ASPECTS OF METABOLIC CONTROL IN ANIMAL CELLS.

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It has been possible to correlate the activity of certain enzymes, phosphatases in particular, with the morphological appearance of cultured cell strains. This finding suggests that some differentiated features may be retained in cell culture. Since the activities of some enzymes, compared with the tissue of origin, were low and variable, enzyme adaptation was investigated as a possible cause of fluctuation. Although there was evidence of spontaneous reversible variation in cultured cells, reversible alterations in the activities of several enzymes suggested that enzyme adaptation might occur. Arginase activity, in particular, was found to differ greatly between samples of the same strain of golden hamster fibroblasts and between samples of L63707 and lymphoma ascites cells taken from the animal and from culture. Attempts to induce arginase activity in HeLa cells did not give unequivocal evidence for substrate induction.

The uptake of arginine was studied as a possible factor influencing substrate induction of arginase. Evidence for active transport was obtained at an extracellular concentration of arginine of 0.02mM but there was little evidence for active transport at higher concentrations. The concentrative effect of this process

may be an integral part of substrate induction of enzymes.

Labelling experiments were undertaken to study the intracellular movement of RNA in an attempt to confirm the existence of a messenger fraction in animal cells, which might be involved in enzyme induction. No conclusive evidence was found for the movement of rapidly-labelled RNA from the nucleus to the cytoplasm in cultured mouse fibroblasts. Low concentrations of actinomycin D (0.1 - 5 µg/ml) did not inhibit RNA synthesis. A higher concentration (100 µg/ml.) inhibited rapid labelling in the cytoplasm and nucleolus. This suggests that RNA synthesized on chromatin does not move directly to the cytoplasm, but requires a further step, perhaps at the nucleolus, to effect transit to the cytoplasm.

These results suggest that the description of metabolic control of enzyme synthesis in bacteria is not directly applicable to animal cells.

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A Thesis submitted for the Degree of Doctor of Philosophy

in the Faculty of Science

by

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INTRODUCTION

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1.1

Animal embryogenesis is accompanied by changes in the enzyme activity of the embryo; e.g. neurulation is followed by a rise in the activity of alkaline phosphatase and cholinesterase in chick brain (Rogers et al. 1960). Such changes may involve the controlled alteration of synthesis and function of messenger RNA in a manner similar to enzyme adaptation in bacteria (Jacob and Monod, 1961). The possibility that differentiation and enzyme adaptation might share a common mechanism prompted the present investigation.

Although enzyme adaptation is reversible, nuclear transplantation experiments of Briggs and King (1952, 1957, 1960) suggest that differentiation may be irreversible. In these experiments, Briggs and King transplanted nuclei from amphibian embryos at various stages of development to anucleate eggs of the same species. They found that nuclei transferred from blastulae allowed development to advanced embryos but nuclei transferred from embryos beyond the blastula stage produced fewer surviving embryos. Older stages of donors gave rise to even more abnormal embryos.

Consequently an understanding of enzyme adaptation cannot be expected to reveal the whole process of the control of differentiation. However, the possibility remains that they may share a common mechanism. The existence of enzyme adaptation in bacteria suggests that it evolved before differentiation, and it may be the basic mechanism which made differentiation possible. Bacterial adaptation is stimulated by the presence of specific metabolites in the culture fluid (Jacob and Monod,

1961). If the activity of animal enzymes can be shown to respond to metabolites, then the available information on bacterial induction and repression may be used to understand the process in animal cells.

The technique of cell culture provides a good system for testing adaptation in controlled conditions. Whole animal responses, such as those of the endocrine glands, can be avoided, and inhibitors of RNA and protein synthesis may be used without the production of unidentified responses in other tissues.

Cultured cells are subject to morphological and metabolic variation which suggests that they may adapt to their environment by genetic selection or by non-genetic alteration of the expression of the genes. Genetic adaptation and non-genetic irreversible adaptation may be considered separately from reversible alterations in the phenotype for the purposes of the present study. Some attempt has been made to establish the nature and occurrence of irreversible genotypic and phenotypic changes in continuous culture, so that they may be distinguished from such reversible changes as enzyme adaptation.

Irreversible changes in cultured cells

The appearance and growth potential of cultured cell strains may change spontaneously in a process known as transformation. Ruddle (1962) observed such a change in a cloned pig kidney strain when epithelial cells growing in a contiguous sheet were replaced by rounded isolated cells. Similarly, typical fibroblastic strains have been replaced by epithelial stra (Ludovici et al. 1962; Castor et al. 1961). Dunnebacke Dixon (1957)

observed a change in susceptibility to polio virus infection during such a transformation. These phenotypic alterations remain stable in subsequent culture.

The tumour forming ability of a cell strain may also change spontaneously. Sanford et al. (1954) found up to 100-fold differences in different strains of mouse fibroblasts derived from one cloned line which did not initially form tumours. The tumour forming capabilities were characteristic of each strain and stable.

