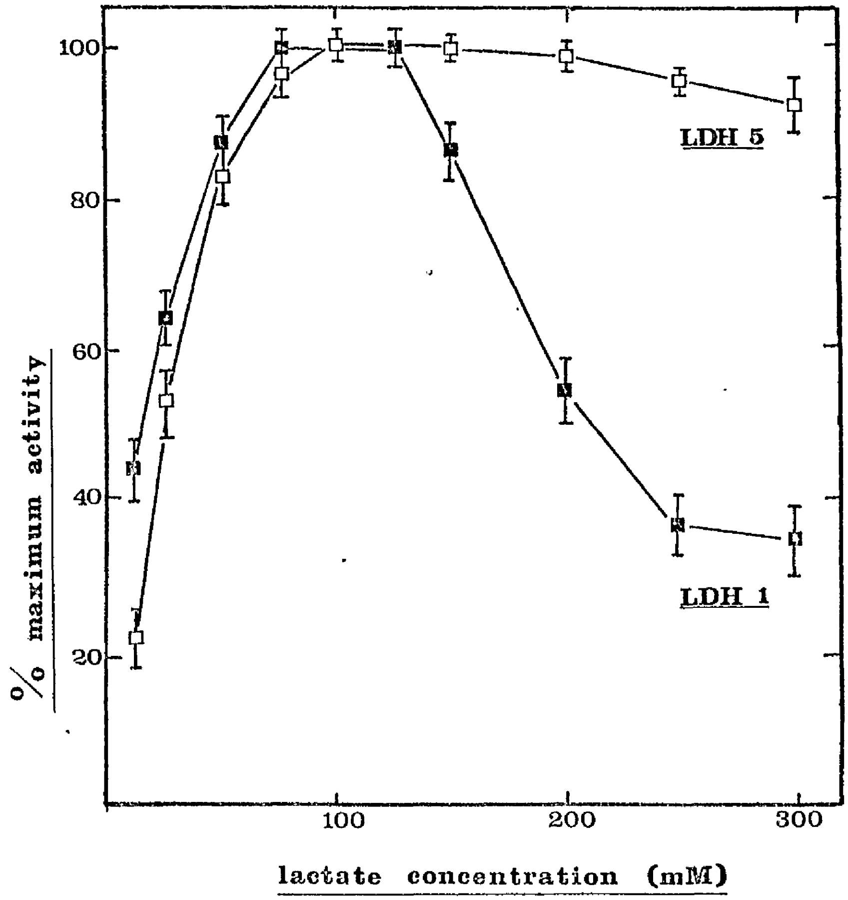
Since the aim of this study was to describe the relative contributions of LDH A and LDH B gene products to total enzyme activity, it was necessary to estimate the relative proportions of a and b subunits

Figure 6.3:- The inhibition of partially purified LDH-1 and LDH-5 activity by lactate.

The assays were performed with sufficient enzyme to convert 0.5 μmoles lactate/min. in 100 mM lactate and 1.3 mM NAD. Each point is the mean ± SD of four estimations.

Figure 6.4:- Calibration curve of the relationship between lactate dehydrogenase isoenzyme activity ratios in low (50 mM) and high (250 mM) concentrations of lactate and the proportion of b subunits in the isoenzyme.

Isoenzyme standards were partially purified by electrophoresis. Each reaction was conducted with 0.5 IU of enzyme activity in 1.3 mM NAD. The points are for 3 independent determinations.


100
80
60
40
20
% maximum activity
100
200
300
lactate concentration (mM)
LDH 5
LDH 1

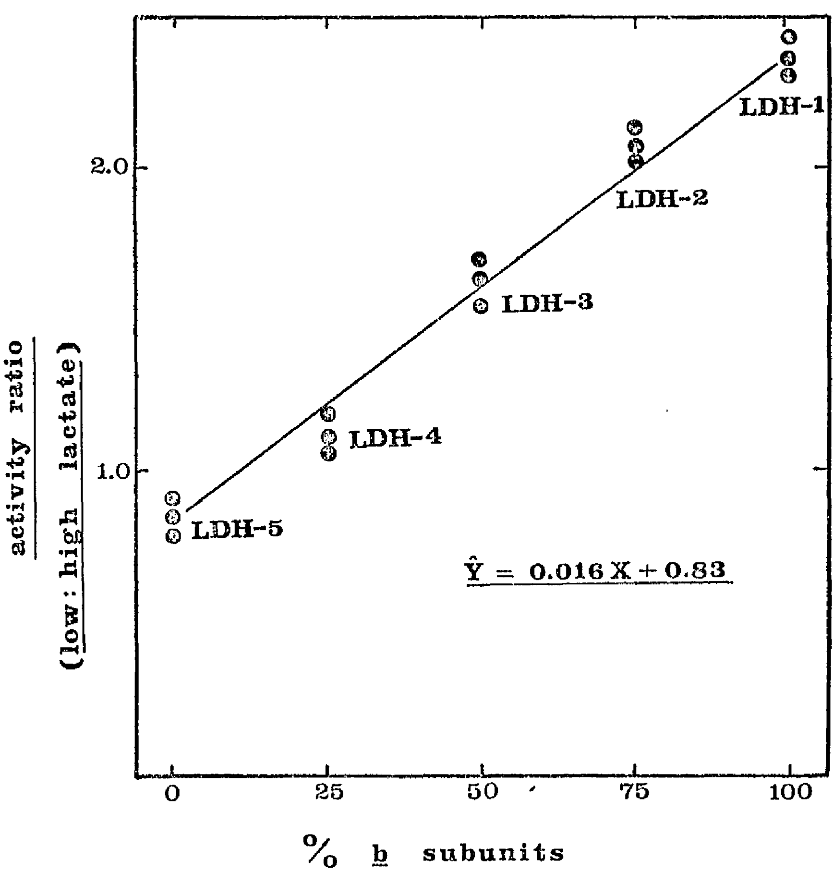

2.0
1.0
activity ratio
(low: high lactate)
0
25
50
75
100
% b subunits
LDH-1
LDH-2
LDH-3
LDH-4
LDH-5
Ŷ = 0.016 X + 0.83

Table 6.4:- The proportion of lactate dehydrogenase b subunits in the hepatocytes, freshly isolated Kupffer cells and various Kupffer cell lines as determined by different methods

The figures in parentheses indicate the number of animals or cell lines used in the determination. Values are means ± SD.

Extract	Proportion of b subunit (%) Method of determination		
	Electrophoresis	Substrate inhibition	Chromatography
Hepatocytes	1.4 ± 1.0 (4)	3.3 ± 3.3 (4)	-
Adult Kupffer cells	22.6 ± 1.9 (5)	27.2 ± 2.9 (5)	18.2 ± 1.7 (4)
Foetal Kupffer cells	43.8 ± 1.3 (3)	50.6 ± 3.5 (3)	-
Kupffer cell lines:-			
Primary adult after			
26 doublings	3.2 ± 0.9 (15)	5.3 ± 2.0 (15)	-
33 doublings	2.3 ± 1.1 (15)	2.4 ± 1.8 (15)	-
69 doublings	2.2 ± 1.1 (15)	3.1 ± 2.3 (15)	0.9 ± 0.4 (6)
SV40-transformed adult	not detected (10)	0.4 ± 0.7 (10)	not detected (6)
Primary foetal	not detected (7)	1.4 ± 1.1 (7)	0.2 ± 0.1 (6)
SV40-transformed foetal	not detected (4)	0.0 (4)	not detected (4)

in each cell extract. Although the proportions of a and b subunits can be calculated from the data presented in Tables 6.2 and 6.3, more direct estimates can be obtained from substrate inhibition studies. Figure 6.3 illustrates the effect of lactate concentration on enzyme activity of partially purified LDH-1 and LDH-5. Whilst LDH-5 activity is barely reduced in the presence of 300 mM lactate, LDH-1 is progressively inhibited by lactate concentrations above 125 mM. After comparison with a calibration curve, the ratio of LDH activity in the same extract at 50 mM and 250 mM lactate provides an estimate of the proportion of b subunits in the extract (Stambaugh and Post, 1967). Figure 6.4 shows a calibration curve obtained from determining the low:high activity ratios for partially purified LDH isoenzymes. There was a high degree of correlation between the two variables ($r = 0.98$) and linear regression revealed that the calibration points could be best described by a line of equation $Y = 0.061\ X + 0.83$, where Y is the estimate of low:high lactate activity ratio and X is the proportion of b subunits in the extract.

In Table 6.4 appear the estimates of b subunit proportion determined by the three methods. After electrophoresis or chromatography, the proportion of b subunits was calculated from the relative contributions to the isoenzyme activities detected. Comparison of b subunit proportions in freshly isolated cell extracts reveals that estimates obtained by the three methods are in general agreement.

The b subunit proportion initially decreases when Kupffer cells are grown in culture, but remains relatively stable at between 2 to 5% of the total activity for at least 40 population doublings in culture. When all the data are considered, it is apparent that b subunits are not detected in SV40-transformed adult and SV40-transformed foetal Kupffer cells in culture. Total LDH activity in SV40-transformed Kupffer cells appears to be the result of only LDH-A gene product activity. The b subunit is present in primary foetal Kupffer cell lines but at a frequency well below that found in primary adult Kupffer cell lines.

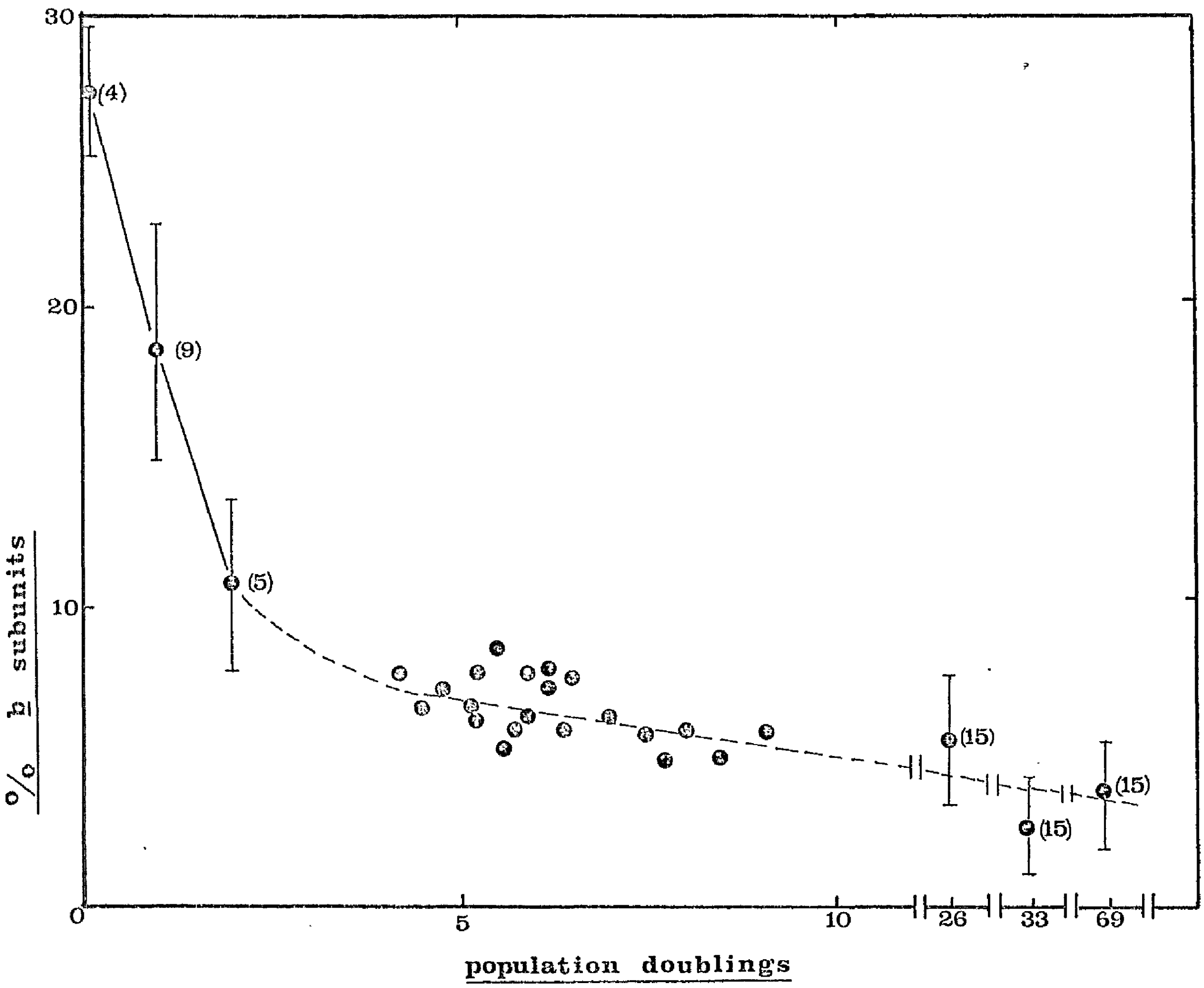


Figure 6.5:- The relationship between the proportion of lactate dehydrogenase b subunits and the number of population doublings for adult Kupffer cells in culture.

The figures in parentheses indicate the sample size and the points illustrate the mean ± SD. The single point observations are the average of estimates for two colonies of equal size.

Since the substrate inhibition method of estimating b subunit proportion yielded values in agreement with those obtained by isoenzyme separation techniques, this method was used to study the kinetics of the reduction in b subunit proportion in cultured adult Kupffer cells. Groups of dividing cells were selected from cultures of freshly isolated Kupffer cells. After 1 division, 18 pairs of cells growing on "Melinex" polyester film were selected and 9 pairs assayed at each lactate concentration. After 2 divisions, 10 groups of four cells were selected and 5 assayed at each lactate concentration. Thereafter, pairs of colonies of equal cell number were selected and enzyme activity in low and high lactate concentrations determined. Colonies varied in size between 20 and 500 cells. The number of cells in each colony was related to the number of cell doublings in culture. Figure 6.5 illustrates the relationship between the proportion of b subunits and the number of population doublings in culture. The estimate of b subunit proportion in freshly isolated cells was based on four independent extracts. Enzyme activities in low or high lactate concentration were not made on the same group of cells in the samples for the first two divisions, and therefore estimates for cells at these stages may not be as reliable as those obtained at later stages when both activities were determined in the one extract. However, the data clearly demonstrate that b subunit proportion decreased rapidly when freshly isolated Kupffer cells were cultured. After approximately 5 doublings the proportion stabilized and remained relatively constant for at least 60 population doublings in culture.

The substrate inhibition method was used to estimate the contribution of the LDH b subunit to total LDH activity in adult Kupffer cells recently transformed by SV40. After 26 population doublings, primary adult Kupffer cells were infected with SV40 as described in section 5.2.1. The cells were seeded onto "Melinex" polyester film in 50 mm plastic petri dishes and colonies allowed to develop to 60-80 cells in size. Those colonies with irregular morphology were considered to be SV40-

transformed (see section 5. 3. 1) and LDH b subunit proportion determined. The proportion of LDH b subunits in 10 pairs of colonies with irregular morphology was 0. 2 ± 0. 3% of the total number of LDH subunits. Within approximately the first 8 divisions since infection with SV40, the proportion of LDH b subunits had fallen from 5% to near zero.

6. 4 Discussion

Since the study presented in this section is largely a diversion from the main theme of phenotypic variation, a brief discussion of the results will be conducted at this stage rather than in section 7. The results of this section provide some information on the biochemical alterations which take place when a Kupffer cell enters the culture environment, and the relationships between the transformed and foetal phenotype.

The distribution of LDH isoenzymes in freshly isolated adult Kupffer cells and hepatocytes from the Chinese hamsters are in general agreement with those described for rat cells by Berg and Blix (1973). These workers demonstrated that all of the rat hepatocyte LDH activity was in the form of LDH-5. In contrast was the distribution of LDH isoenzymes in rat Kupffer cells where the activity was distributed between LDH-5 (46%), LDH-4 (37%), LDH-3 (14%) and LDH-2 (3%). Thus, in spite of the species difference, rat and Chinese hamster Kupffer cells demonstrate a similar distribution of LDH isoenzymes. The data based on separation techniques presented in this section suggest that the only difference between these two species with respect to Kupffer cell LDH isoenzyme distribution is a marginal increase in LDH b subunit proportion in the Chinese hamster.

The data demonstrate that total LDH activity decreased when a Kupffer cell entered the culture environment. Associated with the decrease in total activity was an alteration in the relative contributions of LDH a and b subunits to this activity. It should be emphasized that the contributions of a and b subunits to total activity are based on catalytic assay and thus only refer to active subunits. The proportions do not necessarily refer to the total number of subunit molecules present. When total LDH activities (Table 6.1) and the proportion of LDH b subunits (Table 6.4) are considered, two phenomena are evident. There was a reduction in the number of active a and b subunits as well as reduction in the proportion of b subunits which contributed to total LDH activity. The reduction in total LDH activity when adult or foetal Kupffer cells entered the culture environment could not be explained by a reduction in only the number of active b subunits.

The changes in the proportions of LDH a and b subunits when Kupffer cells are cultured are presumably the result of changes in the physiological environment. The consistent decline in the proportion of b subunits could certainly be interpreted as being of metabolic significance. Everse and Kaplan (1973) have suggested that cells relying on glycolysis have mainly LDH-5, whereas cells with predominantly aerobic metabolism possess a large proportion of LDH activity in the form of LDH-1. One of the main functions of the Kupffer cell is phagocytosis (see section 3.1), a process which is dependent on aerobic metabolism (Karnovksy, 1968). Berg and Blix (1973) thus suggested that the presence of LDH b subunits in Kupffer cells is a reflection of their predominantly aerobic metabolism. The rapid decline in the proportion of LDH b subunits when Kupffer cells are cultured may reflect both alteration in cellular metabolism to an emphasis on anaerobic glycolysis and an increased rate of mitosis (Papaconstantinou, 1967). The decreased phagocytic capacity of cultured Kupffer cells noted in section 3 could also be explained in terms of these changes in basal metabolism.

With regard to LDH subunit proportions, the response of adult Kupffer cells to the culture environment is rapid. Within the first five cell divisions in culture the contribution of LDH b subunits to total activity is reduced to a value which is subsequently maintained during continued culture. It is not possible to determine whether the alterations in total LDH activity and subunit proportions are the result of altered synthesis or degradation of the subunits. Certainly, the data on primary adult Kupffer cells do not necessarily provide evidence for alterations in LDH _A_ and LDH _B_ gene activities when Kupffer cells enter the culture environment. Vesell and Fritz (1971) reviewed evidence which suggested that degradation plays as significant a role as synthesis in establishing LDH isoenzyme patterns. Their data demonstrate that degradation of LDH isoenzymes proceeds with great specificity. Thus, the changes in the proportions of LDH _a_ and _b_ subunits in Kupffer cells as they are established in culture could be interpreted on the basis of differing degradation rates.

Transformation of Kupffer cells by SV40 resulted in a change in LDH isoenzyme pattern and _b_ subunit proportion. Such a result has also been reported by Prasad _et al._ (1972) who demonstrated that tumours induced in hamsters by SV40 exhibited a decrease in _b_ subunit proportion. Caltrider and Lehman (1975) observed that there was a shift towards a decreased _b_ subunit proportion in Chinese hamster embryo cells transformed by SV40. The results of this study demonstrate that transformation of Chinese hamster Kupffer cells, whether adult or foetal, by SV40 effectively eliminates _b_ subunit activity. In these cells all detectable LDH activity is the result of the _a_ subunit. In such cases it is tempting to conclude that the LDH _B_ gene is no longer active. The reduction in _b_ subunit activity is a rapid process such that 8 divisions after infection of cells with SV40, transformed cells did not possess any _b_ subunit activity.

Caltrider and Lehman (1975) have interpreted the shift in isoenzyme pattern after transformation by SV40 to be a reversion to a more foetal pattern of protein synthesis. The studies on Kupffer cells presented in this section suggest that SV40-transformation results in a phenotype different from that possessed by foetal Kupffer cells. Cells from primary adult and primary foetal Kupffer cell lines possess both a and b subunits, the only difference being a reduction in the proportion of b subunits. Cells from SV40-transformed adult and SV40-transformed foetal Kupffer cell lines possess only a subunits. The relationship between b subunit proportions in cultured adult and foetal Kupffer cells is different from that found between freshly isolated adult and foetal Kupffer cells. While there existed a greater proportion of b subunits in freshly isolated foetal Kupffer cells, the reverse was true for cultured foetal Kupffer cells. The reason why the b subunit proportion should be reduced to a greater degree when foetal Kupffer cells enter culture remains obscure. Transformation of adult Kupffer cells with SV40 thus resulted in a change in LDH isoenzyme pattern in the opposite direction to that expected if there were to be a reversion to the foetal pattern.

The studies reported in this section indicate that when a Kupffer cell is cultured, changes in an enzyme total activity are also accompanied by changes in the proportions of its component molecules. Both changes occur immediately the cell enters the culture environment and a new, relatively stable state is achieved within the initial divisions in culture. SV40-transformation of the cultured adult Kupffer cells did not result in reversion to an LDH isoenzyme pattern possessed either by foetal Kupffer cells *in vivo* or in culture. The transformation appeared to result in the extinction of LDH-B gene products, although the possibility that b subunits were being produced at a level below the limits of detection cannot be excluded. Whether similar changes to those reported for LDH isoenzymes in this section occur for the other enzymes studied in this dissertation, remains to be ascertained.

SECTION 7

GENERAL DISCUSSION AND CONCLUSION

The results presented above encroach upon many areas of cell biology and could be approached from several angles. Since it is not possible to consider all aspects exhaustively, a few general areas directly related to the aims of this study will be discussed. More specific comment or brief discussion of the results can be found in the relevant sections.

Although it appears to satisfy the aims of this study, the system employed is currently at a disadvantage by being without precedent. Thus, a major point to be stressed is that caution is required in the generalization of the described phenomena to other cell culture systems. The system used in this study is unique in two ways.

Firstly, it utilizes cultured Kupffer cells, a cell type apparently not previously cultured for extended periods. Relatively little is known about Kupffer cells in vivo or in culture and any attempts at comparison with phenomena reported for other cultured cell types must recognize that we are dealing with an essentially unknown system.

The second reason why comparison with other studies must be cautious relates to the method of cell line isolation and the subculture routine. All Kupffer cell lines used in this study were initiated with a primary cloning step. The usual way to initiate monolayer cell lines is to explant the required tissue into culture and eventually clone those cells which grow out from the piece of tissue (see Kruse and Patterson, 1973). While both methods yield cloned cell lines there exist important biological and practical differences. By virtue of cloning both methods result in cell lines derived from a single cell. However, the selection pressures imposed upon this single cell or its predecessors during the initial culture period are probably different. Primary cloning results in selection for cells capable of attachment and division in culture immediately after isolation. Such cells do not have the opportunity of competition or interactions with other cells of the tissue population. Cloning of cells which grow out from a tissue explant presents a different situation. The cells which grow out may possess different phenotypes, have the opportunity to interact and those which divide with the most rapidity will dominate the culture. Thus, it must be emphasized that primary cloning may yield a different class of cell line to that obtained when cloning is performed at a later stage. In the absence of information it is possible to present an argument for greater heterogeneity between cell lines after either type of cloning. The fact that all classes of Kupffer cell line were maintained in exponential growth and not allowed to achieve confluence may influence evolutionary processes within the cell lines.