Spontaneous variation in the karyotype of cultured cells

Maintenance of mammalian cells in culture often leads to an alteration in the karyotype (Sax and Passano, 1961; Ruddle, 1961; Yu, 1963). These alterations may be produced by the failure of daughter chromatids to separate during division (non-disjunction) or by fragmentation and chromatid exchange in one or more chromosomes. Karyotypic changes sometimes follow a regular pattern (Levan and Biesele, 1958; Hsu and Merchant, 1961); freshly isolated cells usually retain the normal diploid number (each chromosome paired as found in most somatic cells), but after several transfers the chromosome number may double (tetraploidy). A few of the tetraploid complement may be lost at this stage resulting in hypotetraploidy. In other cell strains the modal number remains near the diploid but tends to deviate from the mode by one or two chromosomes (Yu, 1963).

Gross chromosomal changes, such as non-disjunction, fragmentation and chromatid exchange may contribute to genetic adaptation to the in

vitro environment, in addition to the possible effect of mutation at the submicroscopic level. However, some cell strains seem able to adapt to in vitro conditions without gross alteration in the karyotype, as in the Chinese hamster lines isolated by Yerganian in 1961.

The most important implication of spontaneous variation is that it may produce heterogeneous cell strains even after the strains have been cloned (i.e. grown up from a single cell). The different levels of enzymes that Westfall et al. (1958) recorded in two strains from one clone of mouse fibroblasts illustrates this. Male and DeSerli (1963) showed that a strain of human epithelial cells contained some cells deficient in alkaline phosphatase and others deficient in β-glucuronidase and glucose-6-phosphate dehydrogenase, showing that this strain is also heterogeneous. Strains with low and high alkaline phosphatase have also been described in HeLa (cultured human cervical carcinoma - Gey et al. 1952) by Konigsberg and Mitowsky (1958) and Cox and MacLeod (1962) and in several other strains by Cox and MacLeod.

If stable variants can arise by alterations in gene expression without actual genetic changes then the mechanism for their formation may have an important bearing on differentiation. However, the part played by mutation must be established first. In the absence of suitable recombination techniques, genetic analysis of cultured cells has been restricted to attempts to correlate changes in enzyme activity and drug resistance with chromosomal aberrations.

Resistance to metabolic analogues

Resistance to metabolic analogues has been produced in some cell lines by repeated subculture in the presence of the analogue. However, only resistant strains produced by sudden discrete changes may be used for genetic analysis.

Szybalski and Smith (1959) have claimed a single step mutation in the resistance of D-98 human bone marrow cells to 5 µg./ml. azaguanine. Resistance to amethopterin in Fisher's L5178 cultured mouse ascites cells is a one step process (Fisher, 1959). In other cases the selection of analogue resistant variants seems to have been gradual, implying a multi-step event (Kit et al. 1963; Szybalski et al. 1961; Metzger and Moskowitz, 1963). Lieberman and Ove (1959) describe the evolution of a puromycin resistent strain of AMK 2-2 adult monkey kidney cells as a sequential process.

In a few cases chromosomal markers have been associated with resistant cell lines (Harris and Ruddle, 1960, 1961; Schrecker et al. 1963; Dodarli and Maio, 1963; Konigsberg and Nitowsky, 1962) but in no case has direct correlation been proved.

Changes in enzyme activity accompany resistance to metabolic analogues in many cases (Szybalski, 1961; Kit et al. 1963; Skold et al. 1962). Increase in D-glucose-6-phosphatase activity has been correlated with resistence to 2-deoxy-D-glucose (Barban, 1962), partial or complete loss of guanosine monophosphatase with resistance to purine analogues (Szybalski et al. 1962), and increase in folic acid reductase

with amethopterin resistance (Schrecker et al. 1963; Biedler 1963). If marker chromosomes could be obtained in such variant strains it might be possible to establish that genes for specific enzymes were on those chromosomes. However, there were no single step events leading up to resistance, or correlations with chromosomal abnormalities.

Nutritional Variants

Alteration of the nutritional environment of cultured cells has provided a further approach to the isolation of variants. A variant of strain HeLa depending on sodium lactate (Chang,1961), mutants of Jensen sarcoma utilising asparagine (McCoy et al. 1959), and increased glucose uptake (Bradley and Syverton,1962) and other variants with altered carbohydrate requirements (Chang,1957) have been derived by this means. Variant lines have also been produced by altering the protein supplement of the medium (Saksela et al. 1961; Okumura et al. 1961). Enzymic changes have not been detected in most of these. Saksela et al. (1961) have noted a correlation of chromosome alteration with growth of HeLa cells in different human sera. (See also Saksela and Saxen,1962), but this lacks sufficient specificity to be of immediate value.

Correlation of enzyme variation and karyotype

There have been many attempts to correlate the deficiency of glucose-6-phosphate dehydrogenase in humans with aberrations in the sex chromosomes (Trujillo et al. 1961; Boutler et al. 1962; Gartler et al.

1962; Marks et al. 1962). Marks et al. (1962) have suggested that the quantitative control of glucose-6-phosphate dehydrogenase may reside in the X-chromosome but that there is a supplementary effect by an autosomal gene. This could account for some of the conflicting reports.

The karyotype of clones of Chang cultured human liver cells with differing alkaline phosphatase activity shows variation in the modal numbers, and specific markers have been attributed to each strain (Konigsberg and Nitowsky, 1962). This is the only direct link of chromosome pattern with enzyme activity so far reported in which a causal relationship of one with the other has been claimed. As such it is very important, but further selection experiments and recloning are necessary to eliminate the possibility that the occurrence of chromosomal abnormalities and enzyme alterations are coincidental.

Melo and DeCarli (1962a, b) adopted a different approach to the problem. They grew clones of a human epithelial line in petri dishes and when the clones matured, overlaid them with agar gels containing different specific chromogenic enzyme substrates. As the enzyme reactions released coloured products each clone was represented by a spot of colour, the intensity of which was proportional to the enzyme activity. Quantitation is difficult by this method, but it is possible to determine which clones have activity, and which have none or very little. If the agar overlay is applied aseptically it may be removed after assay, and the clones harvested and grown up for eventual karyotypic analysis. This provides a convenient method for screening many different cell strains

for many enzymes, and selecting the most pronounced differences for chromosome analysis.

Lines selected by this procedure are at present being analysed for correlations of enzyme and karyotype. Results so far indicate that the deficiency of alkaline phosphatase from some strains may be due to chromosomal variation rather than to point mutations, but as yet only preliminary reports are available (DeCarli and Meio, 1963).

Szybalska and Szybalski (1962) transferred the ability to synthesise inosinic acid pyrophosphorylase (IMPPase) to a cell line deficient in this enzyme, by incubation of the deficient line with DNA from an IMPPase-positive line. However, there are not sufficient data to be sure that this system is completely analogous to DNA transformation in bacteria. Further genetic analysis and suitable recombination techniques will be required before the effects of mutations can be distinguished from epigenetic effects or contamination by viruses.

The possibility remains that differentiation may be produced by gene repressors which are bound to the gene in the undifferentiated state, but dissociate from it during differentiation. A purely genetic basis cannot be claimed for irreversible changes in enzyme activity until such an unmasking process is disproved.

However, the existence of variation in enzyme activities between strains in a population has a more immediate application in the present work. The possible occurrence of spontaneous changes must be kept in mind in discussing the adaptive nature of enzyme fluctuations.

1.2

The reversible adaptations in cultured cells considered here are concerned only with changes in enzyme activity. Only those changes in activity brought about by alterations in protein synthesis can be regarded as adaptive within the definition of the term as used in bacteria. The current theory of protein synthesis is based chiefly on evidence obtained from bacteria and bacteriophage and may be described briefly as follows.

The coding for the sequence of amino acids in the polypeptide chains of protein is determined by the sequence of bases in DNA. The function of transferring information from DNA to the site of protein synthesis is attributed to RNA and the fraction responsible is called messenger RNA.

The structure of DNA and the genetic code

Watson and Crick (1953, 1954) described the structure of the DNA molecule as a polymer of deoxyribonucleotides joined by phosphate diester linkages in which two strands are held together by hydrogen bonding between the base components of the nucleotides to form a double helix. The bases in the two strands are arranged in such a way that adenine pairs with thymine and guanine with cytosine.

Recombination studies employing mutations in bacteriophage T2 have provided evidence that information may be carried in DNA by a code of nucleotide triplets (Crick et al. 1961). Each triplet is held to represent the incorporation of an amino acid during protein synthesis.

Nirenberg and Matthaei (1961) and Ochoa's group (Tongyai et al. 1961; Speyer et al. 1962a, b; Basilio et al. 1962; Nebha et al. 1962; Gardner et al. 1963) have produced cell-free polypeptide synthesis in the presence of E. coli ribosomes, an energy producing system, and a synthetic polynucleotide primer. Their results suggest a triplet code of nucleotides, and have led to the postulation of many triplet nucleotide sequences for specific amino acids.

RNA synthesis

In cell-free systems DNA can act as a primer for RNA synthesis (Ochoa et al. 1961; Chamberlin and Berg, 1962). The RNA produced has similar base ratios to the priming DNA (Chamberlin and Berg, 1962). The drug actinomycin D, which inhibits DNA-primed RNA synthesis (Goldberg et al. 1962), inhibits all synthesis of RNA in cultured cells (Reich et al. 1962), although it does not inhibit the synthesis of RNA in cells infected with RNA viruses (Reich et al. 1962; Eason et al. 1963). This suggests that all DNA synthesis in animal cells is DNA primed, although actinomycin may also affect the stability of RNA; evidence for decreased RNA synthesis can also be interpreted as increased RNA degradation. This will be discussed in a later chapter.

There are three main categories of naturally occurring RNA. One of these is the chief constituent of the ribosomes, and has base ratios dissimilar to the total DNA. The synthesis of this fraction is probably directed by specific segments of DNA (Yanofsky and Spiegelman, 1962).

After the ribosomes have been centrifuged out of a preparative

sucrose solution, a further RNA fraction remains in the supernatant. This is soluble or transfer RNA and to it has been attributed the function of an adaptor molecule between amino acids and messenger RNA as described below.

A further RNA is detectable by isotope labelling. It is rapidly labelled, of a base composition similar to DNA, and can be made to hybridise with DNA. It is this fraction which has been described as messenger RNA.

Protein synthesis

The present hypothesis concerning the mechanism of protein synthesis is discussed in reviews by Campbell (1962) and Simpson (1962). Single stranded messenger RNA unites with ribosomes, which either exist free in the cytoplasm or loosely bound to the endoplasmic reticulum. Transfer RNA, under the influence of activating enzymes, becomes associated with amino acids. One transfer RNA molecule attaches to one amino acid and the reaction is catalysed by one specific activating enzyme. The transfer RNA-amino acid complex then attaches to a specific site on the messenger RNA molecule, which is itself attached to a ribosome. Following this a further RNA-amino acid complex attaches to the messenger RNA polymer in an adjacent position to the first. Attachment then proceeds sequentially, amino acids being arranged along the messenger RNA molecule.