The advantages of primary cloning lie in the fact that the progress of a single cell can be monitored as soon as it enters culture, it can be isolated from other cells, whether different or not, and its histotypic identity may be established. Primary cloning is of course restricted to those tissues amenable to dissociation.

Another point to note before the results are considered is the measure of "population doublings" in culture. This parameter is only an approximation of cumulative population doublings and as the culture period proceeds will probably come into greater error. Under the subculturing routines used in this study, "population doublings" is a more suitable parameter of age in proliferating culture than the number of subcultures. "Population doublings" is not meant to imply a precise increase in population size in culture, but rather to give an approximate reference to the culture stages considered.

With these considerations in mind, some general aspects of the results will now be discussed with no great attention being paid to individual enzyme activities. The discussion will be divided into four aspects of phenotypic variation in the Kupffer cell lines. These are:- (1) variation of enzyme activities, (2) correlation of enzyme activities, (3) the relationship between adult, foetal and SV40-transformed Kupffer cell line phenotypes and (4) the karyology of the Kupffer cell lines. A brief résumé of the results will be presented and at appropriate points suggestions for future work will be made. Finally, some concluding comments will be made. Differentiated functions and culture of primary adult Kupffer cells have already been considered in section 3.4. The study of Kupffer cell line lactate dehydrogenase isoenzymes was discussed in section 6.4 and illustrated the complexities which may underlie a total enzyme activity.

7.1 Variation of enzyme activities in Kupffer cell lines

In section 1 it was noted that there exist several examples of enzyme activity variations in tumours and cultured mammalian cells. Due to diverse and often unidentified origins of the cells and environmental influences *in vivo* (see section 1.3), the extent or basis of this variation is unknown. Whether the occasional examples of "anomalous" enzyme activities are genetic or epigenetic variations is difficult to determine. As a preliminary attempt to examine phenotypic variation, one of the major aims of this study was to describe the extent and distribution of enzyme activity variation in cell lines derived from identical material. A cell culture system was developed whereby it was possible to isolate cell lines derived from identified material and maintain them under constant environmental conditions. All cell lines used in this study were derived from closely related animals and, with the exception of the primary adult cell lines, each class of cell line was derived from one animal.

It seems likely that the enzyme activity variations to be discussed below were real phenotypic variations in a single cell type and not due to error in method. The identity of all cell lines was established by the presence of specific Kupffer cell functions and all analyses of variance revealed that variation due to method was a minor component when compared with the variation between cell lines, whether primary or SV40-transformed. Although it is assumed that the catalytic assays were of physiological relevance, it must be remembered that they only measured the rate of a particular reaction and it is possible that several molecular species may have contributed to this activity. The assays were of total activity based on cell homogenates and thus potential changes in sub-cellular distribution or isoenzyme forms would have been overlooked. For example, the studies described in section 6 indicated that when LDH iso enzyme distributions were considered, complexities underlay the total LDH activity.

The major study on variation of enzyme activities was conducted in primary adult Kupffer cell lines. All enzyme activities demonstrated a common, general pattern of change, although some were more variable than others. When freshly isolated adult Kupffer cells entered culture all of the enzyme activities were reduced to a fraction of the activities in vivo. This reduction in activity occurred within the first few divisions in culture and as culturing proceeded, further reduction was less dramatic and after 40 population doublings a level was reached which was stable until at least 70 population doublings. After this stage, some enzyme activities deviated and proliferation ceased in many cell lines.

The pattern of change in variation of activities between primary adult cell lines was similar for all enzymes. Variation between Kupffer cells in vivo was small but activities in colonies derived from single freshly isolated Kupffer cells rapidly diverged until the stage of approximately 8 cell divisions. After this stage the variation in enzyme activities between cell lines remained similar. There was a slight decrease in variation between 26 and 40 population doublings to a value which was stabl until at least 70 population doublings, whereafter variation increased.

All SV40-transformed adult cell lines were isolated from primary cell lines derived from a single animal. Analysis of enzyme activities in SV40-transformed adult cell lines revealed even greater heterogeneity between the cell lines. Upon reflection, the design of the study on enzyme activities in SV40-transformed cell lines was inadequate. An SV40-transformed cell line was isolated from a single cell within a primary cell line. For an adequate description of phenotypic variation after SV40-transformation to be made, many SV40-transformed cell lines need to be isolated from the one primary cell line. The design of the present study was expected to generate greater heterogeneity and increase the probability of the isolation of a cell line with an "anomalous" enzyme activity. This point will be returned to below. Any heterogeneity caused by SV40-transformation would be superimposed upon heterogeneity between cells within the primary cell line and heterogeneity between primary cell lines. Hence, the chances of obtaining a heterogeneous sample of cell lines are greatly increased. In spite of the limited experimental design the data allow some observations to be made.

Transformation of adult Kupffer cells by SV40 resulted in a rapid change in enzyme activities. The changes which were evident 26 population doublings after SV40 infection occurred within the first 8 cell divisions after infection. Only the activity of G6PDH remained unchanged. Although there were fluctuations in LDH, IDH and G6PDH activities, enzyme activities were generally stable until at least 90 population doublings after infection with SV40. During this culture period there was a marginal increase in the variation of enzyme activities between the cell lines.

Primary foetal cell lines demonstrated enzyme activities less than those in primary adult cell lines. Unfortunately, data for enzyme activities in freshly isolated foetal Kupffer cells are not available and thus it is not possible to determine whether the differences between the in vivo and in culture activities for adult Kupffer cells occur for foetal Kupffer cells. The variation in enzyme activities between primary foetal cell lines, although significant was less than that observed between primary adult cell lines. Six enzyme activities maintained a stable value until at least 97 population doublings. Catalase, MHO and peroxidase activities decreased between 26 and 40 population doublings in culture, but only MHO activity continued to decrease upon subsequent culture. During the culture period there was a gradual increase in enzyme activity variation between primary foetal cell lines.

Enzyme activities and their patterns of variation in SV40-transformed foetal cell lines were indistinguishable from those in SV40-transformed adult cell lines.

In spite of the considerable variation of enzyme activities between cell lines within each class, no steady-state "anomalous" enzyme activity was detected. The study of genetic regulation in mammalian cells has been hampered by a lack of regulatory mutations and in the course of this study it was hoped that cell lines with "anomalous" enzyme activities would be isolated. By analogy with the mouse (see Paigen, 1971; Paigen et al., 1975) such "anomalous" enzyme activities may be the first signs of the existence of a regulatory mutation. The variation which arises from neoplastic transformation (see section 1.5) was to be exploited in an attempt to isolate cell lines with "anomalous" enzyme activities. While SV40-transformation may have increased the variation of enzyme activities between cell lines, no "anomalous" activity was detected.

Although several studies have reported cell lines or tumours with "anomalous" activities (see Pitot, 1966; Schimke, 1969) the frequency of these cases is not known. As the studies described in this dissertation proceeded it became apparent that a major problem in the detection of an "anomalous" enzyme activity was to describe the distribution of activities when identical cells are cultured. Thus the experimental design was directed towards a basic description of variation so that in future studies with this system an "anomalous" activity could be recognized. To illustrate this point, a situation could be envisaged where only two cell lines were studied from the one animal and happened to have an enzyme activity at opposite extremes of the normal range. The results of this study suggest that this range may be several fold depending on the enzyme and thus recognition of an "anomalous" activity must be approached with consideration of statistical sampling. If the aim of this study had been to just isolate cell lines with "anomalous" activities, a more suitable experimental design would have been to isolate small numbers of cell lines from each of a large number of animals, preferably of different strains. This would be the equivalent of the approach adopted by Paigen who screened 63 strains of mice for β-glucuronidase activities (see Paigen et al., 1975). With the design employed here cell lines were derived from the same genetic origin and if the "anomalous" activity were to have a genetic basis, then all the cell lines would presumably possess a similar enzyme phenotype (unless we are dealing with an individual heterozygous for a sex-linked mutation).

The problem of the frequency of "anomalous" enzyme activities in cell lines remains unanswered. The large degree of variation between cell lines presumably with the same initial genotype suggests that an answer to the problem would require a large experiment considering cell lines from many strains of animal. If an "anomalous" enzyme activity were to arise in the system employed in this study then it must be a relatively rare event based on either mutation or an infrequent epigenetic change.

The fact that there was a sharp decline in all enzyme activities considered when adult Kupffer cells enter culture supports the general belief among cell culturists that enzyme activities decline when a cell enters the culture environment (see Davidson, 1964; Green and Todaro, 1967; Terzi, 1974). In this study, the use of identified freshly isolated cell suspensions eliminated problems of cell type and introduced the chance of a primary cloning step. In this way identified cells could be observed from their initial entry into culture and the problem of overgrowth by other cell types was eliminated. Thus on first impressions this system would appear ideal for the examination of possible enzyme activity decline when a cell enters culture.

Although it is considered likely that a reduction in enzyme activity does occur, disadvantages of the methods make it possible to argue that doubt still exists. The enzyme activities recorded during the initial decline in culture (see section 4. 3. 4) were determined on colonies of varying size. Since it was not possible to assay enzyme activity in the same colony on successive occasions the decline could only be deduced from study of colonies containing cells which had experienced different numbers of division in culture. While it is not rigorous proof, the fact that a consistent trend in the data occurs when colonies of increasing size were considered indicates that there was a real decline. It is also possible that the single freshly isolated Kupffer cells assayed were not the class of Kupffer cell which gave rise to colonies when placed in culture.

The colonies could have been derived from a sub-population of Kupffer cells with low enzyme activities. Thus the initial in vivo enzyme activities in the cells which give rise to colonies and subsequent cell lines remain in doubt. The best that could be attempted were estimates of enzyme activity in homogenates of large numbers of Kupffer cells, and activities in a small sample of single cells. The difference in activity between these two groups of estimates was small.

While some enzymes show variations in activity during the cell cycle (see Schimke, 1969; Klevecz, 1969) variations in the proportion of cells in the various stages of the cell cycle may have affected enzyme activity when the colonies were small. The fact that lysosomal enzyme activities do not fluctuate during the cell cycle (Berg et al., 1975) suggests that the apparent reduction in β-glucuronidase activity was not the result of cell cycle influences. Since the decline in activity of the other enzymes studied followed a pattern similar to that of β-glucuronidase, it is concluded that variations due to the cell cycle were small when compared with the apparent decline due to other causes.

It would appear that there was a biphasic loss in enzyme activity as adult Kupffer cells were cultured. There was a rapid decline within the first 8 divisions in culture, a stable period, and then a further decline between 26 and 40 population doublings. Whether the loss is really composed of two phases is a problem of resolution and must await further data. It is possible that the first decline represented an immediate response to the culture environment whilst the second decrease was the result of population changes and the emergence of cells with a lower enzyme activity.

The basis and mechanism for the rapid decline in enzyme activity are not clear. The decline was apparently rapid, indicating an immediate response of the cell to the culture environment. Such a response could have been the result of removal of in vivo stimuli, or a rapid adaptation to the culture environment. Either way there was an alteration in the enzyme realization process to result in a different activity. Whether this was due to decreased synthesis, increased degradation or regulation due to structural modification is not known. The studies of Yagil and Feldman (1969) suggest that G6PDH is not continuously degraded in cultured cells. If G6PDH is not available for the degradative system so important in determining enzyme activities (Rechcigl, 1971; Mellman et al., 1972) then major alterations in activity must be brought about by changes in synthesis. Thus the decrease in G6PDH activity when adult Kupffer cells entered culture may have been the result of decreased synthesis. Whether the other enzyme activities were altered by changed rates of synthesis or degradation remains to be demonstrated. In terms of cellular economy it would be more logical for a long term decrease in enzyme activity to be accomplished by decreased synthesis rather than increased degradation. Whatever the mechanisms of the change, the results indicate that it occurs immediately a Kupffer cell first divides in culture and affects both total enzyme activities and probably isoenzyme distributions (see section 6). The observation that the initial decline in most enzyme activities appeared to follow simple dilution kinetics indicates that the enzyme realization process is immediately altered upon entry into culture and that a new steady-state is achieved after dilution of enzyme produced under the previous steady-state.

Another aspect of phenotypic variation between Kupffer cell lines concerns the uniqueness of each cell line. It was noted in section 1 that cell lines may often possess similar enzymic constitutions (Davidson, 1964; Terzi, 1974) and it was suggested by Terzi (1974) that long-term patterns compatible with growth in culture tend to be similar and thus cell lines show convergence of properties. It would seem that much of the uniformity observed in the older established cell lines may, in fact have been the result of the adaptation to, or the selection of cells for, standard culture regimens, as recent modifications of culture techniques have permitted the establishment of cell lines with more individual characteristics (Auersperg and Finnegan 1974; Gunn et al., 1976). Several cases of phenotypic variation were cited in section 1, but once again the specific origins of the cell lines are generally unknown and the variation may have been due to consideration of cell lines derived from different cell types.

The results of this study demonstrate considerable variation between Kupffer cell lines. This variation was evident between cell lines derived from the same cell type and presumably with the same initial genetic information. Each cell line was probably unique, but the fact that we are dealing with continuous variables makes discrimination between cell lines at a similar point in an enzyme activity distribution difficult. However, when nine continuous variables are considered our degree of discrimination is greater and presumably if the number of variables were increased the uniqueness of each cell line would become apparent.

By and large, the phenotypic variation of enzyme activities between Kupffer cell lines involved quantitative and not qualitative variations. A proportion (11%) of primary adult cell lines did not possess arginase activity and at later stages MHO activity was lost in several cell lines. With the exception of these cases, each class of Kupffer cell line contained cell lines which were qualitatively identical with respect to the presence or absence of the enzyme activities considered. That qualitative variations exist between the Kupffer cell lines is probable. Kaighn and Prince (1971) observed that individual clones derived from a human liver cell culture were unique in the spectrum of serum proteins synthesized. With an approach which could be used for future studies with the Kupffer cell lines, Moir and Roberts (1976) used antisera to demonstrate that closely related Drosophila cell lines and subclones of a clone were unique in their protein spectra.

The final point is a brief comment on the ageing of Chinese hamster Kupffer cells in culture. The primary adult cell lines demonstrated many of the classic symptoms of ageing of human fibroblast cultures (Hayflick and Moorhead, 1961; Hayflick, 1965) and the increased variation between primary Kupffer cell lines in the later stages of culture could be ascribed to the ageing process. In would appear that for many primary adult Kupffer cell lines 70 population doublings in culture marked the beginning of the senescent stage. At approximately this stage there were often alterations in plating efficiency, population doubling time, karyotypes, and some enzyme activities changed and their variation increased. A sub-population of cells with low activity of some enzymes also emerged in the one cell line examined for this type of heterogeneity. It was after 70 population doublings in culture that the primary adult cell lines progressively ceased to proliferate. Changes in culture kinetics of ageing human fibroblast cultures have been reported (Hayflick and Moorehead, 1961; Hayflick, 1965) as have karyotypic changes (Thompson and Holliday, 1975) and enzyme activity changes (see for example Wang et al., 1970; Srivastavi, 1973; Turk and Milo, 1974; Fulder and Tarrant, 1975; Sun et al., 1975). Thus the increased heterogeneity between the primary adult Kupffer cell lines after 70 population doublings was probably the result of senescence. The primary foetal cell lines possessed a finite lifespan of approximately 130 population doublings, although heterogeneity was not considered immediately prior to this stage. Variation of enzyme activities after 97 population doublings was greater than at earlier stages and as culturing proceeded there were karyotypic changes. It was not determined whether the SV40-transformed cell lines possessed a finite lifespan. However, during the course of study, there were no changes in mean enzyme activity and only slight increases in variation of enzyme activity between the SV40-transformed cell lines.

It is not possible to establish either the basis or the mechanism for the observed phenotypic variation. With the cautions noted above, it is reasonable to assume that the primary foetal cell lines were derived from identical cells. The primary adult cell lines were derived from three inbred sibs and no difference could be detected in the distribution of enzyme activities between cell lines from these animals. Even when only primary cell lines from the one adult animal were considered extensive phenotypic variation was apparent. Thus it seems reasonable to argue that, if the original cells were identical, the variation arose as a result of each cell's individual response to being isolated from the tissue and placed in culture. When the cells were placed in culture two processes occurred, first a decline in enzyme activity and secondly an increase in enzyme activity variation between clones derived from the freshly isolated cells.

The extent and distribution of the enzyme activity variation suggests that we are probably not observing the effects of mutation or elimination of genetic material, but rather, we are observing heritable regulatory phenomena involved in enzyme realization. Once this conclusion is reached we are then facing one of the major problems in biology, the need to explain stable alterations in cellular phenotypes, i. e. differentiation. However, with respect to this study a few indulgent comments could be made.

It is most probable that the stimulus for a change in the Kupffer cell phenotype upon culturing is the altered environment. Such alterations could be the disruptions of inter-cellular relations or the removal of molecular stimuli (e. g. hormones or chalones). This drastic change in the cell's environment would induce a "reprogramming" of many enzyme realizations. In the case of the Kupffer cells this change would appear to be immediately when the cell enters culture. In some respects the "reprogramming" observed in this study is not that normally associated with differentiation. Differentiation is usually interpreted to be the result of selective masking of genes (Maclean, 1976) to bring about qualitative changes in the cellular phenotype. When the Kupffer cells enter culture the change in the enzyme activities was quantitative and presumably the structural genes were still transcribed. The only exception to this was the occasional "repression" of arginase activity. The fact that the degree of reduction of the in vivo enzyme activities was different indicates adaptation to an environment requiring altered metabolic patterns.

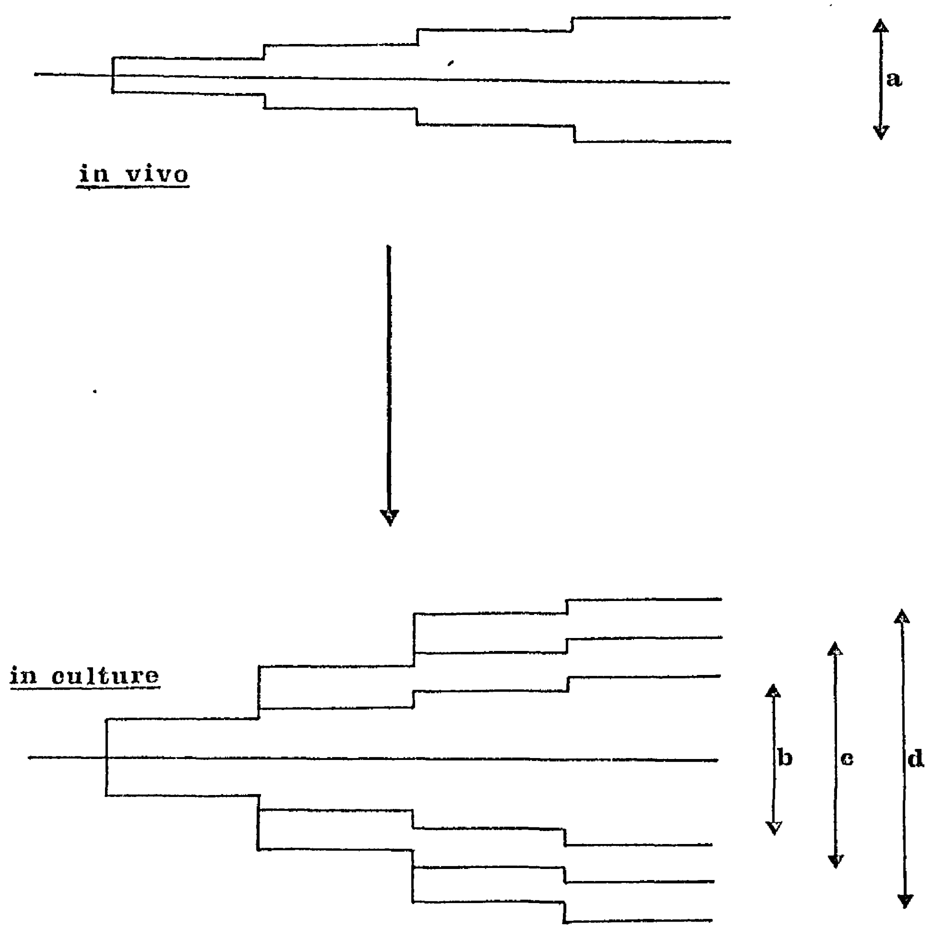


Figure 7.1:- A hypothetical model providing a basis for the phenotypic variation between Kupffer cell lines.

Successive regulation steps in an enzyme realization process *in vivo* are set with a finite tolerance in a population of cells. Upon entry into culture, the enzyme realization process is altered by changes in regulation which are achieved in a population of cells (clones) with an increased tolerance. The diagram illustrates four degrees of variation between cells *in vivo* or their clones in culture.

a - variation after four regulation steps all of 5% tolerance.

b - variation after four regulation steps, one of 10% tolerance, three of 5% tolerance.

c - variation after four regulation steps, two of 5% tolerance and two of 10% tolerance.

d - variation after four regulation steps, one of 5% tolerance and three of 10% tolerance.

The site(s) in the enzyme realization process (see Figure 1.1) at which this regulation is achieved is unknown. On the basis of economy, the most efficient way to achieve a long-term reduction in enzyme activity is to reduce the rate of enzyme synthesis. Section 1.2 indicated that there exist many points at which regulation of enzyme synthesis could be effected. It is precisely this large number of points of regulation which could lead to the phenotypic variation. Certainly, control at the transcriptional level would be possible and an explanation based on the Britten/ Davidson or Georgiev models of control would be feasible (see Davison and Britten, 1971, 1973; Georgiev et al., 1972). However, in section 1.2 it was suggested that post-transcriptional control brought about quantitative adjustments to a pattern of protein synthesis determined by the synthesis of new message. Pitot et al. (1974) demonstrated that phenotypic variation between hepatomas could be a manifestation of translational control. Translational control of catalase activity in hepatomas has been demonstrated by Uenoyama and Ono (1973) who isolated two proteins which bind to polyribosomes synthesizing catalase. One protein is an inhibitor and the other an activator of translation and control is achieved by a balance between the two. This system may be responsible for the reduction in enzyme activity and if it were common for groups of enzyme activities it would also explain correlated activities.

It is possible that the variation is established because the site(s) of regulation are several steps before active enzyme is apparent. An alteration in an early regulation step, or steps, could result in a cascade effect (Maclean, 1976) and subsequent increased variation. A hypothetical scheme is presented in Figure 7.1. One would expect that in a population of cells regulatory steps are set with a finite limit of precision, and as the number of steps in a sequence increases, a cascade effect results in increasing variation. In such a way cells possessing an identical "setting" of regulatory steps would demonstrate variation. If a regulation step is altered, for example in this study as the result of entry into culture, and the level of precision is decreased, i.e. each cell responds in an individual way, the degree of variation is increased. A change in environment as drastic as that when a cell enters culture may well be beyond the normal interpretative capacities of a particular differentiated state. When confronted with this challenge the cell may have to adapt via processes other than those permitted by its differentiated state. This new situation may mean that the response of the cell when "resetting" the regulatory steps may not be as accurate as that which would occur within the particular differentiated state. As the number of regulation steps altered by the response to the new environment increases so does the degree of variation between cells.