When an amino acid becomes attached by its transfer RNA molecule to a specific site on the messenger RNA, peptide bonding occurs between it and the adjacent amino acid on the messenger RNA. As fresh amino acids

are attached, the polypeptide chain grows and separates from the messenger RNA as the transfer RNA molecules become detached. As the polypeptide is only attached at the site of polymerisation, several polypeptides may be attached to one messenger RNA at one time.

Amino acid incorporation into protein proceeds at a higher rate when ribosomes are associated in groups known as polysomes (Wettstein et al. 1963; Steehelin et al. 1963; Noll et al. 1963; Zimmerman, 1963; Penman et al. 1963; Goodman and Rich, 1963). Electron micrographs confirm the association and show a thin strand connecting the ribosomes in some cases (Noll et al. 1963). It is thought that this represents serial synthesis of protein by successive ribosomes proceeding along a messenger RNA strand (Goodman and Rich, 1963). When polysome preparations are treated with RNAse the association breaks down leaving single ribosomes (Zimmerman, 1963; Wettstein et al. 1963). Actinomycin D ultimately produces a decline in the number of polysomes, implying that a labile, DNA primed RNA holds the ribosomes together (Steehelin et al. 1963).

The synthesis of different proteins is stimulated by messenger RNA synthesised at specific regions of the DNA and transported to the ribosomes. The sequence of bases in the messenger RNA determines the nature of the protein to be synthesised.

Bacterial messenger RNA

When bacteriophage infected bacteria are exposed to radioactive precursors of RNA a rapidly labelled element can be identified (Volkin

and Astrachan, 1956) and this eventually associates with the ribosomes (Brenner et al. 1961). After the appearance of activity at the ribosomes synthesis of phage specific protein begins (Brenner et al. 1961). Infection is therefore followed by rapid synthesis of a phage specific messenger which stimulates the synthesis of phage specific protein.

A similar active fraction, of molecular weight comparable to phage messenger, has been found in bacteria (Brenner et al. 1961; Gros et al. 1961). This RNA is labile and will hybridise with homologous DNA; at the appropriate magnesium concentrations, it will form an association with bacterial ribosomes (Gros et al. 1961). There is strong evidence to suggest that this RNA is bacterial messenger (Tissieres and Hopkins, 1961).

Messenger RNA in animal cells

When cells are incubated with radioactive RNA precursors and autoradiographs are prepared at intervals after the introduction of isotope, a regular pattern emerges (Goldstein and Micou, 1959, 1960; Amano and Leblond, 1960; Fitzgerald and Vinicheikul, 1959; Prescott, 1960; Perry, 1961). Initially activity is spread over the whole nucleus, but very shortly (ca. 30 mins. to 1 hour) it becomes particularly concentrated over the nucleolus. Gradually (1 hour onwards) the activity appears in the cytoplasm and soon increases, but it remains below that of the nucleus (Srinivasan et al. 1962; Harris, 1960). Similarly, if samples are taken at regular intervals after labelling and fractionated into nuclei and cytoplasm, an accumulation of activity is observed first in the nucleus and

later in the cytoplasm (Paul and Struthers, unpublished).

If RNA is extracted from cells, which have been incubated with radioactive precursors, and centrifuged in a sucrose density gradient, the label in early samples appears in the 35S-45S region of the gradient (where S = sedimentation rate in Svedberg units) and in later samples it appears in the 28S, 14S, and 4S regions (Scherrer et al. 1963). These last three regions correspond to ribosomal and transfer RNA, which make up the bulk of the RNA of the cell, and are the stable RNAs involved in the machinery of protein synthesis. The 35S-45S sedimenting RNA probably corresponds to the rapidly labelled nuclear fraction seen in autoradiographs.

The kinetics of RNA formation and accumulation in various parts of the cell permit postulation of a nuclear origin of RNA in most cases. It is, however, not possible to prove the precursor relationships of different fractions until independent synthesis in nucleus, nucleolus, and cytoplasm, at different rates, has been disproved. To eliminate the synthesis of labelled RNA whilst examining the fate of a short pulse labelled fraction, many workers have returned the cells to unlabelled medium containing an excess of the unlabelled precursor to dilute the labelled precursor pool (e.g. Goldstein and Micou, 1959; Taylor, 1960). Most "pulse-chase" experiments show a similar pattern to that described above, and the kinetics are compatible with nuclear origin of cytoplasmic RNA.

These results are subject to certain reservations. The effective

dilution of radioactivity in the RNA precursor pool during the chase depends on the assumption that freshly absorbed unlabelled precursor is incorporated into RNA as readily as nucleotides liberated into the pool by RNA degradation. If RNA synthesis uses degradation products preferentially, then synthesis of labelled RNA will continue during the chase. Therefore, the appearance of label in different RNA fractions does not necessarily mean the movement of undegraded RNA to these fractions.