Such a model, although purely hypothetical, would provide a simple explanation for the variation between cell lines and is based on a cell's individual response to the culture environment. The fact that enzyme activities which demonstrate close correlations between cell lines also demonstrate similar degrees of variation suggests correlated regulation steps in the process of increasing variation. The aspect of correlation will be considered below.

A logical way to obviate any explanation for the increased variation would be to argue that the variation pre-existed in vivo. It is known that a heterogeneity of syntheses exist amongst hepatocytes (Rappaport, 1963; Tsanev, 1975) and it is possible that Kupffer cells demonstrate such heterogeneity. The results of this study indicate that 35 Kupffer cells from the one animal were relatively homogeneous with respect to the enzyme activities examined, but it is not known whether these were from the class which gave rise to the cell lines.

7.2 The correlation of enzyme activities in Kupffer cell lines

The observed variation of several enzyme activities in the Kupffer cell lines makes it possible to study inter-relationships between the activities. One would expect that there is a biological need for co-ordination and correlation of many enzyme activities. Such control would be required not only for differentiation and development but also for maintenance of homeostasis. Distinction must be made between those enzymes whose appearances in development coincide (Greengard, 1971) and those enzymes whose activities are correlated as a result of metabolic inter-relationships. Thus, there is a difference between qualitative and quantitative enzyme activity co-ordination. The relative activities of different enzymes must be correlated with each other in order to effectively integrate the regulation of intermediary metabolism and the assembly of intra-cellular structures. This correlation would be expected to be greatest in enzymes catalysing successive steps in a metabolic sequence.

The number of studies which have considered the relative activities of individual enzymes in mammalian cells is small. Dagg et al. (1964) showed that genetically determined differences in ^{14}C-uracil metabolism to $^{14}CO_2$ as studied in the intact mouse, were associated with comparable differences in activities of three enzyme activities involved in pyrimidine degradation. There appears to be a co-ordinated control either at the level of synthesis or organelle assembly for the various cytochrome proteins (Chance and Hess, 1959). Five enzyme activities involved in carbohydrate metabolism are closely correlated (see Paigen, 1971) and Meir and Cotton (1966) have suggested that this may result from synthesis of all five enzymes from one polycistronic messenger. The levels of a number of mitochondrial enzymes appear to be present in constant relative proportions in mitochondria from widely different sources (Mahler and Cordes, 1971). Knox (1972) has presented detailed analyses of quantitative correlations between enzymes in foetal, adult and neoplastic rat tissues.

The presence of constant ratios of some enzyme activities has been noted in cell culture studies devoted to medical diagnosis (Tedesco and Mellman, 1969; Tandt and Schaberg, 1973; Hall and Neufeld, 1973; Young et al., 1975). Rosenblatt and Erbe (1973) have recorded reciprocal changes in the level of functionally related folate enzymes during the culture cycle of human fibroblasts. Stern and Krooth (1975) did not observe quantitative relationships between the three enzymes of the Leloir pathway during the culture cycle of human fibroblasts.

It appears that enzyme activity relationships have not been considered in systems similar to the one employed in this study. In this system the relationships were observed at the same stage of the culture cycle in cell lines of similar or identical tissue and genetic origin.

Calculation of correlation coefficients revealed significant quantitative correlations between several enzyme activities. These correlations were stable during the culture period considered for each class of Kupffer cell line. The correlation coefficients indicate that not only pairs, but groups of enzyme activities possess consistent quantitative relationships and that these relationships may be different in primary adult, primary foetal and SV40-transformed adult Kupffer cell lines. The fact that enzyme activity correlations were observed in all classes of Kupffer cell line and that there were differences in patterns of correlations between the classes suggests that they are of biological significance and not artifacts of assay or culture methods. While the results do not give any indication as to the basis of these correlations, other studies suggest likely metabolic interactions of several of the enzyme activities.

The presence of enzyme activity correlations is interpreted as being a case of genetic regulation. Since the environment for all cell lines was assumed to be the same, and large variations in enzyme activity existed in cell lines of identical or similar origin, then we are probably not observing just simple regulation effected by substrates and products. Although this type of regulation is important in determining enzyme activity, it is superimposed upon processes involved in the production of a potentially active enzyme molecule. The basic extent of expression is defined by these processes and is probably set by the mechanisms responsible for the correlations.

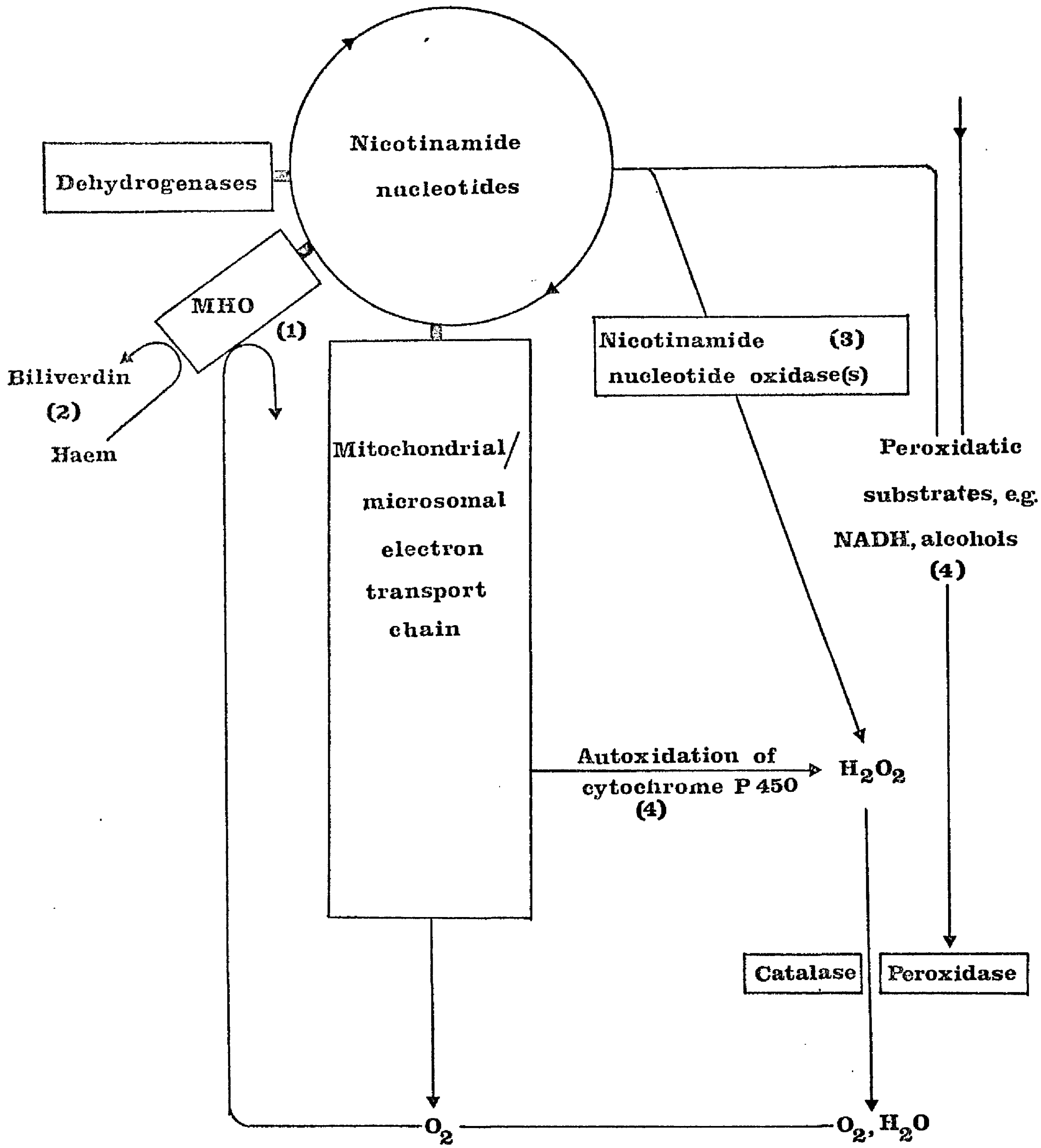


Figure 7. 2:- Metabolic relationships between dehydrogenase, MHO, peroxidase and catalase activities.

A general account of the reactions can be found in Mahler and Cordes (1971). The figures in parentheses refer to specific references. (1) Schacter et al., 1972; (2) Tenhunen et al., 1969; (3) Thurman et al., 1972; (4) Nicholls 1962.

Although the dehydrogenases catalyze diverse reactions, the common factor is their requirement for nicotinamide nucleotide cofactors. Thus, their activity is dependent on the state of the reduced and oxidized nicotinamide nucleotide pools and can influence the redox state of the cell (Krebs, 1967). Gumaa et al. (1971) have reviewed evidence which reveals association between the redox state and the activities of major metabolic pathways. Redox state alterations will influence glycolysis, gluconeogenesis, Krebs cycle activity and pentose phosphate pathway activity (Gumaa et al., 1971). Hence cofactor influences may explain a correlation between the dehydrogenase activities. It is important to note that acetyl CoA is a common product of pathways containing all the dehydrogenases considered in this study and may also effect a correlation by virtue of its position at the focus of central metabolic pathways. It is tempting to conclude that the dehydrogenase activities will influence and be influenced by the redox state of the cell and in a steady metabolic state exist in constant proportion to each other. If the redox state of the cell is taken as a unifying influence on dehydrogenase activities, it is apparent how MHO, peroxidase and catalase activities should be correlated with dehydrogenase activities. In Figure 7.2 appears a scheme of metabolic relationships between these enzymes. Such a scheme demonstrates the importance of the redox state in influencing the enzyme activities and may provide an explanation for the activity correlations. Hokama and Yamagihara (1971) demonstrated additional direct regulation of catalase or peroxidase by nicotinamide nucleotides. Nicotinamide nucleotides are particularly important in determining hydrogen peroxide levels in phagocytic cells (Paul and Sbarra, 1968). The nicotinamide nucleotide oxidase(s) catalyze the reaction and the hydrogen peroxide participates in detoxification of ingested material (Paul and Sbarra, 1968).

The correlated variations in dehydrogenase, MHO, peroxidase and catalase activities suggest a "reprogramming" of the homeostatic redox state of Kupffer cells when they enter culture. The data do not allow a statement as to whether specific correlations are the cause or result of the proposed alteration in redox state. It could be that instead of direct regulation of all these enzyme activities, they are sub-ordinate to regulation of another activity.

Arginase activity is not related to the above reactions and was shown not to be correlated with any enzyme activity studied. The fact that arginase activity was not detected in a number of primary adult cell lines suggests that it is not essential for growth in culture and that the presence or absence of this activity is under independent control to that of the other enzyme activities. Interpretation of the data for β-glucuronidase is more difficult. β-Glucuronidase does not participate in any of the above reactions and appears to possess an activity independent of the other enzymes in primary adult cell lines. However, in primary foetal and SV40-transformed cell lines, β-glucuronidase became correlated with the dehydrogenase activities. The situation is complicated by the inverse correlations in primary foetal cell lines. The basis for this change is not clear, but at the risk of being teleological, regulation of β-glucuronidase in primary foetal and SV40-transformed cell lines is probably closely integrated into the altered differentiated or developmental state.

The data on enzyme activity correlations probably provide evidence of some general co-ordinate regulatory mechanisms in mammalian cells. It is proposed that these regulatory mechanisms are associated to varying degrees. Dehydrogenase activities are regulated co-ordinately in all the classes of Kupffer cell line. Closely associated with this regulatory "unit" is associated regulation of the Kupffer cell functions MHO and peroxidase activities. Since MHO activity is eventually lost after prolonged culture of primary adult cell lines co-ordinate regulation of MHO and peroxidase is not obligatory. Both MHO and peroxidase activities are lost upon SV40-transformation without disruptions of correlations between the dehydrogenase activities. When peroxidase activity is abolished by SV40-transformation, catalase activity becomes independent of the dehydrogenase activities. Such a change may be related to

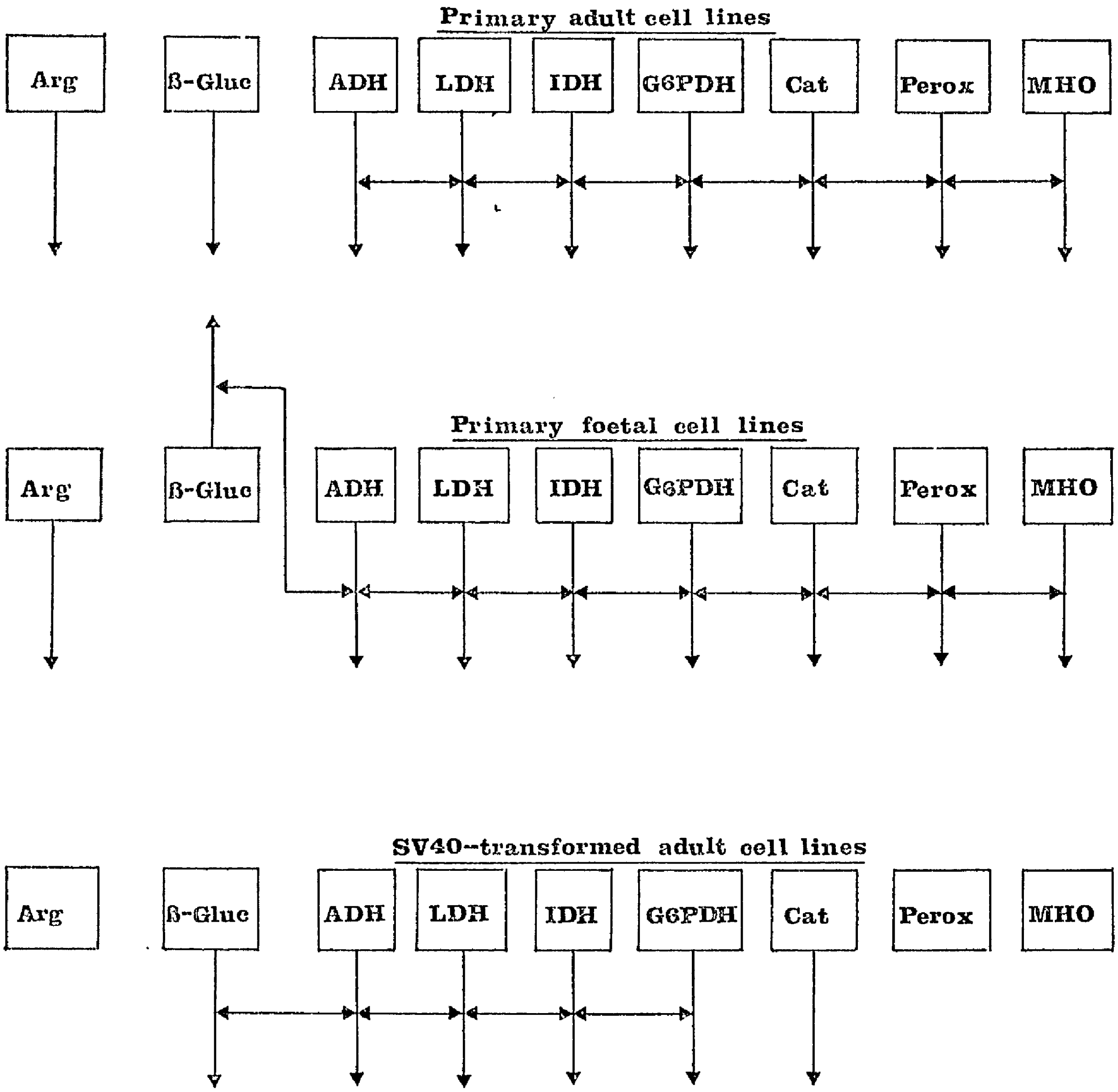


Figure 7.3:- A scheme for co-ordinate regulation of some enzyme activity realizations in cultured Kupffer cells.

Vertical arrows indicate realization of enzyme activity. Horizontal arrows indicate those enzyme activities demonstrating co-ordinate regulation. Arrows above the boxes indicate inverse co-ordinate regulation. Those vertical arrows not in contact with horizontal arrows indicate independent realization. The boxes indicate the potential for realization and do not necessarily indicate genes. The absence of vertical arrows indicates that enzyme activity is not detected and presumably that realization is not achieved.

a general change in the redox state of the transformed cell and reduced hydrogen peroxide formation because of the extinction of phagocytic activity.

Regulation of β-glucuronidase is distinct from regulation of the other activities. However, under certain differentiated or developmental states, the regulation becomes co-ordinated with that of the dehydrogenase activities. Such co-ordination may be the result of regulation associated with a particular "developmental programme" or differentiated state. This view is supported by the inverse correlations in foetal and SV40-transformed Kupffer cell lines. In contrast to these patterns of co-ordinate regulation is the behaviour of arginase activity. Arginase activity is independent of all others studied.

A scheme for co-ordinate regulation of enzyme activity realization in cultured Kupffer cells is presented in Figure 7.3. This scheme presents a possible interpretation of the data and apparently represents a new approach to the study of enzyme regulation. Whether it is valid can only be determined by future experiments. It must be stressed that the scheme does not distinguish between any of the individual steps of enzyme realization and co-ordinate regulation could be effected at any of the stages considered in section 1.2. In order to establish the validity of this type of interpretation, further experiments must be devoted towards expanding the number of enzyme activities considered as well as probing the realization process of each enzyme activity and demonstrating that the proposed co-ordinate control is exerted at the same point. The studies of Oenoyama and Ono (1973) provide an interesting starting point for such an investigation. These workers demonstrated that specific protein molecules control the translation of catalase messenger by binding to the polyribosomes responsible for catalase synthesis. It is possible that such molecules could effect co-ordinate regulation of several enzyme syntheses by binding to the relevant polyribosomes.

Isolation of Kupffer cell lines in medium containing compounds relevant to the enzyme activities may provide information on the regulation.

The presence of toxic compounds may influence enzyme activities and it would be of interest to observe whether only specific enzyme activities are affected or whether co-ordinate regulation still occurs. Examples could be the isolation of cells in medium containing hydrogen peroxide, with direct relevance to catalase and peroxidase activity and indirect effects on redox state, or dehydrogenase substrates which are either themselves toxic, or result in toxic products.

7.3 Enzyme patterns and the phenotype of SV40-transformed and foetal Kupffer cell lines.

The results of this study allow us to consider some aspects of neoplastic transformation and its relationship to the foetal state. In section 1.5 it was apparent that the extent to which neoplastic tissues resemble their foetal origins is not clear. It is the purpose of this section to briefly consider the extent to which the SV40-transformed and primary foetal Kupffer cell lines were similar and interpret the results in the light of the general theories expounded in section 1.5. Implicit in this discussion is the assumption that transformation of cultured cells by SV40 is an analogous process to neoplastic transformation _in vivo_. While available data indicate this to be true (Tooze, 1973) definite evidence for the system employed in this study must await tumourigenicity experiments.

At the stage when primary foetal cell lines were initiated, the foetal Kupffer cells possessed all the functions associated with adult Kupffer cells. Kupffer cells of the mammalian foetus apparently possess a phagocytic capacity at a very early stage in development. The emergence of phagocytic activity and presumably the other Kupffer cell functions occurs shortly after the 25 somite stage of development and coincides with the development of an extensive circulation system (Du Bois, 1963). The foetuses used in this study were at a stage of development considerably past the 25 somite stage. With respect to the enzyme activities considered in this study, the primary foetal cell lines were qualitatively identical to the primary adult cell lines.

The primary foetal cell lines possessed both similarities and differences in properties when compared with SV40-transformed cell lines. Primary foetal and SV40-transformed Kupffer cell lines often possessed lower enzyme activities, less variation in enzyme activities as culturing proceeded, shorter population doubling times and higher saturation densities when compared with primary adult Kupffer cell lines. There were also obvious differences between primary foetal and SV40-transformed Kupffer cell lines. Primary foetal cell lines possessed a different culture morphology, expressed Kupffer cell functions, demonstrated less variations in enzyme activity between cell lines and differences in enzyme activity correlations between cell lines. Primary foetal cell lines possessed Kupffer cell functions for a longer period in culture than the primary adult cell lines and also all possessed arginase activity (in 11% of primary adult cell lines this activity was not detected). In addition, only SV40-transformed cell lines expressed T-antigen. The only apparent difference between SV40-transformed adult and SV40-transformed foetal cell lines was in the greater proportion of T-antigen in the latter, in all other respects these two classes of Kupffer cell line appeared to be similar.

Thus, the Kupffer cell lines used in this study can be divided into three distinct phenotypic classes - primary adult, primary foetal and SV40-transformed cell lines. In some respects the primary foetal cell lines possessed a phenotype intermediate between those expressed by primary adult and SV40-transformed cell lines. However, the data do not provide convincing evidence for extensive reversion to the foetal phenotype after transformation of cultured Kupffer cells by SV40. It is obvious that the stage at which foetal material is isolated could greatly influence the phenotype of the resultant cell lines. If potential foetal Kupffer cells were isolated before they expressed Kupffer cell functions, then the absence of Kupffer cell functions in the resulting cell lines would provide evidence for a similarity between foetal and transformed cells. Such a consideration may explain the conflicting views on the extent of foetalism in neoplasia (see for example Knox (1972, 1974) and Wu (1973)). Since the foetal period of development spans a long period during which rapid changes occur, variations in the stage at which foetal material is studied will affect the degree of similarity between foetal and neoplastic material. In this study it would not be possible to make a true comparison with early foetal material because precursor Kupffer cells not possessing their ultimate histotypic functions could not be recognized. The most that can be said of the material used in this study was that it was foetal in origin and represented an intermediate stage in development between precursor cells and the Kupffer cells in adult liver.

Transformation of both foetal and adult Kupffer cells by SV40 produced a consistent phenotypic change. There was a production of cell lines which were qualitatively identical but demonstrated quantitative differences with respect to the enzyme activities studied. The qualitative changes in enzyme activities brought about by SV40-transformation can be seen in Figure 7.3. SV40-transformation resulted in the loss of arginase, MHO, peroxidase and LDH b subunit activities. Although T-antigen is probably a product of the SV40 A gene (Tenen et al., 1975) the results of Ting et al. (1973) suggest that several host specific proteins emerge as a result of SV40-transformation. Thus while this study only described loss of specific enzyme activities, it is likely that there was also the emergence of new proteins and enzymes. It would be interesting to utilize immunological techniques to establish whether SV40-transformed Kupffer cell lines produce some unique proteins or proteins only found in foetal Kupffer cell lines.