From the results of experiments designed on the pulse-chase principle, Harris (1959, 1962, 1963a) claims that the bulk of nuclear RNA decays without passing to the cytoplasm, and the label that appears in the cytoplasm is independently derived (Harris and LaCour, 1963). Harris (1963b) maintains that there is a specific RNA degrading enzyme located in the nucleus, and absent from the cytoplasm. He has suggested (1962) that the predominant function of this labile nuclear fraction may be to act as the epigenetic repressor substance as in the scheme of Jacob and Monod (1961). He also suggested that if a part of this fraction functions as a messenger it does so by transcription of the message at the nuclear membrane on to an RNA fraction synthesised in the cytoplasm. Such a transcription process, being RNA primed, would be expected to be actinomycin resistant, but no synthesis compatible with this has been demonstrated in the cytoplasm or at the nuclear membrane, except in cells infected with RNA viruses.

Actinomycin D inhibition of RNA synthesis has been used to limit the period of incorporation of labelled precursor to a short pulse, so that the movement of the labelled fraction could be traced in the absence

of further RNA synthesis. 100 µg./ml. of actinomycin must be used for 15 minutes to obtain 95% irreversible inhibition of RNA synthesis in cultured L cells (Paul and Struthers, 1963). Even after this treatment incorporation of RNA precursors into acid insoluble material may continue in both nucleus and cytoplasm. Much of this may be terminal addition of cytidine on to existing transfer RNA (Morits, 1963; Paul and Struthers, unpublished), but subsequent studies have shown incorporation into ribosomal RNA in varying amounts (Paul and Struthers, unpublished; present results).

Scherrer et al. (1963) and Tamaoki and Mueller (1963) obtained an increase in ribosomal activity accompanying a decrease in that of 35S-45S sedimenting RNA in the presence of 5 µg./ml. actinomycin. They interpreted this as a transfer of undegraded polymer from the rapidly labelled fraction to stable ribosomes. However, at the concentration of actinomycin that they used, RNA synthesis can continue, and the re-incorporation of degraded fragments or mononucleotides cannot be excluded.

Comparison of the base ratios of the rapidly labelled fraction with those of DNA (Scherrer et al. 1963), and the formation of hybrids between rapidly labelled RNA and homologous DNA (Scherrer et al. 1963; Bacon et al. 1963) suggests that this fraction may be messenger RNA. Its correspondence to the bulk of the DNA would enable it to carry a replicate of the DNA code, obtained by base pairing in a similar manner to DNA replication (Watson and Crick, 1954).

Before rapidly labelled RNA of animal cells can be considered analogous to messenger RNA in bacteria, its movement to the cytoplasm must be proven.

1.3

Changes in enzyme activity that do not involve protein synthesis

In addition to non-specific effects of pH, temperature, substrate concentration, and the presence of co-factors, enzyme activity is subject to other forms of specific control that do not require any alteration of protein synthesis.

The product of a reaction or series of reactions may inhibit the activity of one or more enzymes in the series. This is known as feedback inhibition and is distinct from repression which involves a diminution in the synthesis of the enzyme. Feedback inhibition leads to reduction of the rate of a reaction when the inhibitory product accumulates. For example, Gerhart and Pardee (1962) described an example of feedback inhibition in the synthesis of cytidine nucleotides. They found that accumulation of cytidine triphosphate inhibited aspartate carbamoyltransferase, which catalyses the formation of carbamyl aspartic acid, a precursor of uridine and cytidine triphosphates. They demonstrated that the inhibitor became associated with the enzyme at a different site from substrate.

It is important to distinguish changes in enzyme activity involving the amount of the enzyme from those involving a change in catalytic efficiency. Concentration changes may be caused by inhibition or stimulation of protein synthesis. The stimulus is often substrate or product, and other non-specific factors, such as hormones and inorganic ions, may also participate. However, changes in the concentration of

enzymes may also be effected by the prevention of degradation of labile enzymes by substrate (Dubnoff and Dimick, 1959), if the enzyme normally exhibits a high turnover rate. Similarly, the active removal of an enzyme (e.g. glutamyl transferase in cultured L cells (Paul and Pottrell, 1963), may lower its concentration. Consequently, distinction between enzyme adaptation and other types of control may only be made by demonstrating an increase or decrease in the rate of protein synthesis in the response to a specific metabolic stimulus.

Finally, spontaneous irreversible changes in enzyme activity, discussed earlier, must be distinguished from reversible adaptation in the presence of a given stimulus.

Enzyme adaptation in bacteria

Evidence for metabolic control of enzyme synthesis has been gained chiefly from bacterial systems such as the substrate induced β-galacto-sidase system in E. coli (Jacob and Monod, 1961) and the penicillinase of Bacillus cereus (Kogut, Pollock and Tridgell, 1959). In these systems and in the repression of tryptophan synthetase (Cohn and Monod, 1953) and arginine synthetase (Vogel, 1961) in E. coli it has been proved conclusively that during adaptation considerable alterations in the synthesis of specific proteins may occur in microorganisms. When β-galactosidase is induced in E. coli by β-galactoside a 10,000-fold increase in enzyme (more than 6% of the total cell protein) is produced.