The alterations in culture morphology and enzyme pattern brought about by SV40-transformation are not interpreted as "deranged" control. Pitot and Cho (1966) suggested that the prime lesion in neoplasia was derangement of control. While it may be a question of degree, the changes apparent in this study are considered to support the idea that neoplasia is a "disorder of normal differentiation" (Markert, 1968; Pierce, 1970;

Weinhouse, 1974) and that transformation of cultured Kupffer cells by SV40 results in a trans-differentiation. The fact that the change was always the same suggests a precise alteration of the differentiated state to produce a new cell type and not random changes in the differentiated state. In order to verify the last point it would be necessary to consider many more cell functions before the non-randomness of change was established. The trans-differentiation caused by SV40-transformation was rapidly achieved and probably occurred within 8 cell divisions of infection with SV40. The fact that apparently differentiated Kupffer cells were transformed by SV40 does not support the suggestion of Pierce (1970) that neoplastic transformation arises from a relatively undifferentiated stem or precursor cell. It is interesting to note that transformation of cultured peritoneal macrophages, cells related to Kupffer cells, by SV40 does not result in a loss of their specific functions of alkaline phosphatase and phagocytic activities until 6 months after transformation (Grabska <u>et al.</u>, 1974).

The fact that the two enzyme activities associated with Kupffer cell functions, MHO and peroxidase, were lost together could support one of the principles of neoplasia suggested by Coggin and Anderson (1974) i.e. that genes are turned on and off in sets. MHO and peroxidase could be regarded as being the products of a set of genes whose expression results in the Kupffer cell phenotype. The loss of LDH <u>b</u> subunit activity after transformation of Kupffer cells by SV40 was associated with the loss of MHO and peroxidase activities. LDH <u>b</u> subunits are associated with an aerobic metabolism (Agostoni <u>et al.</u>, 1966), a metabolic condition necessary for phagocytic activity (Paul and Sbarra, 1968). Thus it could be envisaged that LDH <u>b</u> subunit, MHO and peroxidase activities are all in the same gene set necessary for Kupffer cell function.

The mechanism by which the trans-differentiation is achieved is not known. Transformation of cells by SV40 is associated with integration of the SV40 genome into the host genome (Sambrook <u>et al.</u>, 1968; Hirai and Defendi, 1975). Such integration could produce distortions in chromatin structure and disrupt transcription of some gene sets or result in the alteration of the pattern of gene masking to allow transcription of new gene sets. Maclean (1976) has recently reviewed the subject of controlled transcription of single genes or sets of genes. A more specific action of SV40 has been implied by the studies of Anderson and Martin (1976) who demonstrated that the SV40 A gene responsible for T-antigen expression (Tenen et al., 1975) was essential for the maintenance but not the initiation of the transformed state. Therefore it seems that integration of SV40 DNA into the host genome results in an instability of the previous differentiated state, followed by a trans-differentiation and subsequent stabilization and maintenance of the transformation by integrated SV40 A gene products.

With respect to the Kupffer cell phenotype, transformation by SV40 results in changes in two separate controls. Firstly, one in which genes are inactivated or activated as described above, and secondly, one in which there is a change in the activity of the products. The basis for the quantitative alteration of the Kupffer cell phenotype is not clear. However, the discussion presented in section 7.1 and the model in figure 7.1 could provide an explanation. The alterations in enzyme levels after transformation by SV40 could be the direct result of a new "programme" imposed by the trans-differentiation, or a secondary adaptation to a greater energetic commitment to mitotic proliferation and increased intercellular contact brought about by the change in culture morphology. With each SV40-infected cell responding to the trans-differentiation with only finite precision, the resulting alteration in control of enzyme realization could produce an increased variation in enzyme activities between the cell lines (see Figure 7.1).

It is considered that the decline in several enzyme activities after transformation by SV40 is a different process to the decline after the entry of a freshly isolated Kupffer cell into culture. The decline of enzyme activities apparent when a Kupffer cell entered culture was probably due to an environmental change and the cell interpreted the change within the limits of the "programme" determining the Kupffer cell differentiated state. Since the environment presumably remained constant, the decline in some enzyme activities after primary Kupffer cells had been transformed by SV40 was probably due to a change in differentiated state.

7.4 Karyology of the Kupffer cell lines

The final aspect of Kupffer cell line variation to consider is that of karyotypic variation. The distinctive karyotype of the Chinese hamster permitted identification of all normal chromosomes. In all categories of cell line, chromosomes with abnormal morphology were rare and did not appear to exhibit a consistent pattern of change. The majority of chromosomes in both primary and SV40-transformed cell lines were of normal morphology. Since the techniques of chromosome examination employed in this study do not allow detection of duplication or deletions of small segments of chromosome material, such an observation is only a crude measure of the gene composition or fidelity of the chromosomes. Although only a few cell lines were examined from each class, for the purpose of this discussion they will be considered as being representative of their class.

Most primary and SV40-transformed Kupffer cell lines were diploid for a considerable number of population doublings. For each class of Kupffer cell line the diploid karyotype was modal and the individual proportion of cells with other combinations of chromosomes was small. Such a situation supports the observation of Terzi (1972b) that Chinese hamster cell lines have a strong tendency to maintain a modal chromosome number equal to the euploid one characteristic of the species.

The results of this study suggest that there was no difference between the degree of diploidy in primary and SV40-transformed Kupffer cell lines. After 90 population doublings 69% of primary adult Kupffer cells were diploid. After 80 population doublings since infection with

SV40, and a total of approximately 106 population doublings in culture, 67% of SV40-transformed Kupffer cells were diploid. These values are means for all cell lines examined in each class.

The cause of the gradual decrease in the proportion of diploid cells in primary and SV40-transformed cell lines remains obscure. In the primary Kupffer cell lines, most of which appeared to possess a finite lifespan, the decrease may have been the result of the ageing process. Thompson and Holliday (1975) suggest that chromosome changes in cultured human fibroblasts are a secondary consequence of the cellular deterioration which leads to ageing. That such a process is the cause of deviation from diploidy in SV40-transformed Kupffer cell lines is unlikely. The SV40- transformed Kupffer cell lines survived long periods in culture and were probably capable of indefinite growth in culture. The SV40-transformed cells may tolerate chromosome change such that many different genome combinations are compatible with growth in culture. A hypothesis could be advanced that the increased karyotypic variation in SV40-transformed Kupffer cells is the result of relaxed selection against some rearranged genomes, and thus producing a cytogenetically polymorphic cell population. With reference to "established" cell lines Terzi (1972a, 1974) has proposed a similar hypothesis and suggests that the polymorphism is achieved by selection for fastest growth and restrictions imposed by the mitotic apparatus. An alternative hypothesis to explain karyotypic variation after extended periods of culture of SV40-transformed Kupffer cell lines could be founded on abnormal cellular control. The two hypotheses are conceptually different and it would prove difficult to obtain evidence for either one. Through cloning experiments it may be possible to determine whether SV40-transformed Kupffer cells have an increased rate of karyotypic change and whether various chromosome combinations are compatible with growth in culture.

It is generally considered that transformation of cells by oncogenic viruses and particularly SV40, results in extensive changes in chromosome number and morphology (see Defendi, 1966; Lehman and Defendi, 1970; Ponten, 1971; Lehman, 1974). Whether the chromosome changes are the cause or the result of transformation is not clear. It has been suggested that the initial step in transformation is the formation of polyploid cells (Lehman and Defendi, 1970; Hirai et al., 1971; Lehman and Bloustein, 1974). Lehman and Defendi (1970) and Hirai et al. (1971) observed that infection of Chinese hamster embryo cells with SV40 resulted in unscheduled DNA synthesis within 48 hours of infection and that this produced polypoid cells. The near diploid cells present in an SV40-transformed cell line are thought to arise from multipolar mitoses (Lehman and Boustein, 1974). However, the results of this study indicate that diploid cells predominate in SV40-transformed Kupffer cell lines and polyploid or near polyploid cells are not frequent (< 10% of all cells). It is possible that eventual chromosome changes had not been produced within the period of study. Girardi et al. (1966) have noted that there may be a long delay in chromosome change.

That the SV40-transformed Kupffer cell lines could in fact maintain a stable predominance of diploid cells is suggested by several studies of transformation by oncogenic viruses. Stable diploid, or occasionally pseudo-diploid transformed cell lines have been obtained from Chinese hamster embryo cells transformed by SV40 (Lavialle et al., 1975) and adeno virus 12 (Brailovsky et al., 1967), and Syrian hamster embryo cell lines transformed by Simian Virus SA7 (Popescu et al., 1974), adenovirus-SV40 hybrids (Black and White, 1967), Polyoma virus (Defendi and Lehman, 1965; Yamamoto et al., 1973), herpes virus and cytomegalovirus (Nachtigal et al., 1974). Kelly (1975) obtained a diploid cell line from SV40-transformed mouse embryo cells. Thus, the results of the above cited studies and those presented in this dissertation would suggest that oncogenic virus transformation of cultured mammalian cells does not necessarily lead to karyotypic disruption and that heteroploidy and polyploidy are secondary to viral transformation.

An explanation of the different results reported from various studies is not possible. However, differences in the method of transformation and origin of the transformed material may both influence the degree of karyotypic change. Nachtigal et al. (1971) observed that differences in karyotypes after SV40-transformation depended on the tissue of origin of the cell culture. Earlier studies on SV40-transformation were based on "established" cell lines which had already undergone karyotypic change before transformation. The results of this study and those of Kelly (1975) suggest that transformation of a diploid cell line is likely to yield a transformed cell line which is also diploid. Kelly (1975) proposes that transformation by oncogenic virus may have a role in maintaining diploidy. He observed that while transformed cell lines remain diploid, the original untransformed cell line deviated to the tetraploid condition as culture continued.

The method of infection and isolation of transformed cell lines may also affect the type of cell line recovered. Infection of Kupffer cells in suspension with high multiplicities of virus might result in growth of only those cells with a diploid complement. Zur Hausen (1968) observed that many cells with virus-induced chromosome abnormalities fail to divide. Chu et al. (1966) found that cultures of cells from a patient with a tumour similar to Burkitt's lymphoma yielded only diploid cells, even though the cells with normal karyotypes made up less than 2% of the original tumour.

Alternatively, the chances of obtaining a diploid SV40-transformed cell line might be influenced by the stage at which cloning is performed. If karyotypic changes are the result, and not the cause of transformation the emergence of an altered karyotype in a cell population suggests selective growth advantages afforded by the changed genome and, hence faster growth of the altered cell at the expense of cells with a normal karyotype. The method of transformation used in this study employed a cloning step immediately after infection and thus a cell capable of division in culture did not have to compete in a population with other cells which may be the progeny of cells with a karyotype altered after infection. Previous studies which did not employ a cloning step immediately after infection may have resulted in the selection of cells which possessed a growth advantage.

With respect to the study of enzyme activity variation in Kupffer cell lines, it is concluded from the chromosome studies that gross changes in karyotype did not contribute to enzyme activity variation. When the detailed examinations of enzyme activities were conducted in primary and SV40-transformed Kupffer cell lines, it was during a period when the cell lines were diploid. The difference in enzyme activities between primary and SV40-transformed Kupffer cell lines were not associated with any obvious karyotypic change. Although the chromosomes of primary and SV40-transformed Kupffer cell lines appeared identical, more extensive studies are required before the possibility of re-arrangements can be excluded. Detailed banding analyses performed by Sachs and his colleagues (see Sachs, 1974) have revealed that malignancy of polyoma virus transformed Syrian hamster cells is controlled by specific chromosome segments, Their success suggests that it may be worth conducting a large study of chromosome banding patterns in primary and SV40-transformed Kupffer cell lines. Such a study may indicate whether specific chromosome segments are involved in SV40-transformation or the enzyme activity changes.

7.5 Conclusion

Central to this study has been the question of the use of cell culture systems for dissection of the complex regulatory phenomena present in the intact animal. The system utilized in this study would appear to be of potential value in the study of enzyme regulation and more general aspects of differentiation and neoplasia.

Several Kupffer cell lines can be isolated from a single Chinese hamster and cultured for a considerable period. These Kupffer cell lines, whether of foetal or adult origin, express Kupffer cell functions and can be transformed by SV40. The methods employed in this study demonstrate that it is relatively simple to isolate cell lines derived from the one cell type and animal, but which demonstrate significant quantitative differences with respect to enzyme activities. These quantitative differences are thought to be due to epigenetic variations and when several cell lines are considered, demonstrate a continuous distribution. The use of micro-techniques for the assay of enzyme activities in single cells or small numbers of cells can expand the range of observations. Not only is it possible to assay enzyme activities in periods immediately after entry of cells into the culture environment but it is also possible to describe the variation of enzyme activities between cells within a cell line.

The studies presented in this dissertation are of a preliminary nature. The ranges and distributions of variation of some enzyme activities have been described and the evidence suggests that there is coordinate control of some enzyme activities. The fact that several cell lines originating from the one cell type can be isolated from the same animal and show several fold, stable, differences in enzyme activity makes such material of potential value in the study of epigenetic variation and several studies are possible. For example, Kupffer cell lines at opposite extremes of an enzyme activity range could be fused together and enzyme activities in the hybrids may provide evidence of control relationships, not only for an individual enzyme activity but perhaps for groups of enzyme activities. "Micro" enzyme assays could be used to examine enzyme activities in heterokaryons immediately after fusion. Such studies could also involve fusions with SV40-transformed or foetal Kupffer cells and could consider control of both Kupffer cell functions and specific enzyme activities.

The sudden transfer of a Kupffer cell from its highly sophisticated physiological environment to the comparatively "sparse" environment of the culture system is probably accompanied by exposure of that cell to severe stress. The Kupffer cells which survive the change in environment appear to respond in individual ways and there is considerable quantitative variation in enzyme activities between the resulting cell lines. While introduction into the culture environment brings about quantitative changes in enzyme activities, transformation of a cultured Kupffer cell by SV40 brings about specific qualitative changes as well as further quantitative changes. It would appear that the regulation processes responsible for the changes after transformation by SV40 are different from those which are responsible for the initial changes when a Kupffer cell enters culture.

Since the variation described in this study arose in cell lines initiated from identical cell types with often identical genetic background, the enzyme phenotype of Kupffer cells in culture is not an accurate reflection of the enzyme phenotype in vivo. Such an observation may be of relevance when considering problems of medical diagnoses based on enzyme activities in cell lines derived from biopsy material. In section 1.6.5 it was noted that, because of enzyme activity variation, heterozygotes with expected intermediate enzyme activities cannot be accurately diagnosed in many cases. It is possible that the variation is due to the processes apparent in this study. Thus, while enzyme activity variation is of interest in enzyme regulation studies, it may limit the possibilities of medical diagnosis. Perhaps the main future for some medical diagnoses based on enzyme activities in cell lines may lie in the use of large cell line samples or in reducing the decline and variation in enzyme activities due to the culture environment. It is possible that modification of the culture environment may result in the maintainance of the in vivo enzyme phenotype.

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APPENDICES

Abbreviations:- Cat - catalase; Arg - arginase; MHO - microsomal haem oxygenase; Glu - β-glucuronidase; Perox - peroxidase; ADH - alcohol dehydrogenase; LDH - lactate dehydrogenase; IDH - isocitrate dehydrogenase; G6PDH - glucose-6-phosphate dehydrogenase.

Appendix 1

Appendix 1.1:- Enzyme activities in 130 primary adult Kupffer cell lines at three stages in culture.

Appendix 1.2:- Enzyme activities in 24 primary foetal Kupffer cell lines at three stages in culture.

Appendix 1.3:- Enzyme activities in 65 SV40-transformed adult Kupffer cell lines at three stages in culture.

In appendix 1 the enzyme activities are arranged in columns. In the columns, numbered left to right, column 1 identifies the cell line; columns 2, 3 and 4 present replicate enzyme activities after 26 population doublings; columns 5, 6 and 7 present replicate enzyme activities after 33 population doublings; columns 8, 9 and 10 present replicate enzyme activities after 40 population doublings. For the primary cell lines, the population doublings were since isolation and for SV40-transformed cell lines, the population doublings were since infection with SV40. The replicate enzyme activities were determined on the one cell extract. All enzyme activities are specific activities (moles/min./mg protein), and the units are specified at the beginning of data for each enzyme activity.

Appendix 1.1

Cat.(x10^{-4})									
001	197	203	212	109	121	107	191	213	187
002	458	472	441	421	403	441	361	324	378
003	468	483	452	351	341	362	208	221	198
004	317	316	314	220	216	230	109	116	104
005	397	383	398	213	226	210	133	130	136
006	448	440	453	304	307	296	197	196	198
007	350	361	363	337	329	342	141	137	145
008	267	272	261	184	193	176	163	167	159
009	275	283	264	209	196	213	170	163	177
010	237	221	246	173	179	163	112	117	108
011	517	503	521	380	391	372	335	334	337
012	335	352	321	189	193	174	134	141	127
013	679	673	682	572	561	579	425	421	429
014	361	353	364	364	372	343	227	219	246
015	363	371	359	188	179	191	115	121	110
016	213	223	207	157	152	163	157	151	159
017	173	181	169	175	173	177	130	126	137
018	333	327	335	217	219	215	163	161	165
019	206	217	193	113	117	108	141	145	136
020	582	593	574	439	401	462	331	303	341
021	409	413	403	343	332	351	262	267	254
022	383	379	386	336	330	339	164	167	159
023	538	546	518	418	409	420	369	347	321
024	254	264	248	135	149	130	127	126	129
025	266	260	269	207	201	215	175	179	169
026	445	432	458	232	216	240	134	138	130
027	246	243	249	137	132	139	132	130	136
028	641	663	641	526	529	543	479	485	476
029	632	647	621	485	474	493	401	417	398
030	479	463	481	450	434	456	373	384	370
031	647	648	657	445	451	439	327	320	334
032	612	610	628	598	592	603	460	478	483
033	388	387	384	342	345	340	220	218	225
034	431	442	430	397	396	399	355	350	359
035	540	547	533	463	468	461	323	328	318
036	337	337	339	243	248	236	157	158	159
037	397	395	401	287	285	289	170	163	175
038	526	528	531	353	358	347	230	241	227
039	379	378	382	357	353	360	286	289	284
040	505	503	510	387	386	385	260	261	265
041	492	482	482	444	461	459	275	274	276
042	200	209	198	117	113	119	132	136	133
043	415	416	410	297	290	299	247	246	248
044	219	213	220	104	109	100	155	149	157
045	392	387	395	264	268	259	126	129	120
046	428	438	420	272	274	268	217	213	219
047	619	623	619	595	590	597	549	542	547
048	423	430	420	353	353	357	258	252	259
049	175	183	170	172	172	174	170	170	179
050	553	558	542	478	483	471	435	435	424
051	419	410	423	370	370	375	300	308	301
052	271	264	278	201	213	197	133	135	134
053	372	377	368	326	331	316	304	305	312
054	401	413	398	376	381	370	334	337	330
055	101	107	096	107	105	109	123	124	120
056	325	331	320	228	227	231	210	230	220
057	235	242	231	195	194	198	172	171	174
058	253	248	258	164	167	163	145	141	147
059	464	472	460	417	423	410	389	374	391
060	322	331	320	139	139	142	157	156	159

061	514	534	498	456	452	460	383	387	275
062	443	438	447	305	300	403	161	169	157
063	283	287	276	183	184	180	160	165	158
064	296	291	294	219	217	220	176	169	178
065	564	583	562	404	408	396	320	331	315
066	424	420	429	317	312	320	195	186	197
067	633	634	638	467	462	470	347	332	350
068	357	356	355	257	259	252	215	216	212
069	375	361	378	362	368	357	275	282	272
070	342	361	340	195	183	199	134	130	138
071	437	429	441	382	387	380	302	313	295
072	393	399	384	329	336	315	227	218	230
073	374	361	379	203	212	198	167	172	160
074	406	413	395	289	286	292	182	180	185
075	390	391	393	348	345	349	237	235	240
076	344	346	344	229	219	235	120	123	117
077	437	435	440	339	336	342	253	250	255
078	405	401	407	360	361	367	305	302	308
079	394	393	395	372	370	375	262	271	256
080	348	351	339	191	193	187	148	142	151
081	401	402	397	330	334	341	247	246	248
082	439	449	427	314	326	307	235	237	230
083	374	362	381	237	246	221	123	127	119
084	130	132	130	171	179	169	175	173	178
085	359	353	362	250	253	247	266	264	273
086	217	213	220	143	144	142	107	104	112
087	587	574	590	513	512	534	379	378	381
088	213	227	209	142	145	142	117	113	124
089	136	134	139	128	128	129	142	140	145
090	141	145	137	090	093	087	132	135	127
091	120	127	113	121	122	122	127	123	128
092	294	330	301	125	127	123	137	133	139
093	687	691	672	536	521	540	547	532	550
094	442	438	447	375	362	378	286	279	287
095	175	178	170	149	138	152	174	173	177
096	452	461	449	399	386	401	305	312	300
097	374	373	381	330	332	328	160	163	160
098	396	392	399	321	336	321	147	143	145
099	412	413	407	392	393	388	345	339	340
100	396	398	372	354	346	361	220	216	213
101	640	637	645	463	467	459	372	383	378
102	510	513	521	382	380	383	273	286	284
103	321	331	316	274	274	283	213	194	201
104	334	324	348	251	256	241	186	191	153
105	637	630	633	516	507	523	430	461	440
106	516	523	501	426	436	420	352	352	360
107	180	172	186	178	163	181	156	154	149
108	234	242	251	192	190	195	175	173	178
109	373	323	391	309	302	314	216	214	219
110	192	193	191	175	172	178	161	165	157
111	396	363	384	350	356	341	272	275	263
112	386	371	392	331	336	324	247	248	246
113	334	335	321	296	294	301	213	226	204
114	486	473	492	436	412	421	405	403	409
115	172	173	178	174	173	176	155	150	159
116	207	210	205	192	198	187	184	189	173
117	274	263	241	256	241	268	217	212	223
118	614	628	604	476	463	481	436	426	418
119	436	438	421	375	387	361	342	363	334
120	142	149	153	126	127	123	114	113	114

121	171	186	183	150	157	153	145	146	147
122	274	287	298	256	241	258	234	228	241
123	439	457	461	416	403	419	372	363	381
124	373	343	371	326	336	341	259	258	261
125	246	238	212	227	230	220	220	223	218
126	192	197	183	178	174	179	150	143	139
127	179	164	171	153	153	156	136	138	134
128	235	243	251	219	218	220	217	217	220
129	329	337	341	317	309	319	262	263	271
130	597	584	572	531	534	527	546	536	526

Arg.($\times 10^{-8}$)									
001	042	046	037	032	039	025	042	049	036
002	064	067	069	060	052	063	055	051	063
003	066	058	071	055	052	058	042	046	050
004	052	053	061	043	047	038	032	037	024
005	059	063	052	042	049	047	034	027	038
006	063	069	056	051	053	050	041	046	038
007	099	103	096	052	059	046	030	020	039
008	000	000	000	000	000	000	000	000	000
009	098	094	090	068	053	051	044	042	047
010	043	052	038	039	036	044	030	038	025
011	102	106	097	089	073	091	054	046	059
012	051	059	046	039	032	041	033	039	027
013	120	118	109	105	113	094	096	103	087
014	000	000	000	000	000	000	000	000	000
015	000	000	000	000	000	000	000	000	000
016	041	032	048	036	040	041	030	036	038
017	039	031	042	040	051	039	036	048	037
018	053	061	045	041	051	038	034	032	036
019	042	048	036	033	037	028	037	030	035
020	114	106	113	080	084	076	065	063	059
021	103	097	113	067	074	061	041	043	038
022	098	094	103	051	056	048	039	038	041
023	000	000	000	000	000	000	000	000	000
024	086	094	084	059	063	054	029	025	022
025	045	046	048	043	040	035	042	036	047
026	089	096	081	050	056	044	038	042	031
027	000	000	000	000	000	000	000	000	000
028	072	084	069	041	036	044	030	027	034
029	091	087	093	050	056	048	049	056	045
030	066	069	060	035	030	041	021	025	017
031	075	069	082	044	043	047	027	029	026
032	119	109	123	086	084	096	050	063	058
033	057	062	056	051	050	056	043	045	038
034	061	076	054	039	049	027	023	026	020
035	077	072	083	043	047	038	025	029	021
036	096	092	100	064	066	059	045	049	041
037	092	102	091	060	050	070	042	049	036
038	101	115	093	067	078	056	040	050	030
039	109	115	096	065	076	054	042	053	041
040	105	103	109	071	086	064	057	055	059
041	107	116	104	066	071	059	044	040	049
042	041	048	040	033	039	026	033	045	023
043	084	086	081	046	049	043	026	034	020
044	043	032	047	041	049	036	028	027	034
045	080	089	074	053	058	047	031	037	029
046	094	096	090	052	058	046	047	042	056
047	083	080	084	081	075	086	072	079	064

048	106	112	096	072	084	065	059	062	051
049	029	036	022	025	035	021	023	031	025
050	111	118	108	092	086	094	063	072	053
051	113	123	106	081	097	074	068	073	061
052	051	062	043	042	041	045	034	038	028
053	104	110	096	070	080	064	056	059	052
054	095	099	093	053	058	049	033	030	037
055	098	103	095	064	068	060	092	097	086
056	050	056	044	046	050	040	042	047	038
057	046	050	040	041	047	035	039	043	035
058	043	045	040	040	046	037	035	038	027
059	076	070	083	041	046	038	031	035	026
060	042	049	037	041	044	038	037	039	034
061	092	095	087	051	056	049	048	054	042
062	036	039	046	036	027	048	036	026	041
063	043	046	040	041	041	053	037	030	042
064	048	046	052	042	049	037	038	030	045
065	096	092	098	054	059	043	034	038	028
066	103	109	097	076	072	079	052	056	049
067	100	113	095	088	093	081	052	063	047
068	047	053	041	046	049	040	042	046	038
069	076	079	074	047	053	041	023	016	029
070	042	051	039	037	042	030	034	027	038
071	106	113	104	062	054	067	045	043	048
072	048	054	042	046	050	041	044	043	047
073	000	000	000	000	000	000	000	000	000
074	108	096	112	075	085	071	051	065	043
075	108	101	111	074	080	068	050	054	046
076	000	000	000	000	000	000	000	000	000
077	094	096	090	058	062	053	037	035	040
078	110	112	111	082	086	077	064	058	065
079	117	110	123	085	076	089	060	065	060
080	045	049	041	039	044	032	036	039	031
081	107	103	109	073	070	077	058	050	063
082	096	098	090	059	054	063	038	036	041
083	042	044	039	039	045	033	032	036	027
084	049	044	053	045	040	050	041	043	039
085	075	074	078	047	043	051	024	026	019
086	110	116	103	096	098	093	078	072	084
087	063	068	057	030	036	024	018	012	021
088	089	085	093	056	050	059	032	037	026
089	041	043	037	038	030	041	034	027	037
090	097	099	094	066	060	071	034	036	030
091	041	042	045	039	032	041	035	033	038
092	047	040	052	039	036	042	035	030	039
093	085	080	089	082	086	077	078	073	084
094	096	099	092	055	057	050	035	043	031
095	091	098	087	069	064	073	041	047	035
096	103	100	107	083	086	080	055	059	049
097	043	047	039	038	032	043	036	039	031
098	104	096	107	078	070	085	054	050	057
099	000	000	000	000	000	000	000	000	000
100	046	049	041	043	047	037	042	048	036
101	117	112	112	087	080	093	051	056	050
102	070	076	063	045	048	040	022	018	025
103	047	040	051	042	043	040	042	047	037
104	050	055	045	045	047	040	029	039	021
105	064	068	060	031	043	024	020	015	023
106	079	072	084	042	049	037	032	038	027
107	088	082	093	053	050	057	039	035	036
108	000	000	000	000	000	000	000	000	000

109	047	041	053	045	056	040	040	045	035
110	099	103	094	069	074	063	027	020	031
111	098	093	104	056	050	062	036	030	039
112	090	098	081	068	061	075	040	030	046
113	000	000	000	000	000	000	000	000	000
114	121	117	118	097	096	090	078	074	083
115	000	000	000	000	000	000	000	000	000
116	097	099	092	063	069	053	031	045	027
117	046	042	053	040	049	035	043	047	035
118	112	109	103	094	097	090	067	060	073
119	105	101	109	063	057	067	046	051	040
120	000	000	000	000	000	000	000	000	000
121	095	097	091	065	068	061	044	047	036
122	097	099	092	050	059	042	039	043	036
123	110	114	102	096	099	092	069	073	061
124	093	097	090	057	063	051	037	032	043
125	049	055	040	046	051	040	042	046	038
126	000	000	000	000	000	000	000	000	000
127	000	000	000	0000	000	000	000	000	000
128	000	000	000	000	000	000	000	000	000
129	079	083	074	048	056	041	025	031	019
130	082	083	079	041	045	034	027	021	031

MHO ($x10^{-12}$)

001	013	014	013	009	008	008	014	016	012
002	019	017	019	019	019	020	018	017	016
003	015	016	015	016	016	016	014	013	014
004	017	019	016	014	015	013	008	008	009
005	019	020	019	014	013	014	010	010	010
006	013	013	012	010	011	012	010	009	010
007	018	017	018	016	015	017	011	010	010
008	016	016	017	012	010	011	010	013	009
009	016	017	014	014	014	015	012	012	012
010	015	016	014	013	012	012	008	009	008
011	017	017	018	017	016	017	016	016	015
012	018	017	018	013	013	013	010	008	011
013	023	022	021	022	021	020	021	021	022
014	012	012	013	012	012	013	012	012	013
015	019	018	020	014	016	015	010	011	011
016	014	017	014	011	011	011	011	010	010
017	014	013	014	014	015	013	014	015	014
018	017	017	018	014	016	015	011	010	011
019	013	014	013	010	013	009	011	013	010
020	020	020	021	019	018	019	017	016	017
021	016	015	018	017	016	017	014	014	013
022	020	022	021	018	017	018	012	012	013
023	019	019	020	016	016	017	017	015	016
024	015	014	015	010	009	009	009	010	009
025	016	017	017	014	015	013	013	012	012
026	021	020	021	014	013	013	010	011	009
027	015	015	016	011	013	010	010	009	010
028	025	026	027	024	025	025	022	021	022
029	018	019	018	016	016	016	019	019	019
030	020	022	023	019	018	019	017	018	017
031	016	017	016	015	014	014	015	015	014
032	024	023	024	025	023	021	021	021	021
033	017	019	019	015	015	014	013	012	013
034	018	019	020	017	017	017	017	016	017
035	016	019	016	018	017	018	014	014	014

036	018	020	017	015	015	014	011	013	011
037	019	020	020	016	017	015	012	013	012
038	013	012	013	013	012	013	012	012	011
039	016	017	017	017	016	017	015	014	015
040	022	024	021	018	017	017	015	015	016
041	021	020	021	020	021	022	016	017	014
042	014	015	013	009	007	008	010	011	010
043	019	018	018	017	017	019	015	017	016
044	015	015	012	008	007	008	011	012	011
045	019	020	021	016	017	015	010	010	011
046	021	020	021	016	016	018	014	013	013
047	024	023	024	024	023	024	023	025	021
048	016	016	017	010	011	010	014	014	014
049	018	019	018	018	018	017	018	017	018
050	024	023	021	021	024	022	020	020	019
051	016	016	017	016	016	017	015	015	015
052	016	015	014	013	013	012	010	010	011
053	017	018	019	017	016	016	016	017	016
054	018	019	018	018	017	017	017	017	016
055	008	006	007	008	009	008	010	009	009
056	017	014	019	015	015	014	014	014	014
057	013	015	014	012	013	012	010	010	009
058	015	013	017	011	011	013	011	011	012
059	020	021	022	017	016	017	018	017	018
060	017	016	017	010	013	009	011	012	011
061	021	020	021	019	018	019	019	018	019
062	020	022	020	018	019	017	012	013	011
063	016	017	017	013	012	013	012	011	011
064	012	013	012	011	013	012	010	010	009
065	017	016	018	016	016	017	015	015	015
066	013	012	013	012	012	013	012	012	011
067	016	017	017	015	015	014	014	013	014
068	019	020	020	015	014	015	013	012	013
069	018	019	019	015	015	016	010	011	010
070	018	019	020	013	015	012	010	010	009
071	016	017	018	017	017	018	015	015	015
072	011	011	012	009	008	011	010	009	010
073	018	018	020	014	015	014	012	012	012
074	020	023	021	016	017	015	013	014	013
075	016	015	016	013	012	013	012	011	012
076	018	019	020	014	015	014	010	011	010
077	020	021	019	017	018	017	016	017	017
078	016	017	016	013	012	013	011	012	011
079	014	013	014	013	013	012	014	015	014
080	018	019	018	013	013	011	011	010	011
081	019	018	019	017	018	019	014	013	013
082	019	019	018	018	019	018	015	014	014
083	018	019	017	014	013	011	010	010	009
084	010	013	010	012	012	011	012	012	012
085	018	017	018	017	018	018	016	016	016
086	014	016	013	011	010	010	008	007	007
087	019	020	019	020	020	020	019	019	020
088	014	015	015	011	011	011	009	009	008
089	010	011	012	010	013	009	011	010	011
090	011	012	011	009	009	009	010	012	009
091	010	011	010	010	011	011	010	010	008
092	017	019	017	010	013	009	011	010	010
093	023	022	023	023	023	023	022	022	023
094	018	016	017	017	017	016	016	015	016
095	013	015	015	011	011	011	013	014	014
096	016	019	015	016	016	017	015	015	015

097	019	020	021	017	017	018	012	013	012
098	021	020	020	019	017	020	011	011	011
099	021	022	021	020	020	021	018	019	018
100	014	013	013	015	015	015	014	014	015
101	017	016	015	016	016	016	018	019	018
102	013	015	011	010	010	011	017	016	015
103	017	016	018	016	016	015	014	014	014
104	018	020	019	015	016	016	013	011	011
105	024	023	022	022	022	023	020	020	019
106	015	014	015	015	016	015	016	016	017
107	013	012	012	013	014	011	012	012	013
108	015	017	017	013	014	013	013	012	012
109	015	014	013	014	014	016	014	014	015
110	014	016	013	013	012	012	011	010	010
111	015	015	016	014	014	013	015	015	015
112	016	017	016	016	016	017	013	012	013
113	018	019	017	016	015	015	014	012	012
114	018	018	017	019	019	020	019	019	020
115	013	013	013	013	014	013	012	011	011
116	014	016	016	013	012	011	013	012	012
117	019	020	020	013	013	012	010	009	010
118	027	027	027	024	024	024	019	018	019
119	025	024	026	019	019	020	017	016	018
120	012	012	013	010	011	011	009	007	007
121	013	012	014	012	013	013	011	010	011
122	013	014	013	013	012	012	013	013	013
123	018	019	018	019	019	020	018	017	019
124	016	016	017	014	015	013	014	013	015
125	014	014	014	015	015	014	013	012	013
126	014	015	013	012	012	013	012	011	012
127	012	013	013	010	011	011	010	009	010
128	010	011	010	011	012	011	012	012	012
129	019	018	019	015	014	014	014	015	014
130	023	022	023	020	020	019	022	021	022

Gluc.($\times 10^{-12}$)

001	391	396	389	362	361	363	358	356	357
002	340	340	347	291	291	294	278	279	277
003	246	241	246	190	196	187	187	186	189
004	268	263	269	193	196	190	156	154	156
005	364	364	362	240	241	241	162	163	167
006	289	284	291	237	238	241	184	182	197
007	352	354	350	274	272	279	206	201	208
008	346	342	345	298	293	291	214	213	217
009	291	293	291	243	240	246	199	198	198
010	246	246	241	198	197	199	160	163	157
011	294	297	293	269	263	273	245	250	248
012	240	236	241	198	197	195	146	153	141
013	393	391	393	329	335	334	275	272	277
014	256	253	259	199	196	196	148	150	149
015	331	332	329	287	284	288	230	231	229
016	363	367	360	306	305	306	254	256	252
017	247	247	251	218	217	216	188	186	189
018	331	330	350	284	286	283	225	224	223
019	350	346	352	269	269	271	218	218	217
020	320	321	318	274	279	271	209	208	205
021	258	256	259	206	203	208	155	156	159
022	372	384	375	305	306	300	264	263	260

023	247	248	243	169	163	162	115	115	116
024	396	391	400	342	342	342	305	307	298
025	396	398	394	342	347	346	291	291	297
026	424	426	420	371	374	376	309	302	308
027	407	409	411	362	361	363	300	301	310
028	399	396	393	364	365	362	312	320	317
029	448	448	447	372	380	378	305	306	303
030	297	294	296	257	256	257	207	203	209
031	163	169	170	197	198	196	168	161	172
032	260	258	263	206	207	208	151	152	143
033	120	121	123	186	187	183	124	123	124
034	213	214	216	172	172	180	126	127	128
035	257	259	255	199	198	200	181	186	184
036	398	396	397	342	346	345	297	296	296
037	368	362	370	313	314	315	262	261	263
038	278	274	283	242	246	242	183	184	187
039	199	199	197	142	143	140	101	103	103
040	240	246	247	196	196	198	147	146	147
041	286	286	285	252	253	256	184	186	190
042	368	365	370	314	314	316	230	239	235
043	279	279	274	240	238	241	155	157	155
044	437	436	432	347	346	348	320	322	317
045	290	294	290	230	236	230	260	257	263
046	328	326	328	254	254	253	226	223	228
047	451	452	453	371	374	366	342	345	348
048	298	294	293	264	263	261	189	183	190
049	413	423	420	445	443	447	413	412	415
050	399	396	394	386	386	390	309	308	307
051	271	256	258	221	217	219	134	136	135
052	385	384	386	340	342	317	291	293	290
053	273	270	271	228	226	234	163	163	162
054	232	236	232	191	187	196	178	179	180
055	359	359	358	334	340	337	285	286	284
056	392	396	393	347	349	348	215	217	219
057	358	359	357	308	309	310	194	195	195
058	429	428	427	363	363	362	317	316	318
059	279	284	280	242	240	239	185	195	190
060	389	387	391	338	336	337	297	293	291
061	234	236	238	184	186	186	150	154	153
062	384	382	387	297	293	295	263	264	267
063	412	410	416	364	365	367	321	320	323
064	427	424	428	394	392	396	281	286	287
065	395	393	396	317	323	321	193	194	195
066	450	452	447	351	351	343	200	197	203
067	203	201	207	150	156	159	124	123	130
068	426	426	428	371	374	362	279	276	279
069	353	356	352	284	285	286	153	156	150
070	328	328	327	296	293	292	188	187	192
071	364	363	364	309	308	312	126	127	128
072	387	388	387	330	334	324	176	176	166
073	434	424	429	385	386	384	338	342	337
074	463	459	460	378	389	385	325	324	325
075	401	407	403	356	357	358	210	213	212
076	369	369	371	340	341	342	228	226	224
077	253	256	251	198	192	197	167	163	165
078	295	294	253	276	274	276	195	194	197
079	513	514	510	403	404	406	144	146	144
080	463	460	463	364	365	360	252	252	257
081	385	386	387	271	270	268	198	199	191
082	235	236	238	141	142	145	142	140	140

083	341	345	347	292	297	295	263	268	264
084	492	495	493	414	413	416	325	323	327
085	379	373	340	260	261	262	194	194	196
086	428	430	431	381	381	372	295	293	290
087	225	227	230	127	127	123	120	123	126
088	472	474	475	392	390	391	307	309	304
089	324	325	326	278	276	273	256	254	253
090	642	643	647	503	509	512	444	443	447
091	463	480	469	415	415	416	327	331	329
092	347	349	350	293	294	297	245	245	241
093	410	419	415	364	368	360	330	332	334
094	197	196	193	153	150	152	114	116	118
095	568	563	561	482	483	486	435	439	438
096	312	314	310	235	236	231	176	174	173
097	508	506	501	410	411	412	368	363	364
098	486	487	483	392	391	397	294	293	290
099	319	317	316	303	302	305	312	312	316
100	324	329	330	276	274	276	258	259	256
101	312	304	309	221	225	227	160	160	162
102	434	436	439	304	306	309	186	185	183
103	393	396	391	321	315	319	293	291	290
104	519	514	521	490	491	497	445	440	439
105	395	390	391	325	325	324	256	259	253
106	211	213	215	178	176	177	101	107	109
107	370	371	369	262	263	264	258	256	250
108	296	295	297	286	286	286	247	246	248
109	342	341	343	245	240	249	262	259	260
110	579	578	580	486	486	487	417	416	418
111	300	306	307	229	223	225	181	181	180
112	263	261	262	263	260	261	196	190	191
113	384	386	381	332	334	336	252	253	254
114	245	240	237	198	197	196	109	106	109
115	491	493	490	389	384	388	297	297	295
116	409	401	406	356	357	353	221	220	225
117	435	419	429	311	318	319	294	293	297
118	313	319	314	262	263	262	205	210	206
119	320	317	322	209	204	209	149	143	150
120	672	674	673	528	523	517	513	506	517
121	614	615	617	574	576	584	537	534	539
122	318	314	319	287	286	283	221	221	234
123	294	294	290	178	163	159	134	135	137
124	295	296	294	296	292	292	202	211	199
125	346	346	341	290	294	286	226	226	228
126	476	476	482	420	425	415	338	334	337
127	399	394	396	300	297	304	256	256	259
128	472	479	485	352	357	348	244	243	245
129	368	362	367	257	256	253	167	169	168
130	484	485	485	414	416	419	412	413	415

Perox.($x10^{-4}$)								
001	084	096		047	053		081	083
002	193	205		182	190		150	156
003	202	216		153	179		095	089
004	138	145		094	096		049	054
005	171	169		093	096		058	052
006	195	197		130	140		085	089
007	149	143		135	137		061	064
008	114	131		080	086		070	074

009	121	127	085	079	074	069
010	102	114	073	068	048	042
011	217	230	163	189	141	137
012	142	153	079	067	058	053
013	282	296	243	230	174	159
014	153	170	156	163	097	089
015	155	155	074	069	053	049
016	088	093	068	064	069	061
017	073	082	071	080	054	050
018	143	151	094	093	069	070
019	089	082	062	067	081	070
020	234	250	188	193	150	142
021	177	184	142	137	110	121
022	168	142	150	139	074	062
023	229	259	179	183	168	142
024	124	136	068	074	074	068
025	142	157	108	123	087	075
026	220	247	097	083	059	055
027	107	113	058	052	057	049
028	289	306	246	271	196	203
029	274	264	200	231	176	169
030	234	236	197	190	164	132
031	300	319	189	196	147	167
032	275	323	257	212	203	174
033	174	183	149	156	127	103
034	187	169	175	168	159	132
035	239	245	208	186	152	132
036	151	169	108	123	071	072
037	168	174	125	130	086	079
038	242	231	158	149	099	082
039	164	178	159	163	120	097
040	234	257	142	168	114	100
041	186	197	189	153	123	098
042	097	096	063	068	059	047
043	180	188	127	126	101	114
044	086	089	049	042	060	043
045	183	209	117	132	061	060
046	186	197	119	134	093	086
047	280	297	231	207	227	199
048	163	190	126	117	088	080
049	087	092	076	068	074	060
050	233	254	206	196	177	182
051	180	196	162	153	147	149
052	126	142	097	089	062	082
053	183	196	141	132	127	126
054	176	184	162	149	140	139
055	052	059	050	045	055	043
056	167	172	093	094	087	086
057	102	123	084	080	079	078
058	117	117	081	096	060	058
059	221	256	193	174	147	153
060	147	162	061	063	065	060
061	227	234	190	186	166	149
062	187	190	139	154	074	073
063	126	136	081	080	069	062
064	127	156	093	084	074	086
065	250	271	183	193	134	136
066	196	234	148	162	086	089
067	265	279	213	200	148	169
068	156	192	130	121	106	114

069	140	151	143	135	119	120
070	145	167	082	079	058	064
071	187	226	162	173	104	132
072	167	194	141	137	086	095
073	169	186	085	080	066	071
074	184	200	123	108	087	093
075	160	186	137	126	068	068
076	141	158	097	086	047	049
077	185	230	141	151	102	143
078	197	199	149	152	133	149
079	171	196	168	168	114	100
080	145	162	087	080	063	075
081	196	215	142	134	096	106
082	187	184	146	139	096	107
083	169	193	106	100	055	059
084	064	092	068	068	067	069
085	142	170	101	103	103	107
086	105	132	063	091	048	062
087	264	260	231	218	171	186
088	101	120	069	062	051	058
089	056	059	059	047	060	066
090	067	069	062	062	065	060
091	052	074	049	058	047	043
092	136	154	059	050	058	056
093	303	310	226	217	217	206
094	186	197	165	143	118	100
095	080	094	058	053	062	055
096	217	216	186	159	141	127
097	162	148	137	142	080	096
098	164	179	130	124	041	059
099	186	185	172	162	139	117
100	167	175	155	140	100	139
101	281	261	213	243	179	203
102	224	256	176	149	122	137
103	149	152	127	131	108	094
104	146	137	126	107	080	080
105	296	268	217	221	184	193
106	202	207	196	159	143	152
107	078	094	077	086	058	052
108	094	098	084	080	078	081
109	159	171	127	134	096	116
110	081	081	094	079	064	072
111	174	171	147	131	126	124
112	169	194	142	121	107	124
113	146	158	127	122	106	103
114	223	223	196	207	172	149
115	096	098	078	079	066	069
116	094	100	094	069	077	074
117	123	091	114	086	089	043
118	274	316	219	204	180	147
119	187	156	164	172	145	168
120	063	069	060	057	047	049
121	079	076	074	070	068	063
122	128	146	114	130	097	104
123	186	194	178	164	156	149
124	160	170	158	163	104	097
125	103	097	091	083	087	084
126	086	097	082	079	070	069
127	089	087	066	063	059	052

128	097	098		090	087		089	087	
129	145	179		142	136		113	089	
130	254	241		207	213		215	209	

ADH.(x10^{-9})