The analysis of mutations in the lac region of the E. coli chromosome indicates that separate genes control the structure and rate

of synthesis of β-galactosidase. Two mutations producing high and low non-inducible levels of the enzyme have been identified at loci different from the structural genes. The wild type of each is dominant. Mutations at these loci as well as controlling the operation of the β-galactosidase structural gene, also control the operation of the structural gene for β-galactoside acetylase, which is closely linked. The ratios of the activities of the two enzymes remains the same in induced and in non-induced states. From recombination analysis of mutants of the above type, Jacob and Monod (1961) have formulated a hypothesis for the regulation of protein synthesis. Subsequent investigations of metabolic control mechanisms have been based largely upon this scheme and it may be stated briefly as follows.

The structural genes determining the sequence of amino acids in individual proteins are located in the DNA of the bacterial chromosome. The synthesis of each enzyme is directed by a messenger from a specific structural gene. Structural genes, determining the synthesis of several enzymes in one metabolic pathway, may be controlled by a single operator gene. The operator gene and the related structural genes are closely linked, and the linkage group is known as an operon.

The action of the operator gene is under the control of a regulator gene. This gene, not necessarily located near the operon, produces a repressor substance, capable of inhibiting the function of the operator. This simultaneously represses synthesis of messengers at all the structural genes in the operon.

Enzyme inducers may inhibit the action of the repressor substance presumably by combining with it, and hence allow the operator gene to initiate synthesis of messengers at the structural genes. Metabolic enzyme repressors may increase the efficiency with which the repressor substance from the regulator gene inhibits the function of the operator gene. Alternatively, metabolic repressors may inhibit the operator gene directly. The interaction of metabolic inducers and repressors with the action of the repressor substance of the regulator gene may provide the mechanism that enables fluctuations in the metabolic pool to alter quantitatively the synthesis of messenger RNAs, and consequently the activity of specific enzymes.

Catabolic reactions are frequently controlled by enzyme induction, while anabolic reactions are more often controlled by enzyme repression. β-galactosidase induction in E. coli is an example of induction of a catabolic reaction while arginine (Vogel, 1961) and tryptophan (John and Monod, 1953) synthetases are anabolic and repressed by arginine and tryptophan respectively.

Enzyme adaptation in animal cells

It is possible that a similar system to that found in bacteria may exist in animal cells to permit them to differentiate and adapt to metabolic fluctuations. However, considerable modification of the mechanism would be required as the range of conditions producing enzyme adaptation in animal cells appears to be much wider and includes hormones and differences in osmolarity (see below). The necessary modifications

may involve the operon and the regulator genes directly, or may be produced by ancillary mechanisms; e.g. the function of the genes may be influenced by the configuration of DNA or the presence of histone, and the function of messenger RNA may be influenced during transport to the cytoplasm, or during attachment to ribosomes. The possible connection between the complexity of animal cell structure and modifications to the adaptive mechanism will be discussed in a later chapter.

Enzyme repression in vitro

The effects to be described later in this work, are all on increased synthesis of enzyme, or induction, but it is worthwhile noting at this stage that repression of enzyme synthesis also occurs. Cox and Heelood (1963) described the repression of alkaline phosphatase by cystine and cysteine in human cell cultures, and Doffare (1958) found that glutamyl transferase of strain Hela was repressed by glutamine. This was investigated further by Paul and Pottroll (1963) who described the kinetics of the system in L strain mouse fibroblasts. D-glutamyl transferase was actively synthesised in the absence of glutamine and actively degraded on the return of glutamine to the medium. The increase in enzyme activity in the absence of glutamine was inhibited by 6-azaguanine and fluorophenylalanine. This suggests that the fluctuations in enzyme activity were due to alterations in protein synthesis and were not due to feedback inhibition.

Fluctuations in enzyme activity in response to diet

Changes in enzyme activity in animal tissues have been produced by alterations in the diet. Changes in the protein fat and carbohydrate content alter the activity of rat pancreatic enzymes (Grossman et al. 1948); a high protein diet causes an increase of all the urea cycle enzymes (Schimke, 1961, 1962a, b, 1963) and of D-amino acid oxidase (Lardy and Feldot, 1950). Changes in the concentration of hexoses in the diet produce fluctuations in the carbohydrate metabolising enzymes (Fitch and Chaikoff, 1959; Freedland and Harper, 1957, 1958), substrate specific in some cases. These will be discussed below.

Most of these reports are of general interest only and lack evidence of protein synthesis which would indicate whether the changes in enzyme activity were due to an increase in enzyme concentration or merely to activation of existing enzymes. However, the results of Schimke (1961, 1962a, b, 1963) on arginase and other urea cycle enzymes suggest that the increased activity is due to *de novo* enzyme synthesis. He was unable to demonstrate any induction by addition of substrates to the diet (Schimke, 1963).

Fluctuations of enzyme activity in response to hormones

Fluctuations of enzyme activity in response to hormones

adrenocorticotrophic hormone (Hill et al. 1963) and triiodothryonine (Halevy and Avivi, 1960) have been shown to increase the activity of glucose-6-phosphate metabolising enzymes, and tryptophan pyrrolase activity has been increased by insulin, independently of substrate and adrenal activity. (Schor and Friedon, 1958). The effect of many hormones and additions to the diet may be due, at least in part, to stimulation of the adrenals, as both groups of adrenal hormones have been shown to influence enzyme activity, both in vitro (Nitowsky and Herz, 1963; Cox and MacLeod, 1962; Maio and DeCarli, 1963) and in vivo (Knox and Auerbach, 1955; Knox, 1951; Rosen and Milholland, 1963; Greengard et al. 1963; Rosen, 1963; Rosen et al. 1963; Dietrich, 1954).