001	153	156	152	145	143	148	105	107	103
002	165	163	168	156	153	157	147	146	148
003	156	157	153	148	146	143	127	123	130
004	094	094	097	109	108	110	103	104	102
005	115	113	118	107	106	107	098	097	099
006	093	092	091	086	083	087	105	103	106
007	115	115	117	081	082	081	111	112	110
008	184	183	186	094	095	095	115	114	118
009	133	132	133	130	132	129	123	124	121
010	118	119	115	084	083	087	096	098	093
011	155	154	156	141	139	144	145	147	146
012	099	094	103	090	091	092	097	094	094
013	170	172	169	167	163	161	159	159	154
014	124	123	121	112	113	111	110	109	110
015	129	129	127	119	118	117	116	116	116
016	140	142	138	138	138	137	123	124	123
017	124	126	122	112	118	112	118	114	123
018	123	123	124	112	110	109	119	118	121
019	127	128	129	119	118	123	114	114	113
020	162	158	165	150	150	151	149	146	151
021	142	145	141	120	122	118	124	125	121
022	126	127	126	121	121	121	126	126	128
023	165	162	168	123	125	120	119	118	117
024	098	094	102	094	093	098	095	098	093
025	135	134	133	134	137	133	127	123	124
026	097	098	096	098	095	099	092	093	098
027	126	123	129	100	104	107	098	098	096
028	175	172	178	168	163	169	160	158	157
029	188	189	188	158	159	156	145	142	141
030	143	141	146	137	134	136	125	124	126
031	170	172	167	158	156	152	141	143	141
032	175	178	172	169	168	166	162	163	163
033	130	132	127	139	132	141	127	123	131
034	189	193	184	179	179	182	165	165	165
035	158	153	158	140	142	138	142	141	139
036	143	142	145	108	108	106	106	108	104
037	151	152	150	137	132	141	116	118	115
038	127	123	128	113	115	110	118	113	123
039	146	142	146	137	136	132	132	121	132
040	136	136	138	123	124	120	111	119	109
041	150	151	150	121	124	119	120	120	121
042	132	135	127	137	136	137	121	123	124
043	155	158	151	142	148	140	132	121	123
044	119	118	121	090	091	094	103	106	101
045	129	123	133	118	119	117	117	117	116
046	132	132	131	143	142	142	121	124	121
047	192	194	192	189	186	192	180	180	189
048	139	138	146	123	126	119	126	124	128
049	119	121	117	118	123	117	107	106	103
050	178	174	178	162	164	160	151	152	158
051	169	168	170	165	163	165	157	158	157
052	119	123	118	123	122	128	113	113	114
053	168	169	173	141	141	143	154	153	156
054	153	152	155	149	150	148	143	147	140

055	125	128	123	119	118	117	110	115	104
056	146	147	146	134	136	130	129	126	129
057	126	124	128	110	112	111	115	115	115
058	096	098	097	093	094	095	099	096	104
059	164	163	161	157	156	159	153	154	156
060	118	119	120	104	102	106	107	108	106
061	142	143	146	136	138	136	147	147	149
062	125	123	125	107	105	109	104	106	102
063	096	098	094	103	104	106	109	104	108
064	123	122	121	110	112	111	123	122	123
065	174	176	172	156	154	158	144	145	145
066	144	148	141	136	138	142	128	131	127
067	160	160	161	156	155	151	151	151	153
068	148	143	148	136	137	139	127	128	127
069	145	139	132	131	133	132	134	134	135
070	110	113	112	105	105	107	106	107	108
071	152	153	154	127	123	126	148	148	148
072	133	132	132	138	137	136	125	127	126
073	118	118	119	116	114	116	105	106	104
074	135	132	131	131	132	132	127	128	130
075	164	163	162	146	147	145	135	132	138
076	109	109	110	083	082	081	082	081	084
077	162	163	164	148	148	146	136	134	136
078	132	133	132	139	137	141	133	133	130
079	153	154	156	149	142	149	155	154	158
080	108	107	107	119	119	118	091	093	094
081	156	156	157	145	147	146	135	132	138
082	154	153	150	147	147	147	141	145	140
083	117	118	116	103	106	104	110	113	112
084	143	142	141	134	132	131	125	126	125
085	152	156	153	138	132	136	136	132	136
086	122	123	126	129	123	125	093	094	095
087	162	163	161	157	156	158	153	152	150
088	113	112	114	123	120	120	117	118	119
089	143	145	140	132	133	131	139	136	138
090	120	127	114	110	112	110	114	115	115
091	117	118	119	117	115	116	110	112	108
092	132	134	130	109	106	109	124	123	123
093	184	186	185	150	153	151	157	156	158
094	157	156	158	098	094	096	140	143	148
095	112	113	113	100	105	099	102	103	102
096	130	132	131	143	142	141	135	136	134
097	127	128	126	123	128	125	129	126	128
098	094	093	092	099	094	096	090	091	090
099	115	116	114	131	136	138	117	118	114
100	155	154	152	146	147	143	127	132	130
101	178	173	178	164	163	170	155	154	153
102	149	146	145	139	139	137	137	138	136
103	155	155	153	149	146	148	142	141	142
104	138	138	145	132	136	136	127	128	129
105	178	173	179	169	163	169	164	163	168
106	156	154	157	141	142	141	147	147	148
107	136	138	134	128	123	128	150	153	159
108	124	125	126	119	118	117	112	113	118
109	153	157	149	142	146	140	122	124	126
110	096	098	103	100	103	100	104	103	107
111	137	138	139	152	154	152	159	158	163
112	148	142	149	135	136	134	143	142	141
113	135	133	135	137	137	136	123	123	127
114	165	165	168	156	157	156	117	116	116

115	118	118	119	123	124	122	127	128	129
116	136	136	138	127	127	129	113	113	118
117	146	141	143	136	135	136	127	129	126
118	172	175	172	164	162	163	160	161	163
119	150	151	152	149	148	149	131	134	136
120	115	116	114	107	110	106	094	095	096
121	115	116	118	110	111	110	119	118	117
122	142	143	144	138	134	136	135	136	137
123	173	174	176	147	143	141	149	149	150
124	135	136	131	129	129	131	122	123	124
125	135	134	136	134	136	132	130	132	135
126	119	118	123	117	118	116	107	107	106
127	098	096	099	094	092	091	093	095	092
128	151	156	152	136	134	133	147	147	148
129	144	143	146	142	148	140	133	132	133
130	136	134	138	129	126	128	154	156	155

LDH, $(\times 10^{-8})$

001	525	525	527	513	514	517	438	436	439
002	578	578	579	550	546	549	516	519	514
003	550	552	548	518	519	521	409	405	408
004	364	367	364	327	329	326	240	239	240
005	581	580	583	445	446	447	294	298	289
006	493	492	492	461	462	461	421	423	420
007	347	347	348	339	339	340	321	323	322
008	678	677	678	446	443	445	349	349	348
009	442	444	441	416	415	417	374	373	374
010	336	336	335	297	292	291	294	290	293
011	696	694	695	628	627	628	546	546	544
012	413	411	414	440	442	443	298	299	300
013	611	610	611	602	602	602	559	558	558
014	437	437	438	434	434	436	376	371	362
015	275	274	273	296	291	297	232	234	233
016	301	306	303	312	313	312	305	305	307
017	163	162	163	230	231	230	263	264	264
018	326	326	328	300	306	303	314	316	314
019	351	352	352	347	349	348	294	296	291
020	574	576	572	513	512	514	509	506	508
021	471	475	471	443	443	444	436	437	438
022	408	405	406	378	378	379	330	327	328
023	578	572	576	542	542	543	512	513	515
024	270	274	276	267	271	268	260	261	261
025	431	431	433	425	426	426	392	392	392
026	259	259	258	284	286	284	273	273	273
027	390	392	394	343	342	341	291	292	293
028	628	627	629	589	584	587	572	572	574
029	748	746	748	737	736	736	549	549	548
030	580	581	580	559	555	556	528	527	524
031	620	620	621	552	552	552	471	475	470
032	654	654	652	631	633	630	579	577	580
033	424	424	424	431	433	431	408	406	407
034	573	574	575	595	595	595	537	536	536
035	547	545	548	541	541	543	527	529	527
036	481	482	480	408	408	409	327	328	329
037	543	543	544	376	376	376	368	369	372
038	428	427	428	408	401	405	377	377	371
039	469	469	467	473	472	474	464	463	464
040	605	602	604	521	526	524	356	357	356
041	520	520	522	481	484	483	442	446	445
042	364	367	364	320	320	321	278	279	278

043	548	546	547	479	479	479	446	448	443
044	445	447	447	373	373	373	328	329	324
045	391	390	391	362	362	361	295	294	291
046	397	397	396	411	412	413	401	401	402
047	783	784	785	742	743	741	648	647	646
048	451	450	450	448	446	446	432	431	431
049	390	395	390	308	307	308	268	267	268
050	664	662	661	615	615	612	584	583	582
051	552	553	552	540	541	540	461	462	460
052	368	367	368	391	391	391	285	284	286
053	437	437	437	407	406	405	452	452	453
054	551	551	553	534	535	534	489	486	488
055	335	334	336	301	302	301	290	291	292
056	493	491	492	423	426	425	409	407	407
057	425	424	423	354	351	352	372	374	373
058	449	448	447	324	321	324	270	270	271
059	582	584	582	545	546	544	526	527	526
060	487	486	487	357	356	357	339	339	337
061	483	483	484	452	453	456	524	523	524
062	398	398	397	324	326	325	317	318	317
063	462	463	465	375	372	375	345	342	346
064	364	364	367	311	310	309	374	375	374
065	638	639	640	550	551	551	489	486	487
066	427	427	428	441	445	443	425	426	428
067	568	567	563	557	557	557	521	523	522
068	518	519	518	445	446	445	404	403	401
069	509	507	506	501	502	503	497	496	495
070	385	387	387	328	326	330	290	287	291
071	523	523	524	502	502	502	464	463	461
072	554	555	552	509	507	506	443	445	446
073	363	362	362	341	341	343	323	324	321
074	358	358	359	354	354	357	376	372	377
075	573	573	574	518	516	517	429	428	429
076	178	174	178	240	243	243	223	223	224
077	572	571	573	500	504	503	447	446	447
078	467	468	469	458	453	451	468	467	468
079	427	428	429	431	431	430	410	411	410
080	508	507	506	388	387	386	281	282	279
081	550	552	549	499	491	500	442	443	444
082	405	406	406	381	382	380	424	424	425
083	519	517	523	424	424	427	275	276	271
084	497	496	495	438	439	433	390	391	391
085	522	523	521	449	449	447	447	447	446
086	396	394	396	317	318	317	216	217	218
087	634	633	633	652	652	651	528	529	531
088	270	274	272	272	272	271	234	230	229
089	287	286	288	265	261	262	250	249	253
090	324	324	321	268	267	266	297	297	291
091	342	348	346	264	265	267	271	273	270
092	268	265	271	253	254	258	274	276	274
093	749	743	747	786	782	781	623	625	628
094	568	563	561	448	448	449	463	461	462
095	374	370	372	317	315	314	336	336	332
096	473	471	473	451	452	451	463	467	468
097	358	359	362	360	361	362	334	336	332
098	365	368	369	324	325	326	286	287	285
099	573	572	575	446	448	449	478	475	474
100	548	543	545	517	519	522	407	409	413
101	652	648	649	577	576	576	534	538	537
102	468	469	462	423	428	426	487	487	487
103	491	494	492	475	473	471	444	445	446
104	530	532	531	461	462	463	407	406	400
105									

105	631	629	629	598	597	596	570	572	574
106	537	536	537	528	527	526	536	535	534
107	257	256	257	261	261	263	327	329	334
108	401	406	397	375	376	374	374	374	374
109	527	521	526	472	470	475	389	384	386
110	319	317	319	321	324	325	309	306	304
111	525	523	521	505	504	503	474	469	468
112	459	458	459	459	457	459	421	423	421
113	467	466	463	493	497	498	430	434	427
114	579	575	577	550	550	554	545	542	544
115	358	356	357	360	364	367	345	344	344
116	493	498	495	419	424	418	362	362	361
117	465	468	467	449	446	450	411	409	407
118	650	652	651	581	582	583	569	567	563
119	523	524	526	528	528	526	463	461	463
120	280	284	282	284	281	283	275	275	273
121	365	365	368	315	312	309	276	274	278
122	470	472	470	449	445	446	441	440	439
123	641	643	648	609	609	600	487	487	487
124	443	448	447	401	400	410	433	432	431
125	445	446	447	425	428	426	419	419	418
126	261	273	268	256	252	253	289	289	289
127	287	283	284	262	267	264	284	280	281
128	583	585	585	561	561	563	372	375	374
129	490	493	491	477	474	476	440	439	441
130	604	604	609	619	617	621	613	612	613

IDH. ($\times 10^{-9}$)

001	517	518	509	496	490	496	533	535	531
002	654	653	649	629	623	631	581	584	579
003	625	628	623	580	585	573	483	482	480
004	449	448	453	317	309	315	334	336	330
005	663	658	667	407	406	410	368	369	365
006	563	562	561	551	548	556	546	543	540
007	471	473	470	458	453	459	493	496	502
008	702	704	698	472	469	478	390	384	392
009	572	572	584	553	554	557	527	523	531
010	422	423	418	407	408	406	374	375	380
011	701	701	693	589	591	593	572	572	571
012	379	384	372	364	368	360	390	386	392
013	675	678	668	671	674	668	625	628	623
014	493	491	492	463	461	453	458	459	453
015	284	286	287	298	291	289	276	274	278
016	390	394	391	515	517	515	393	391	396
017	483	486	474	468	463	465	373	376	372
018	414	419	423	505	509	507	409	407	410
019	440	446	440	546	543	548	374	376	372
020	550	551	558	536	538	533	509	513	506
021	556	549	556	532	536	527	512	503	515
022	478	473	481	478	475	479	425	423	426
023	654	652	657	620	624	616	580	583	579
024	378	372	384	361	362	360	358	359	357
025	463	467	461	421	423	428	372	374	376
026	390	393	386	370	368	375	364	365	357
027	425	423	427	417	409	419	391	390	393
028	683	685	686	658	662	651	649	643	651
029	742	742	751	669	662	673	593	591	592
030	654	653	652	630	635	626	608	617	603
031	689	687	691	631	632	624	550	546	549
032	715	709	718	692	693	695	660	653	662

033	609	603	612	554	563	550	518	519	517
034	660	652	667	671	659	663	583	586	579
035	631	634	626	600	604	607	598	592	603
036	386	384	391	413	412	401	429	426	432
037	578	572	581	563	561	564	461	460	464
038	504	503	507	492	492	495	472	469	470
039	531	526	533	526	528	527	501	504	497
040	682	684	680	513	503	509	493	499	492
041	534	532	537	518	509	517	513	509	513
042	309	305	313	334	336	339	307	306	304
043	627	629	625	560	564	558	524	523	527
044	436	432	430	423	425	421	405	407	408
045	558	559	559	587	584	589	350	348	353
046	563	561	567	542	546	548	523	526	520
047	723	728	720	715	723	709	697	691	686
048	530	532	532	513	520	517	548	546	551
049	427	425	431	385	382	384	361	361	359
050	741	736	743	732	730	728	648	647	649
051	573	575	570	584	585	578	528	532	529
052	464	469	461	482	483	486	436	432	433
053	568	568	563	533	534	532	546	542	547
054	603	600	609	584	581	587	571	580	569
055	437	430	432	423	420	428	417	423	416
056	574	578	576	507	506	501	487	482	489
057	469	463	470	472	476	470	465	463	466
058	377	375	381	351	350	356	384	382	384
059	660	654	662	624	627	620	611	613	617
060	569	563	571	456	459	453	448	443	451
061	670	671	670	662	668	660	605	602	607
062	437	432	436	442	443	439	431	436	429
063	417	412	419	445	447	443	423	421	427
064	573	575	512	607	609	609	464	461	467
065	592	593	590	528	523	524	472	470	470
066	601	604	599	572	571	570	553	558	550
067	591	596	587	574	570	574	547	546	546
068	592	593	592	530	528	531	487	489	493
069	621	623	618	590	591	593	603	602	601
070	368	369	363	326	326	328	349	347	349
071	572	575	563	547	546	546	536	532	530
072	613	612	618	683	683	681	507	506	506
073	421	423	418	417	421	420	435	432	431
074	546	547	555	507	504	503	448	449	450
075	502	507	500	463	460	467	497	492	499
076	323	320	326	307	303	309	332	331	335
077	654	656	648	580	573	584	530	531	532
078	586	583	589	547	542	549	540	536	544
079	460	463	461	452	456	449	486	489	483
080	585	581	587	431	432	436	399	391	401
081	627	628	624	579	578	580	520	523	517
082	526	523	525	513	514	518	506	504	509
083	592	596	587	511	506	512	306	305	305
084	441	441	443	417	413	420	436	432	437
085	600	604	595	532	532	535	523	528	523
086	461	468	465	453	450	457	446	442	441
087	690	692	689	624	623	624	590	593	589
088	280	284	280	278	274	281	263	260	266
089	365	363	369	389	387	393	373	379	365
090	382	383	387	354	350	359	389	392	390
091	347	349	344	329	320	335	334	339	330
092	347	342	351	412	408	417	387	387	389
093	621	621	623	653	653	658	649	647	653

094	631	632	631	546	543	549	538	534	541
095	459	458	460	434	436	431	447	446	449
096	554	556	550	537	532	540	542	540	547
097	489	487	489	458	459	456	364	368	361
098	374	376	373	399	392	402	390	392	389
099	583	583	585	589	584	592	542	541	544
100	626	627	630	592	591	597	487	486	488
101	700	704	702	658	653	659	620	623	618
102	720	721	719	614	615	614	533	534	536
103	579	574	581	571	573	570	564	567	560
104	396	392	398	487	486	482	478	477	481
105	708	710	713	675	678	673	640	647	634
106	593	594	597	640	642	638	589	587	590
107	390	392	396	374	376	370	388	384	389
108	367	364	371	466	460	470	486	486	482
109	611	613	618	553	554	555	467	467	469
110	386	389	384	436	432	438	413	415	412
111	601	617	593	546	542	548	584	587	583
112	527	521	523	521	523	521	493	496	490
113	554	556	555	567	568	568	540	542	540
114	663	660	662	627	625	631	623	628	619
115	437	438	439	429	423	430	401	405	397
116	569	563	570	538	530	541	469	467	471
117	543	544	546	527	526	521	493	495	491
118	705	709	693	662	663	668	647	648	647
119	643	644	647	708	704	709	575	574	579
120	443	449	440	422	425	420	393	391	395
121	462	461	463	441	448	437	437	432	445
122	550	541	542	525	526	523	522	530	518
123	789	783	786	792	790	795	648	642	653
124	469	463	469	527	524	529	536	532	537
125	526	527	528	509	518	505	502	504	509
126	427	427	423	465	463	467	447	453	450
127	372	378	367	386	387	384	378	372	379
128	529	531	525	517	523	514	537	530	537
129	575	574	577	562	563	567	520	529	518
130	628	629	628	647	643	650	654	658	652

G6PDH. $(\times 10^{-10})$

001	068	069	070	057	056	056	065	065	063
002	096	098	097	091	092	093	085	084	083
003	090	094	092	084	086	083	068	067	068
004	045	043	044	039	038	037	036	035	034
005	097	096	091	041	040	041	046	042	043
006	081	081	082	079	079	079	076	075	074
007	069	069	062	067	065	063	065	065	065
008	075	078	072	071	071	073	068	065	066
009	074	072	073	071	071	070	073	074	076
010	058	059	058	056	052	054	053	054	055
011	084	083	085	098	099	099	093	094	093
012	052	056	054	059	060	060	047	043	045
013	101	102	103	099	098	099	092	091	090
014	071	070	071	063	062	063	058	058	058
015	031	032	034	028	027	029	024	023	024
016	051	052	051	053	054	053	050	052	052
017	028	029	029	040	040	039	044	045	046
018	068	067	068	067	066	065	064	065	063
019	061	060	061	055	052	055	057	056	058
020	103	102	101	094	095	097	102	100	101
021	081	080	081	084	086	085	065	062	064

022	065	064	066	068	068	069	064	063	064
023	096	095	097	088	087	088	084	083	084
024	045	046	045	044	043	044	043	042	042
025	069	069	070	069	068	068	071	070	069
026	053	055	054	050	050	049	042	042	043
027	040	041	042	049	048	047	047	046	045
028	105	103	104	099	096	098	093	090	093
029	106	104	102	193	190	190	098	099	100
030	095	093	092	092	090	092	086	084	085
031	103	102	101	090	092	091	077	073	075
032	106	105	106	104	102	102	094	095	095
033	071	073	072	072	070	071	055	054	055
034	084	085	084	082	083	084	087	086	085
035	083	082	081	097	096	097	084	081	084
036	056	055	054	053	050	052	050	050	051
037	089	087	088	062	063	063	051	050	051
038	072	073	072	069	068	067	070	071	073
039	082	083	082	083	084	085	074	073	072
040	098	097	096	086	087	086	057	057	056
041	065	064	063	062	060	060	068	065	065
042	064	063	063	059	058	058	060	061	059
043	090	092	091	079	078	079	073	072	072
044	072	073	071	062	063	063	054	054	053
045	049	048	049	047	048	047	046	045	044
046	126	120	124	121	121	123	103	103	103
047	097	096	098	118	112	118	089	087	084
048	061	061	061	067	063	066	054	052	054
049	053	052	051	040	042	041	031	030	031
050	130	130	127	150	150	151	098	099	099
051	081	082	081	080	084	083	083	082	081
052	061	064	063	064	062	062	048	047	047
053	072	070	071	066	063	064	075	074	074
054	088	089	089	095	094	093	082	085	084
055	055	054	056	051	052	054	049	049	050
056	081	080	081	070	073	072	067	064	065
057	053	053	052	061	064	062	056	057	055
058	061	063	061	053	054	053	045	046	047
059	095	093	091	088	089	089	086	085	084
060	044	042	043	042	041	042	047	046	045
061	104	100	103	098	097	098	100	098	098
062	060	061	063	067	068	068	058	057	059
063	057	056	054	052	053	054	044	042	043
064	060	061	059	052	050	050	063	062	061
065	105	101	104	091	090	089	071	075	073
066	071	072	072	073	073	072	071	070	070
067	093	098	096	091	090	092	086	087	083
068	084	085	087	073	070	074	067	070	069
069	071	072	070	086	085	085	077	073	075
070	048	046	047	042	042	043	046	046	045
071	086	087	087	084	083	084	076	072	074
072	074	075	075	073	070	071	065	065	062
073	060	061	062	056	058	057	054	056	055
074	070	072	071	068	063	068	063	063	063
075	063	063	062	055	054	054	049	047	049
076	063	062	061	055	056	057	039	038	039
077	078	078	078	076	074	074	074	075	074
078	070	072	071	074	073	075	079	079	080
079	072	071	073	072	074	073	068	069	067
080	062	063	061	050	052	048	043	043	042
081	091	093	090	082	084	083	073	074	075

082	098	099	099	096	094	095	082	080	080
083	082	083	082	069	069	068	046	047	048
084	078	079	079	072	073	071	075	076	076
085	085	083	084	074	075	076	074	072	071
086	065	064	063	051	050	050	035	035	035
087	109	108	107	096	095	095	087	086	086
088	045	044	045	046	046	047	038	037	038
089	059	057	057	057	056	057	040	040	039
090	065	063	066	065	065	063	061	060	060
091	063	063	062	067	065	062	052	052	051
092	054	053	052	050	049	049	043	044	043
093	103	100	101	104	102	101	088	089	087
094	094	096	094	094	094	093	087	087	088
095	074	075	074	063	064	063	058	059	057
096	082	081	080	084	083	082	070	072	078
097	077	078	075	072	075	073	067	068	069
098	048	049	050	055	052	051	042	043	042
099	093	092	091	083	086	084	086	087	086
100	089	090	090	084	083	082	066	065	066
101	106	104	105	094	093	094	087	086	086
102	093	090	092	095	094	094	090	091	089
103	080	085	081	078	077	079	072	072	073
104	058	056	052	060	060	060	065	065	064
105	106	105	104	099	098	098	094	093	094
106	073	072	073	080	081	081	089	088	090
107	064	063	065	058	059	062	060	057	057
108	065	066	066	064	065	064	059	057	056
109	086	086	085	076	074	073	064	065	064
110	057	058	058	058	056	056	043	042	041
111	088	089	088	087	086	086	090	087	086
112	070	070	072	076	073	073	073	072	072
113	059	058	057	062	060	060	066	065	064
114	095	094	094	090	089	087	096	094	093
115	060	064	063	060	061	060	062	060	060
116	080	078	077	067	066	066	054	053	054
117	076	074	075	074	073	073	066	065	065
118	106	105	104	096	094	095	094	093	093
119	078	079	079	070	071	072	077	076	076
120	047	046	046	048	047	048	046	047	046
121	065	064	063	055	056	055	061	062	063
122	078	076	077	074	074	073	072	070	072
123	105	103	104	106	102	102	104	100	102
124	079	077	078	075	076	076	068	069	067
125	073	072	073	070	069	069	068	067	067
126	057	056	057	050	052	051	049	049	048
127	059	058	057	053	052	052	042	041	041
128	099	096	097	097	094	094	104	100	098
129	082	083	082	078	078	078	071	072	071
130	096	096	095	095	096	095	097	097	097

Appendix 1.2

Cat. ($\times 10^{-5}$)

301	510	523	505	493	486	481	473	482	479
302	326	307	314	300	299	312	304	296	296
303	468	472	453	437	448	441	435	429	431
304	537	542	531	502	506	510	513	509	505
305	584	596	602	583	579	573	577	569	572
306	359	321	337	320	326	327	327	328	331
307	412	405	417	390	377	378	382	379	381
308	491	492	483	491	484	485	487	476	483
309	532	542	540	523	521	527	512	509	517
310	548	556	537	526	515	512	531	518	526
311	563	562	561	547	548	553	543	542	546
312	596	587	603	600	601	592	593	598	587
313	407	412	413	402	401	395	401	396	392
314	513	523	519	507	503	498	496	506	491
315	487	472	478	453	451	447	462	459	456
316	523	526	517	505	512	510	503	506	502
317	428	413	417	412	400	405	407	414	409
318	521	526	526	498	483	489	503	479	483
319	517	512	518	503	500	487	502	493	491
320	553	567	583	547	546	546	541	543	540
321	475	472	476	453	450	463	459	461	458
322	502	500	497	478	469	471	479	471	474
323	448	446	437	435	432	436	427	421	423
324	509	501	498	500	501	499	502	495	496

Arg. ($\times 10^{-9}$)

301	036	041	033	042	039	036	035	042	043
302	048	051	046	042	058	047	046	033	037
303	021	019	015	029	021	017	037	017	023
304	027	026	029	033	019	025	021	037	032
305	052	047	057	063	043	051	041	039	039
306	023	029	031	031	038	042	036	042	031
307	041	042	045	040	035	046	033	042	040
308	043	035	030	036	027	034	024	023	038
309	052	063	049	037	046	042	031	045	033
310	045	042	051	033	046	035	043	041	027
311	027	019	031	024	038	033	032	023	024
312	037	036	042	046	037	035	028	029	036
313	042	048	036	034	039	045	042	047	053
314	046	035	035	029	033	040	042	029	038
315	023	024	021	033	020	036	039	042	035
316	029	030	033	042	027	034	025	029	026
317	013	018	017	023	014	027	018	020	029
318	051	059	053	040	046	051	028	047	041
319	052	063	049	037	058	043	036	038	021
320	043	047	039	041	042	037	039	039	045
321	039	031	036	038	051	046	037	030	034
322	033	038	039	032	029	041	028	037	034
323	045	049	040	036	035	039	042	033	032
324	048	040	039	042	051	035	038	034	037

MHO ($\times 10^{-13}$)

301	047	051	043	043	039	045	042	048	041
302	027	021	033	025	026	019	021	018	019
303	043	050	040	039	035	043	036	037	036
304	048	054	047	046	040	049	047	049	051
305	053	056	047	054	049	055	051	045	052
306	027	023	031	025	033	020	026	023	029
307	035	038	031	038	036	031	033	033	030

308	043	049	045	043	035	037	041	037	046
309	049	052	054	047	048	040	045	043	037
310	051	052	050	052	058	051	046	046	045
311	053	057	048	054	048	053	049	050	045
312	057	065	051	060	063	061	053	057	052
313	037	038	045	033	033	038	034	037	039
314	046	051	047	044	048	039	040	037	035
315	042	047	045	040	042	038	040	039	036
316	048	047	048	042	045	046	045	049	050
317	037	033	038	036	037	035	037	032	039
318	048	045	051	041	050	045	040	043	036
319	053	049	042	046	041	047	045	049	050
320	053	051	053	048	049	051	047	054	042
321	043	046	045	046	048	043	048	055	046
322	046	041	047	045	038	037	043	049	037
323	035	038	043	036	047	041	036	035	031
324	042	046	042	044	043	048	040	044	036
Gluc.($\times 10^{-12}$)									
301	091	084	096	112	123	109	103	108	106
302	165	172	158	161	173	165	172	159	167
303	098	073	084	113	126	109	123	103	108
304	083	072	069	091	078	071	087	083	084
305	087	089	096	093	104	101	082	087	079
306	137	146	141	146	139	128	128	126	131
307	142	145	153	136	132	137	129	136	136
308	113	105	118	115	117	103	098	084	106
309	093	095	108	104	103	112	086	110	105
310	115	103	108	112	107	121	113	113	125
311	072	069	068	064	057	078	068	069	075
312	087	093	092	091	101	100	090	085	093
313	126	128	133	118	127	114	113	116	119
314	096	108	103	088	096	091	084	079	086
315	113	123	118	118	126	121	112	107	118
316	086	091	083	082	072	075	094	095	083
317	127	137	133	133	124	121	136	142	125
318	103	113	098	096	084	081	092	093	095
319	089	103	108	084	083	089	071	072	065
320	075	084	081	089	072	069	083	068	069
321	107	108	113	101	104	097	108	097	098
322	101	106	101	109	113	118	113	126	112
323	153	168	143	148	142	142	139	137	143
324	118	121	125	129	122	118	129	115	125
Perox.($\times 10^{-5}$)									
301	573	543	586	512	493	547	486	513	456
302	247	278	263	219	185	243	204	206	241
303	471	526	448	438	417	450	427	481	463
304	577	601	583	530	507	553	542	583	517
305	695	683	659	668	687	643	653	671	672
306	261	291	243	246	271	213	241	283	251
307	392	375	413	363	381	353	351	321	389
308	504	506	517	518	558	496	493	471	482
309	566	574	601	531	527	514	524	528	512
310	609	628	583	539	534	537	573	596	581
311	644	649	658	601	593	631	597	587	613
312	706	732	687	699	693	702	681	693	671
313	390	359	383	374	394	351	355	386	312
314	531	527	568	520	568	493	496	542	468
315	465	483	441	442	448	473	453	418	482

316	557	587	531	531	589	521	313	313	331
317	400	409	398	385	381	397	374	391	356
318	541	582	527	502	523	499	489	485	472
319	537	583	498	518	495	497	493	472	506
320	656	653	671	604	587	593	589	561	567
321	482	463	471	452	458	459	451	436	461
322	529	533	552	508	501	498	503	515	495
323	440	436	452	412	417	401	391	407	403
324	545	581	572	524	528	505	503	523	496
ADH. ($\times 10^{-10}$)									
301	281	274	269	263	271	253	260	263	259
302	213	238	227	224	248	236	218	207	198
303	259	263	248	245	257	253	247	263	241
304	273	298	278	268	265	272	265	279	271
305	294	290	311	283	281	268	291	294	282
306	213	197	225	234	238	243	227	241	235
307	246	268	251	257	259	268	239	245	241
308	263	238	242	275	279	282	249	263	231
309	275	286	282	283	298	272	271	265	273
310	281	296	284	253	247	261	271	284	269
311	269	273	264	271	283	261	264	271	249
312	271	283	287	265	273	255	298	291	313
313	241	241	237	235	246	251	243	264	253
314	257	271	263	268	251	253	260	278	265
315	264	253	248	258	269	271	263	262	272
316	284	293	277	275	271	274	272	265	263
317	243	251	247	237	239	227	236	238	241
318	285	283	295	255	287	272	245	258	257
319	253	271	265	271	271	279	261	258	263
320	297	299	304	305	312	305	280	289	275
321	259	254	241	251	263	281	258	263	271
322	264	291	284	252	251	247	241	258	263
323	243	247	249	257	253	241	240	245	233
324	275	281	274	281	283	281	237	249	241
LDH. ($\times 10^{-8}$)									
301	231	246	229	217	206	225	213	204	246
302	128	138	126	133	131	143	124	109	117
303	213	231	227	196	183	204	183	191	196
304	246	237	229	225	223	217	228	234	221
305	273	259	246	261	268	271	254	243	240
306	168	169	172	132	151	156	145	145	149
307	183	189	201	166	174	183	174	194	187
308	217	234	196	223	235	231	226	242	237
309	251	258	239	237	227	228	231	246	225
310	228	237	222	236	239	221	247	256	237
311	271	284	293	256	251	237	251	271	263
312	283	283	269	279	265	268	274	259	263
313	198	199	213	184	197	192	195	191	207
314	232	256	247	228	219	213	201	195	212
315	219	223	228	223	236	217	213	219	229
316	237	242	231	231	221	225	226	209	213
317	186	199	196	169	147	153	175	184	190
318	217	213	221	219	218	207	233	250	243
319	231	226	238	238	246	247	230	219	226
320	259	259	263	236	248	243	237	230	242
321	203	207	195	196	192	201	192	208	210
322	225	236	228	226	220	235	213	231	219
323	196	172	184	209	234	226	201	193	184
324	216	219	208	218	220	221	196	198	200

IDH. ($\times 10^{-9}$)

301	190	206	184	178	183	172	184	189	187
302	123	136	117	128	134	137	106	094	113
303	185	193	198	168	174	182	167	179	175
304	198	203	187	183	176	179	192	184	189
305	213	224	204	218	203	214	209	226	213
306	150	147	163	128	138	121	131	127	142
307	167	159	146	150	153	168	158	152	163
308	178	187	163	189	184	172	193	185	203
309	208	213	201	200	193	195	198	196	207
310	183	198	185	180	172	185	203	193	213
311	243	255	247	221	228	219	225	236	224
312	229	231	233	236	238	241	220	219	236
313	175	184	167	163	167	156	171	170	170
314	209	231	196	183	193	200	181	176	187
315	187	183	193	193	180	186	179	195	183
316	195	203	185	186	192	174	177	185	171
317	163	172	183	149	151	162	168	170	154
318	175	165	157	179	189	193	201	213	175
319	186	195	176	207	184	191	193	177	201
320	217	213	205	204	194	193	200	206	218
321	168	172	153	165	151	150	169	171	172
322	193	185	203	191	198	201	185	174	197
323	171	183	181	182	185	192	164	183	169
324	176	181	167	183	172	175	166	169	174

G6PDH. ($\times 10^{-10}$)

301	052	055	046	047	043	051	049	048	055
302	033	036	027	034	029	037	026	021	033
303	050	063	042	044	048	041	043	045	037
304	053	063	061	048	051	055	049	043	046
305	057	058	051	059	064	051	055	053	046
306	036	029	028	033	027	035	033	023	036
307	044	048	040	040	037	046	043	042	040
308	043	043	046	052	053	051	051	060	047
309	055	053	057	052	046	057	050	056	043
310	052	053	050	053	056	057	055	053	050
311	060	065	054	057	053	056	061	061	064
312	062	061	057	061	053	055	059	057	058
313	047	046	049	046	042	048	047	046	047
314	054	055	053	050	046	052	049	058	051
315	045	043	047	046	042	048	041	037	089
316	054	051	053	051	045	057	047	050	051
317	045	043	048	037	031	035	043	045	039
318	046	049	045	048	048	043	053	052	047
319	050	055	051	046	047	049	043	048	052
320	057	053	060	053	061	060	052	057	052
321	047	043	044	042	041	040	041	046	043
322	054	059	057	051	056	047	048	045	045
323	043	047	050	047	050	053	049	050	052
324	046	050	043	047	047	043	044	040	042

Appendix 1.3

Cat.	$(x10^{-5})$								
401	124	137	163	091	087	046	138	173	146
402	280	296	258	296	317	256	184	183	196
403	231	246	206	188	147	233	136	139	127
404	225	248	231	172	176	143	196	213	178
405	176	171	186	146	112	173	231	276	217
406	079	049	057	047	036	031	086	089	072
407	087	099	073	098	092	087	046	059	027
408	096	084	123	136	146	153	087	073	091
409	136	121	130	187	156	208	201	264	237
410	157	159	200	143	171	154	141	176	153
411	206	236	244	217	196	205	184	187	136
412	234	271	268	195	172	184	168	164	159
413	190	192	194	153	121	119	207	221	187
414	143	174	162	157	132	186	163	145	153
415	100	117	083	056	059	068	118	132	102
416	168	184	173	169	153	172	217	210	189
417	172	163	192	153	172	184	168	137	141
418	198	200	213	215	217	268	206	200	184
419	251	283	274	284	268	263	287	281	280
420	243	240	236	223	251	232	231	235	220
421	117	132	129	119	134	107	112	146	123
422	126	141	132	173	193	187	108	100	095
423	134	136	147	168	193	187	157	163	161
424	151	172	163	163	152	149	131	149	137
425	163	171	159	146	156	141	198	206	219
426	095	093	078	043	026	071	106	134	095
427	083	097	115	078	076	093	069	051	084
428	069	086	051	073	076	100	079	086	091
429	094	095	096	123	142	131	115	106	132
430	071	068	076	046	037	055	107	103	111
431	087	086	072	123	151	136	064	057	071
432	142	156	123	156	171	168	172	168	192
433	137	172	158	134	116	117	168	132	151
434	186	197	159	234	238	247	187	170	169
435	223	234	237	246	262	259	278	278	297
436	251	258	263	232	231	227	213	200	207
437	173	186	164	148	172	169	153	147	142
438	168	183	180	119	112	121	123	126	120
439	172	184	193	168	160	161	168	171	177
440	155	173	169	172	173	184	196	209	215
441	109	123	097	088	086	075	103	115	106
442	173	191	185	194	193	186	203	217	223
443	184	196	203	171	163	159	193	187	203
444	245	246	237	247	263	231	236	256	243
445	111	099	106	098	087	043	074	096	084
446	248	237	221	293	301	269	249	240	227
447	168	160	174	134	137	152	148	161	140
448	125	137	117	136	129	143	184	183	191
449	096	092	086	067	043	051	068	068	072
450	091	098	092	092	085	072	070	083	087
451	087	134	127	048	068	059	099	083	123
452	117	132	126	063	047	073	098	126	087
453	109	121	136	092	087	141	123	137	149
454	176	203	184	184	203	196	206	233	221
455	234	268	251	200	187	152	198	223	241
456	236	236	253	187	172	151	154	172	135
457	127	146	131	095	072	103	133	142	153
458	168	184	173	176	192	183	170	165	161

459	142	163	147	137	123	163	142	127	135
460	136	134	129	191	181	208	137	146	151
461	179	184	193	142	117	123	119	108	100
462	131	117	093	146	121	117	148	131	117
463	115	106	095	113	127	131	127	146	133
464	183	169	171	198	192	223	209	216	235
465	100	085	096	068	053	071	097	083	105

Arg. no detectable activity

MHO no detectable activity

Gluc. ($\times 10^{-12}$)

401	227	231	226	217	209	213	231	236	241
402	384	389	372	356	358	361	396	402	405
403	417	406	409	405	413	412	401	397	394
404	436	430	437	432	437	446	458	451	557
405	298	299	305	301	307	310	313	309	317
406	314	316	326	284	293	297	326	321	329
407	356	357	368	332	329	321	361	365	357
408	187	193	179	156	151	163	147	142	139
409	384	386	369	372	362	368	396	391	398
410	381	396	392	398	397	391	332	341	338
411	417	415	417	400	413	412	427	423	421
412	426	434	437	403	414	416	484	479	473
413	348	337	339	318	319	322	341	356	351
414	378	383	387	396	392	391	402	407	404
415	411	413	419	384	372	379	371	364	372
416	264	268	274	249	247	256	258	263	267
417	227	229	236	213	225	219	231	223	221
418	238	246	241	226	221	223	251	259	261
419	410	415	409	431	436	431	486	492	487
420	329	326	330	319	327	324	329	321	322
421	334	347	345	336	333	331	317	318	309
422	361	362	367	368	351	353	351	357	360
423	386	392	388	374	372	369	360	365	369
424	397	398	401	358	352	347	361	370	365
425	283	284	287	283	281	278	251	248	242
426	251	256	263	261	270	263	237	242	246
427	220	215	213	189	196	183	236	237	241
428	184	186	191	176	173	181	159	147	146
429	275	273	284	237	231	253	249	246	241
430	256	263	259	278	281	283	278	279	284
431	232	237	236	243	251	250	256	247	243
432	296	306	302	284	283	296	269	271	278
433	307	302	299	279	274	276	286	283	281
434	315	317	321	338	346	349	298	273	284
435	371	383	385	391	398	400	362	372	369
436	448	463	451	468	462	470	478	473	480
437	198	197	193	137	137	136	176	172	167
438	286	289	295	296	291	293	271	274	290
439	298	284	279	284	286	275	302	308	313
440	342	351	346	337	335	340	361	365	369
441	368	375	372	328	327	319	347	342	346
442	373	374	382	396	398	400	383	380	374
443	247	263	258	240	246	251	259	253	254
444	284	287	283	281	291	289	271	273	286
445	319	323	325	306	317	316	296	293	291
446	213	214	217	195	183	187	206	201	200
447	400	397	405	371	372	384	389	379	379

448	284	286	281	279	283	284	256	263	252
449	336	341	342	327	325	321	317	316	319
450	338	340	346	320	317	319	346	328	331
451	406	406	412	426	427	428	450	453	451
452	301	306	309	321	320	315	327	322	329
453	196	205	207	176	173	171	184	184	183
454	203	209	204	189	187	183	169	171	173
455	184	182	178	137	139	143	151	149	145
456	228	225	223	256	253	251	236	243	245
457	246	246	251	241	247	245	256	258	263
458	251	253	261	229	229	222	271	276	271
459	263	264	267	246	249	251	221	223	228
460	284	291	290	299	295	291	296	293	291
461	233	238	237	256	254	268	237	236	237
462	411	407	410	413	412	410	401	402	403
463	320	328	329	317	316	318	296	291	286
464	309	304	312	287	286	291	274	278	285
465	300	310	307	341	345	346	320	332	327

Perox. no detectable activity

ADH. ($x10^{-10}$)

401	117	131	127	183	168	171	152	149	157
402	216	223	241	234	209	217	197	183	194
403	216	231	223	210	210	207	234	236	241
404	263	271	261	249	258	247	248	256	259
405	156	153	159	173	178	184	195	161	143
406	174	171	189	168	172	161	163	142	141
407	183	191	196	171	163	164	194	193	201
408	043	027	036	078	059	063	059	067	072
409	109	113	097	136	128	142	127	118	123
410	220	241	236	239	237	231	216	218	203
411	234	242	251	195	187	183	225	209	203
412	272	283	287	281	271	279	267	283	256
413	153	147	163	185	181	172	177	178	169
414	136	127	140	142	136	131	125	117	110
415	172	168	174	184	153	161	193	198	200
416	118	117	130	109	107	115	101	106	114
417	084	081	056	093	072	087	079	069	073
418	046	027	033	059	046	041	042	038	033
419	251	261	265	246	253	267	243	231	229
420	096	083	087	102	072	084	093	101	085
421	176	186	190	153	143	148	147	147	138
422	193	194	187	184	186	172	179	163	161
423	193	197	198	196	172	184	201	210	210
424	191	196	179	187	187	174	175	169	185
425	107	113	123	096	084	079	087	103	101
426	084	086	081	093	098	101	072	104	083
427	043	053	051	038	052	047	031	029	036
428	058	063	057	063	072	071	054	043	037
429	136	142	137	123	117	129	118	123	129
430	149	163	147	152	163	147	137	148	153
431	109	115	106	096	087	092	098	086	079
432	180	186	191	173	163	178	170	161	176
433	175	184	169	153	168	169	162	147	163
434	193	192	203	184	197	191	186	189	193
435	242	246	237	271	284	259	234	237	253
436	263	271	249	202	213	209	207	213	216
437	037	025	033	029	036	021	046	049	036
438	073	084	093	071	072	083	079	096	091
439	190	209	200	206	213	226	232	231	229

440	236	253	246	248	261	252	226	237	217
441	097	096	101	104	103	109	103	093	098
442	260	271	253	213	219	226	224	231	232
443	156	163	161	172	184	182	162	169	172
444	173	184	159	184	188	193	159	163	171
445	123	113	123	156	161	168	149	142	137
446	103	113	117	056	072	071	078	084	069
447	289	273	281	304	315	298	312	306	297
448	134	141	143	127	132	131	119	126	129
449	156	163	170	183	172	181	172	176	172
450	173	178	170	161	163	169	165	157	162
451	263	271	271	241	249	263	223	227	217
452	183	183	192	189	185	193	172	169	178
453	028	037	032	034	029	025	040	036	031
454	073	078	084	069	065	072	065	069	072
455	024	026	015	017	012	018	029	032	023
456	073	083	096	084	081	079	091	087	086
457	083	096	094	087	085	080	086	087	093
458	107	115	123	093	090	106	110	118	117
459	112	133	127	118	106	123	100	104	096
460	173	136	141	168	168	171	159	157	169
461	139	126	121	142	149	137	148	147	152
462	351	368	371	302	313	316	346	349	352
463	193	191	187	201	213	221	187	183	172
464	204	209	213	213	214	217	198	186	195
465	180	186	193	173	179	186	184	189	190