Increases in tryptophan pyrrolase in response to corticosteroids have been obtained in rat liver (Knox, 1951; Knox and Auerbach, 1955; Rosen and Milholland, 1963) and inhibition of this effect with actinomycin D suggests that it involves protein-synthesis (Greengard et al. 1963). Similarly, cortisone has been shown to induce tyrosine aminotransferase (Greengard et al. 1963; Rosen and Milholland, 1963) and alanine aminotransferase (Rosen et al. 1963). Increases in the last could not be produced by substrate in the absence of hydrocortisone (Lin and Knox, 1957), but the effects of substrate and corticosteroids on tryptophan pyrrolase seem to be independent (Knox and Auerbach, 1955). Similarly, induction of xanthine oxidase occurs independently with xanthine, histamine, and cortisone, and is abolished in each case by ethionine (Dietrich, 1954).

Alkaline phosphatase activity has been increased by prednisolone in cell cultures from several species (Cox and MacLeod, 1963; Melo and DeOerli, 1963), and Nitowsky and Rozz (1963) showed that this increase was puromycin sensitive and not due to activation.

These findings are of importance not only in their own right, but also in analysing results from *in vivo* experiments as they imply that many hormones can affect enzyme synthesis. Consequently, care must be taken to exclude the action of the endocrine system when additions are made to the diet of experimental animals. Many forms of experimental procedure may cause stress, and cortical hormone suppression may confuse the interpretation of *in vivo* experiments.

Changes in enzyme activity produced by substrate

Increases in enzyme activity can be produced by both substrates and hormones. Although hormonal induction may play an equal or greater part in animal metabolic regulation, it is easier to compare substrate effects with induction in bacteria. If enzyme induction in animals was derived from a similar mechanism to that found in bacteria, then substrate induction should be found to be the basic process in animal cells, and hormonal induction may be a modification of it. For this reason the experiments of the present report were limited to the effects of substrate.

The increased activity of xanthine oxidase in the presence of xanthine (Dietrich, 1954) has been shown to be due to protein synthesis, but increases in tryptophan pyrrolase activity produced by substrate do not appear to involve protein synthesis above the normal level (Rosen and

Milholland, 1963; Greengard et al. 1963). Adenosine deaminase increases in chick embryos on injection of adenosine into the air sac (Gordon and Roder, 1953), and glucose-6-phosphatase and fructose -1, 6- diphosphatase of rat liver increase with high glucose, fructose and galactose in the diet (Freedland and Harper, 1957, 1958; Fitch and Chaikoff, 1959). There was no proof of an increase in protein synthesis in any of these.

Increases in the activity of the enzymes of cultured cells

Eagle et al. (1957) reported that the metabolism of cultured cells was subject to adaptation in a freely reversible manner, and demonstrated that an increase in phenylalanine hydroxylase activity of a subline of HeLa cells was inhibited by tyrosine. Weissman et al. (1960) reported increased activity of thymidine, thymidylic, and thymidine diphosphate kinases in L strain mouse fibroblasts grown in thymidine.

Acetylcholinesterase activity increases 2-6-fold *in vitro* during incubation of chick lung with acetylcholine for 8 days (Burkhalter, 1957b). Acetylcholine and some of its derivatives prevent the breakdown of acetylcholinesterase in explanted chick intestine (Burkhalter, 1957a), but this esterase does not appear to be comparable with the brain acetylcholinesterase (Burkhalter, 1957a). A spontaneous increase in acetylcholinesterase has been obtained in chick intestine *in vitro* and appears to be due to *de novo* protein synthesis (Burkhalter, 1963). It is possible that the spontaneous increases obtained in cultured HEp cells (Burkhalter pers. comm.) are due to similar adaptive changes.

Pottrell (1962) has shown that spontaneous fluctuation of acid phosphatase activity obtained in cultured L cells is correlated with the growth cycle of the cells. Activity is low after transfer, rises during log phase, and remains constant during stationary phase. The increase of alkaline phosphatase activity of several cell lines grown in media with different solute concentrations is apparently due to changes in osmotic pressure, rather than alterations in the ionic balance of the intra- and extracellular fluid (Mitowsky, Herz and Geller, 1963).

Alkaline phosphatase activity also increases in the presence of organic monophosphates (Maio and DeCarli, 1963). This increase follows the same pattern as that produced by hydrocortisone; strains with low uninduced activity exhibit the increases whereas strains with high uninduced activity do not. The increased activity in high osmotic tension (Mitowsky et al. 1963) was observed with both high and low activity lines. The enzyme known as alkaline phosphatase is probably a group of enzymes with pH optima in the alkaline range, and this, in combination with the apparent diversity of conditions that may affect alkaline phosphatase activity, makes alkaline phosphatase a complex system to work with.

Although feeding arginine to rats does not produce increases in arginase activity (Schimke, 1963), substrate can be shown to induce arginase in cultured HeLa and KB liver cells (Schimke, 1962c, 1964). The increases in activity that Schimke obtains vary from 1.5X in unclonod HeLa cells to 8X in HeLa clone S3-1. The absence of signs of activation

in assays of mixed extracts from substrate treated and control cells implies that true induction is involved.