LDH. ($\times 10^{-8}$)

401	172	184	176	137	136	139	142	147	150
402	204	210	207	215	216	219	206	204	208
403	256	257	259	247	255	257	239	236	229
404	279	274	278	246	239	239	251	256	250
405	207	209	213	208	213	211	201	203	198
406	216	217	221	214	206	209	213	217	219
407	220	225	226	220	219	223	206	204	203
408	072	063	069	084	086	087	083	080	081
409	206	204	203	209	206	209	215	217	218
410	223	226	229	214	213	217	206	202	203
411	246	249	245	240	237	236	237	229	231
412	258	253	261	259	265	254	250	249	243
413	197	190	204	203	193	197	200	198	205
414	169	175	163	158	157	159	152	142	147
415	184	186	187	183	180	179	175	172	171
416	136	132	136	131	127	126	142	141	139
417	106	110	103	100	102	102	098	103	107
418	108	107	106	104	106	109	093	087	088
419	196	193	200	196	201	198	206	204	203
420	187	184	179	184	193	190	181	187	187
421	188	189	193	174	180	179	169	171	173
422	203	207	210	204	203	203	207	204	200
423	206	201	204	213	216	212	216	209	209
424	210	214	214	214	213	217	215	209	210
425	144	147	151	140	139	138	137	142	141
426	126	134	125	113	117	118	117	123	120
427	112	117	115	094	091	093	097	090	093
428	094	098	093	093	094	100	087	084	086
429	153	147	149	136	138	142	139	141	140
430	161	169	165	163	164	167	161	158	163
431	142	143	149	143	141	150	147	152	153
432	186	187	193	186	193	191	189	193	191

433	192	197	193	190	195	187	184	180	187
434	198	203	200	203	207	209	210	214	211
435	243	246	240	246	251	253	258	263	260
436	251	255	256	261	261	259	273	263	261
437	093	090	094	099	101	106	104	103	109
438	191	197	190	193	197	203	187	184	193
439	187	186	195	175	172	163	190	194	203
440	180	193	201	175	173	171	172	178	174
441	196	193	203	199	191	201	200	193	191
442	221	230	227	226	228	234	217	209	210
443	149	153	157	137	138	142	131	136	131
444	163	178	175	174	172	171	179	184	181
445	178	179	186	176	171	169	172	172	178
446	097	090	092	090	083	086	083	084	079
447	236	231	230	224	229	228	217	224	226
448	165	163	168	169	161	162	170	164	163
449	200	213	218	193	198	200	197	193	204
450	175	184	180	176	172	178	170	170	179
451	251	256	256	234	238	239	239	240	242
452	180	174	173	183	178	169	172	163	175
453	078	084	076	061	063	068	069	069	065
454	086	087	082	084	086	089	082	089	087
455	069	065	064	072	072	070	075	070	068
456	108	109	112	113	113	115	114	101	113
457	123	127	129	126	123	131	109	104	113
458	137	136	130	132	138	138	139	141	140
459	146	146	140	146	142	140	141	140	136
460	158	151	153	153	150	149	148	142	143
461	160	167	161	152	154	153	149	153	154
462	298	304	300	293	301	300	301	300	297
463	224	230	237	217	216	219	206	204	203
464	217	218	213	219	224	226	223	223	228
465	206	204	209	209	208	200	200	197	198

IDH. $(\times 10^{-9})$

401	084	079	086	075	072	077	076	072	073
402	097	101	095	098	100	094	093	093	091
403	107	109	103	108	109	111	101	099	105
404	114	117	112	105	106	101	107	113	105
405	093	094	096	094	098	090	093	087	098
406	097	096	091	096	093	099	095	099	091
407	099	104	089	098	105	093	093	085	098
408	055	062	054	061	063	058	060	063	065
409	093	104	086	096	098	093	098	089	106
410	099	104	095	094	094	093	093	095	089
411	107	111	105	107	108	105	106	109	104
412	107	106	106	107	108	103	106	104	105
413	091	094	087	092	095	086	093	093	095
414	084	089	079	079	076	078	077	076	080
415	090	083	092	089	092	086	085	087	081
416	074	078	073	075	080	072	072	072	073
417	066	068	064	064	063	067	065	061	068
418	066	069	071	063	060	060	061	065	064
419	091	096	087	093	087	095	094	096	091
420	090	083	095	090	096	091	093	095	087
421	087	086	089	088	083	091	085	086	087
422	093	096	086	091	090	093	091	090	090
423	094	097	088	095	087	096	096	096	089
424	096	102	094	097	104	093	096	099	099
425	076	072	071	076	076	078	074	075	073
426	072	070	068	068	065	062	067	067	072

427	066	070	063	063	060	065	061	058	065
428	060	062	061	061	063	059	059	057	063
429	079	084	075	072	073	069	071	069	072
430	081	084	084	083	085	079	080	083	076
431	075	076	076	076	070	078	073	078	075
432	090	095	086	087	085	093	089	087	095
433	091	093	092	090	091	076	088	084	071
434	093	092	106	092	087	085	093	107	106
435	104	103	101	105	105	108	103	107	111
436	106	107	109	107	103	109	108	108	112
437	063	068	072	062	060	058	064	065	068
438	090	093	087	091	090	085	089	085	092
439	087	096	081	086	093	091	089	095	092
440	086	090	093	085	087	090	084	083	079
441	091	103	087	093	091	093	092	094	086
442	103	107	108	102	106	101	101	103	110
443	079	078	081	076	084	069	074	073	079
444	084	083	086	083	081	084	085	086	093
445	082	081	087	084	086	080	083	085	079
446	062	065	058	063	061	059	059	058	063
447	101	103	098	100	097	105	099	092	091
448	081	086	075	082	087	079	084	084	083
449	092	096	087	090	090	096	089	087	088
450	086	084	081	084	083	079	082	087	078
451	107	098	113	105	101	109	104	103	097
452	087	084	093	089	095	073	086	087	080
453	058	063	057	052	055	046	053	047	056
454	062	063	068	060	059	061	061	065	059
455	058	049	063	057	055	060	056	051	063
456	065	068	061	068	067	073	068	071	070
457	070	068	080	071	071	075	068	061	073
458	074	074	075	073	071	072	074	078	067
459	076	083	071	075	073	080	072	071	075
460	081	075	089	079	073	084	075	076	077
461	083	086	077	081	081	083	079	074	083
462	121	123	117	118	113	125	116	115	118
463	099	087	111	093	093	095	090	086	093
464	096	096	098	098	091	093	096	102	104
465	092	093	089	093	091	087	091	093	094

G6PDH ($\times 10^{-10}$)

401	076	072	073	065	069	071	071	068	063
402	085	084	091	080	083	076	086	085	082
403	093	094	098	094	093	087	090	086	088
404	101	115	107	093	098	092	096	094	097
405	081	083	078	086	079	085	084	083	086
406	079	072	071	083	084	083	086	089	083
407	081	072	076	079	075	076	075	078	072
408	063	068	059	069	067	065	071	072	063
409	085	084	087	080	079	083	075	072	076
410	085	086	083	085	087	081	081	079	083
411	089	093	085	093	096	091	086	084	083
412	103	106	108	097	092	095	094	090	091
413	075	073	078	072	070	071	070	069	067
414	070	081	075	063	068	063	068	068	072
415	079	074	076	079	078	073	075	076	070
416	064	065	069	063	068	065	068	064	065
417	059	058	060	063	061	063	061	065	062
418	058	057	052	055	053	054	063	060	057
419	075	078	080	069	063	068	068	067	062
420	080	081	087	081	082	083	084	080	078

421	076	072	079	083	080	075	074	074	072
422	082	086	081	086	083	079	086	079	079
423	079	084	081	078	075	078	080	083	081
424	081	086	083	083	085	086	084	086	088
425	065	063	060	063	058	057	065	063	061
426	069	070	072	073	070	069	064	065	063
427	058	062	061	056	057	054	057	053	053
428	055	058	061	056	061	053	063	068	067
429	087	083	084	083	089	089	088	085	081
430	077	083	086	075	070	071	072	073	072
431	068	065	070	065	063	060	067	059	059
432	077	081	078	084	083	081	072	075	076
433	070	074	071	069	068	063	073	071	075
434	081	084	080	080	075	083	082	086	082
435	089	091	093	087	085	095	091	096	087
436	093	094	091	089	083	086	089	083	081
437	055	054	053	051	052	051	049	046	048
438	074	075	074	075	072	080	069	063	071
439	077	078	081	074	073	076	068	068	062
440	080	072	075	076	072	071	074	075	077
441	093	086	089	090	087	085	077	073	079
442	083	086	085	081	081	081	076	072	069
443	068	062	063	062	067	069	063	068	065
444	074	072	071	072	073	078	071	072	069
445	073	068	062	078	082	069	074	072	071
446	057	056	057	054	053	056	048	049	053
447	076	072	078	081	080	084	080	079	078
448	074	070	069	069	061	065	065	063	067
449	081	082	081	083	082	080	082	086	085
450	075	072	073	072	075	073	078	077	081
451	093	096	091	085	087	083	082	087	081
452	075	081	080	075	077	076	073	076	074
453	057	063	060	054	057	057	063	065	062
454	054	057	055	071	069	068	063	067	066
455	048	050	053	056	052	058	055	056	056
456	065	063	064	063	062	063	059	061	062
457	065	067	060	066	063	059	062	059	061
458	067	069	063	062	065	067	060	062	065
459	071	069	067	073	071	070	078	078	075
460	072	071	068	068	067	073	065	068	071
461	073	071	070	070	069	074	072	078	071
462	113	113	112	110	113	109	097	091	097
463	096	093	094	087	080	079	088	087	086
464	085	083	081	083	084	083	078	072	073
465	080	080	083	084	084	081	085	086	088

Appendix 2:- Extended studies of enzyme activities in various Kupffer cell lines.

$\bar{x}$ = sample mean, s.d. = standard deviation, CoV = coefficient of variation, n = sample size.

Appendix 2.1:- Extended study of nine enzyme activities in primary adult Kupffer cell lines.

The population doublings are since isolation of the cell lines.

	Population doublings						
Enzyme	26	33	40	55	69	83	97
Catalase ($\times 10^{-3}$ moles/min./mg protein)							
$\bar{x}$	39.04	32.27	26.64	27.05	26.84	26.65	15.95
s.d.	15.99	14.68	11.97	9.82	9.00	8.54	9.90
CoV	41.0	45.5	44.9	36.3	33.5	32.0	62.1
n	15	15	15	15	15	15	8
Arginase ($\times 10^{-8}$ moles/min./mg protein)							
$\bar{x}$	87.1	64.5	48.4	44.9	38.7	24.1	8.9
s.d.	22.7	19.5	18.8	19.9	19.6	18.8	11.0
CoV	26.4	31.3	39.6	44.4	51.3	79.2	122.2
n	14	14	14	14	14	14	8
MHO ($\times 10^{-12}$ moles/min./mg protein)							
$\bar{x}$	16.8	15.7	14.4	13.1	10.4	5.9	2.1
s.d.	5.1	5.2	4.0	5.0	5.6	4.7	3.0
CoV	30.4	33.1	27.8	38.2	53.9	79.7	142.9
n	15	15	15	15	15	15	8
β-Glucuronidase ($\times 10^{-11}$ moles/min./mg protein)							
$\bar{x}$	36.2	30.2	23.5	23.3	23.1	23.4	29.0
s.d.	8.9	8.2	8.3	8.6	8.4	8.1	8.2
CoV	24.6	27.2	35.3	37.1	36.4	34.6	28.3
n	15	15	15	15	15	15	8
Peroxidase ($\times 10^{-3}$ moles/min./mg protein)							
$\bar{x}$	18.2	13.8	11.2	11.0	11.1	10.5	6.4
s.d.	7.3	6.1	4.8	4.2	3.7	3.3	3.2
CoV	40.1	44.2	42.9	38.2	33.3	31.4	50.0
n	15	15	15	15	15	15	8
ADH ($\times 10^{-8}$ moles/min./mg protein)							
$\bar{x}$	14.8	13.8	13.3	13.3	13.3	12.9	7.7
s.d.	2.2	2.1	2.2	1.6	1.8	2.0	3.5
CoV	14.9	15.2	16.5	12.0	13.5	15.5	45.4
n	15	15	15	15	15	15	8
LDH ($\times 10^{-7}$ moles/min./mg protein)							
$\bar{x}$	50.6	47.1	43.5	43.2	42.9	41.7	27.1
s.d.	11.5	11.1	10.4	10.0	10.3	9.4	4.8
CoV	22.7	23.6	23.9	23.1	24.0	22.5	17.7
n	15	15	15	15	15	15	8

cont.

Enzyme	26	33	40	55	69	83	97
	Population doublings						
IDH (x 10^{-8} moles/min./mg protein)							
$\bar{x}$	57.7	56.3	52.3	52.1	51.3	48.1	45.4
s.d.	13.5	13.4	11.1	10.7	10.9	10.2	9.2
CoV	23.4	23.8	21.2	20.5	21.2	21.2	20.3
n	15	15	15	15	15	15	8
G6PDH (x 10^{-10} moles/min./mg protein)							
$\bar{x}$	80.3	77.4	71.5	71.1	69.3	54.3	49.6
s.d.	21.5	19.2	19.3	17.9	16.1	14.8	12.4
CoV	26.8	24.8	27.0	25.2	23.2	27.3	25.0
n	15	15	15	15	15	15	15

Appendix 2.2:- Extended study of six enzyme activities in 15 SV40-transformed adult Kupffer cell lines.

The population doublings are since infection with SV40 which occurred 26 population doublings after initiation of the cell line.

Enzyme	Population doublings				
	26	33	40	74	90
Catalase (x 10^{-3} moles/min./mg protein)					
$\bar{x}$	1.62	1.48	1.59	1.54	1.58
s.d.	0.60	0.77	0.70	0.82	0.62
CoV	37.1	52.0	44.0	53.2	39.2
β-Glucuronidase (x 10^{-12} moles/min./mg protein)					
$\bar{x}$	328	327	329	330	330
s.d.	77	74	90	88	88
CoV	23.5	22.6	27.4	26.7	26.7
ADH (x 10^{-10} moles/min./mg protein)					
$\bar{x}$	168	168	165	163	157
s.d.	68	75	72	80	66
CoV	40.5	44.6	43.6	49.1	42.0
LDH (x 10^{-8} moles/min./mg protein)					
$\bar{x}$	187	183	183	186	180
s.d.	47	47	49	49	62
CoV	25.1	25.7	26.8	26.3	34.4
IDH (x 10^{-9} moles/min./mg protein)					
$\bar{x}$	88	87	87	86	82
s.d.	13	13	14	24	15
CoV	14.8	14.9	16.1	27.9	18.3
G6PDH (x 10^{-10} moles/min./mg protein)					
$\bar{x}$	78	76	75	70	70
s.d.	12	10	10	13	14
CoV	15.4	13.2	16.0	18.6	20.0

Appendix 2.3: Extended study of nine enzyme activities in 5 primary foetal Kupffer cell lines.

The population doublings are since isolation of the cell lines.

Enzyme	Population doublings 26	33	40	69	97
Catalase (x 10^{-5} moles/min./mg protein)					
$\bar{x}$	469	453	450	435	434
s.d.	108	114	114	116	118
CoV	23.0	25.2	25.3	26.7	27.2
Arginase (x 10^{-9} moles/min./mg protein)					
$\bar{x}$	39	39	36	35	33
s.d.	6	6	3	8	8
CoV	15.4	15.4	8.3	22.9	24.2
MHO (x 10^{-13} moles/min./mg protein)					
$\bar{x}$	43	41	39	32	25
s.d.	12	14	14	14	13
CoV	27.9	34.2	35.9	43.7	52.0
β-Glucuronidase (x 10^{-12} moles/min./mg protein)					
$\bar{x}$	119	117	119	114	117
s.d.	35	35	32	44	48
CoV	29.4	29.9	26.9	38.6	41.0
Peroxidase (x 10^{-5} moles/min./mg protein)					
$\bar{x}$	492	466	415	396	390
s.d.	170	183	181	182	182
CoV	34.5	39.3	43.6	46.0	46.7
ADH (x 10^{-10} moles/min./mg protein)					
$\bar{x}$	265	257	254	254	235
s.d.	25	14	34	46	38
CoV	9.4	5.4	13.4	18.1	16.2
LDH (x 10^{-8} moles/min./mg protein)					
$\bar{x}$	213	207	201	184	191
s.d.	57	52	55	64	59
CoV	26.8	25.1	27.4	34.8	30.9
IDH (x 10^{-9} moles/min./mg protein)					
$\bar{x}$	180	182	170	164	159
s.d.	40	40	44	37	40
CoV	22.2	22.0	25.9	22.6	25.2
G6PDH (x 10^{-10} moles/min./mg protein)					
$\bar{x}$	49	46	44	40	39
s.d.	11	9	11	13	11
CoV	22.4	19.6	25.0	32.5	38.2

Appendix 2.4:- Extended study of six enzyme activities in 4 SV40-transformed foetal Kupffer cell lines. The population doublings are since infection with SV40 which occurred 26 population doublings after initiation of the cell lines.

	Population doublings				
Enzyme	26	33	40	74	90
Catalase (x 10^{-5} moles/min./mg protein)					
$\bar{x}$	148	146	154	132	123
s.d.	34	28	15	33	29
CoV	23.0	19.2	9.7	25.0	23.6
β-Glucuronidase (x 10^{-12} moles/min./mg protein)					
$\bar{x}$	333	319	299	323	318
s.d.	71	54	74	77	80
CoV	21.3	16.9	24.7	23.8	25.2
ADH (x 10^{-10} moles/min./mg protein)					
$\bar{x}$	108	128	122	122	121
s.d.	25	27	26	36	9
CoV	23.1	21.1	21.3	29.5	7.4
IDH (x 10^{-9} moles/min./mg protein)					
$\bar{x}$	75	86	92	82	73
s.d.	21	28	34	22	30
CoV	28.0	32.6	37.0	26.8	41.1
G6PDH (x 10^{-10} moles/min./mg protein)					
$\bar{x}$	65	62	63	67	70
s.d.	19	16	19	14	15
CoV	29.2	25.8	30.2	20.9	21.4
LDH (x 10^{-8} moles/min/mg protein)					
$\bar{x}$	220	172	197	204	209
s.d.	27	24	38	48	22
CoV	12.3	13.9	19.3	23.5	10.5

Appendix 3:- Distribution of six enzyme activities in 65 SV40-transformed adult Kupffer cell lines after 26, 33 and 40 population doublings since infection with SV40.

Catalase ($\times 10^{-5}$ moles/min./mg protein)

Doublings	Class activity limit										
	50	70	100	125	150	175	200	225	250	275	300
26	0	3	8	8	10	10	12	1	8	4	1
33	3	5	8	5	11	11	11	2	5	2	2
40	1	3	6	10	11	11	7	9	5	0	2

β-Glucuronidase ($\times 10^{-12}$ moles/min./mg protein)

Doublings	Class activity limit							
	150	200	250	300	350	400	450	500
26	0	4	12	13	15	11	9	1
33	2	6	9	15	14	11	7	1
40	2	4	11	16	12	12	4	4

ADH ($\times 10^{-9}$ moles/min./mg protein)

Doublings	Class activity limit							
	5	10	15	20	25	30	35	40
26	6	9	13	22	7	7	0	1
33	5	13	9	22	8	6	2	0
40	6	12	12	20	11	2	2	0

LDH ($\times 10^{-8}$ moles/min./mg protein)

Doublings	Class activity limit										
	75	100	125	150	175	200	225	250	275	300	325
26	3	4	4	7	8	15	12	6	4	1	1
33	2	5	5	9	9	12	13	5	4	1	0
40	2	6	5	9	9	10	15	5	3	1	0

IDH ($\times 10^{-9}$ moles/min./mg protein)

Doublings	Class activity limit							
	60	70	80	90	100	110	120	130
26	3	8	9	14	21	8	1	1
33	2	10	11	14	18	9	1	0
40	2	10	12	12	18	10	1	0

G6PDH ($\times 10^{-10}$ moles/min./mg protein)

Doublings	Class activity limit							
	50	60	70	80	90	100	110	120
26	0	7	12	21	17	5	2	1
33	0	8	15	17	20	4	0	1
40	1	3	22	18	17	4	0	0

Appendix 4:- Distribution of nine enzyme activities in 24 primary foetal Kupffer cell lines after 26, 33 and 40 cumulative population doublings in culture.

Catalase (x 10^{-4} moles/min./mg protein)

Doublings	Class activity limit						
	30	35	40	45	50	55	60
26	0	2	0	4	4	10	4
33	0	2	2	3	7	8	2
40	1	1	2	3	8	7	2

Arginase (x 10^{-9} moles/min./mg protein)

Doublings	Class activity limit								
	20	25	30	35	40	45	50	55	60
26	2	1	3	1	6	4	3	2	2
33	0	2	1	6	6	4	4	1	0
40	0	1	4	5	10	3	1	0	0

MHO (x 10^{-13} moles/min./mg protein)

Doublings	Class activity limit									
	20	25	30	35	40	45	50	55	60	65
26	0	0	2	0	3	3	9	6	1	0
33	0	1	1	0	5	6	7	3	0	1
40	1	0	1	2	5	6	7	2	0	0

β-Glucuronidase (x 10^{-12} moles/min./mg protein)

Doublings	Class activity limit										
	70	80	90	100	110	120	130	140	150	160	170
26	0	2	3	4	6	2	2	1	2	1	1
33	1	2	3	3	2	5	4	2	1	0	1
40	1	2	4	3	3	5	2	2	1	0	1

Peroxidase (x 10^{-4} moles/min./mg protein)

Doublings	Class activity limit										
	25	30	35	40	45	50	55	60	65	70	75
26	0	2	0	2	2	3	4	6	1	3	1
33	2	0	0	3	2	2	11	1	1	2	0
40	1	1	1	3	2	6	5	3	0	2	0

ADH (x 10^{-9} moles/min./mg protein)

Doublings	Class activity limit										
	21	22	23	24	25	26	27	28	29	30	31
26	0	1	1	0	4	4	3	2	7	1	1
33	0	0	0	3	1	5	6	6	2	0	1
40	1	0	0	3	3	4	7	3	2	0	1

cont.

LDH (x 10^{-8} moles/min. /mg protein)

Doublings	Class activity limit 120	140	160	180	200	220	240	260	280	300
26	0	1	0	1	3	5	8	3	2	1
33	0	1	2	1	3	2	10	3	2	0
40	1	0	1	0	6	3	8	3	2	0

IDH (x 10^{-9} moles/min. /mg protein)

Doublings	Class activity limit 110	130	150	170	190	210	230	250
26	0	1	0	4	8	6	3	2
33	0	1	1	4	10	5	2	1
40	1	0	1	2	11	6	3	0

G6PDH (x 10^{-10} moles/min. /mg protein)

Doublings	Class activity limit 30	35	40	45	50	55	60	65
26	0	2	0	2	7	6	5	2
33	0	3	0	3	7	6	5	0
40	0	1	0	5	5	9	2	1