Klein (1961) maintained that RNA (yeast) is required for the induction of arginase *in vitro* by substrate. She suggested that it might be required to make up a deficiency of templates found *in vitro*. The injection of hydrocortisone and yeast RNA, or nucleotides in similar proportions to the nucleotides of yeast RNA, produces a greater increase in tyrosine α-ketoglutarate transaminase of rat liver than hydrocortisone alone. (Diamondstone and Litwack, 1962). Niu (1963) claimed that liver RNA induced tryptophan pyrrolase and glucose-6-phosphatase activity in ascites cells normally lacking these enzymes, and kidney RNA selectively induced L-amino acid oxidase. He maintained that the effect was heritable, and produced by interaction of the foreign RNA with the native DNA in such a way as to cause the DNA to start synthesising specific templates. The significance of these different findings is hard to determine.

In the course of a survey of enzyme activities (reported in the Results section) arginase was found to be lacking in cultured L5178Y lymphoma cells whilst it was present in the same cells grown as an ascites tumour in DBA mice. Paul et al. (1964) were able to repeat this observation and found that the increase in activity occurred in the first 24 hours after inoculation into the mouse peritoneum. When the cells were returned to culture *in vitro* he found that the arginase activity was gradually diluted out.

As arginase could not be induced in this tumour *in vitro* (Fottrell, pers. comm.) attempts were made to induce it in other cultured cell strains. Arginase activity in strain HeLa increased 2-3-fold in the presence of arginine alone and over 10-fold in a combination of arginine and either citrulline or ornithine. A similar increase was obtained with arginine, glutamic acid, and uridine (Fottrell, 1962). It was concluded that the presence of citrulline, ornithine, glutamic acid and uridine may stimulate the synthesis of other enzymes of the urea cycle and associated pathways, thereby influencing the rate of the whole cycle, including arginase.

If the culture medium was changed in the course of incubation with substrate, a three-fold diminution in the final arginase level was observed (Fottrell, 1963). A difference was also observed in the degree of induction at different times under similar culture conditions (Fottrell, 1962 and "Results").

The variations in the amount of induction obtained on different occasions, and the degree of difference between arginase activity *in vitro* and *in vivo*, implies that arginine is not the sole agent responsible for fluctuations in arginase activity. Either arginine is prevented from producing full induction in culture, or else it is a minor component of the necessary stimulus.

As specific systems have been demonstrated for the transport of metabolites in bacteria it is possible that the activity of such a system in animal cells could control the uptake of arginine. This may represent the unidentified component referred to above, and for this reason the uptake of arginine has been studied in strain HeLa, the same line of cells as used for arginase investigations.

1.4.

Control of metabolism by the regulation of entry of metabolites

The transport of metabolites across the cell membrane of bacteria against a concentration gradient occurs at specific sites (Cohen and Monod, 1957). Part of the function of the sites is performed by inducible proteins known as permeases. The number of metabolites that are concentrated by bacteria, and the high specificity of each permease, implies that many such systems must exist. There is a specific permease for the transport of β-galactosides and it is independent of intracellular binding or hydrolysis of galactosides. The permease system is energy dependent since uptake is inhibited by uncoupling agents such as DNP and azide.

β-galactoside permease may be induced by β-galactosides. In the non-induced state very little β-galactoside enters the cell and the activity of the enzyme β-galactosidase, which is inducible by the same substrate, remains low.

Separate mutants lacking the permease and β-galactosidase have been used to show that the genes for the two proteins are closely linked. A further mutation abolishes the inducibility of both proteins, and produces high constitutive levels of each. This type of relationship would justify the inclusion of the permease gene in the operon containing the β-galactosidase gene, and its possible identification with β-galactoside acetylase has been suggested (Monod et al. 1962).

Other permease systems have been described for the concentration of glucuronides (Stoeber, 1957) and glucosides (Monod et al. 1957) in

E. coli and for citrates and other Krebs cycle intermediates in Pseudomonas (Barrett et al. 1953; Kogut and Podosky, 1953) and Aerobacter (Davis, 1956). The accumulation of amino acids in E. coli is almost certainly controlled by a similar type of system (Cohen and Rickenberg, 1956, 1955; Britton et al, 1955; Gale, 1947, 1954), although the permeases do not appear to be inducible in these cases. The amino acid permeases are sensitive to uncoupling agents, and similar amino acids such as valine, leucine and isoleucine, may compete for the same stereo-specific system (Cohen and Monod, 1957). The presence and concentration of amino acids within the cell are under the control of specific entry mechanisms and consequently their metabolic effects, such as the stimulation of enzyme induction, are indirectly controlled by the membrane permeases.

The implications of the presence of specific transport mechanisms for cellular metabolites on the control of differentiation in higher organisms have been mentioned by Cohen and Monod (1957) in their review of bacterial permeases and will be discussed in a later chapter. Since many specific transport mechanisms have been described in animal cells it is an interesting speculation that a considerable degree of control is exercised on enzyme adaptation by alterations in membrane permeability by such mechanisms as have been described in bacteria.

Much of the previous work in the field of membrane transportin animal cells has dealt with the flux of inorganic ions, particularly sodium, potassium, and chloride in blood and nervous tissue (Harris, 1960).

A specific "sodium pump" has been described in nervous tissue (Hodgkin, 1951). The study of hexose entry also suggests that there is a specific system which may be concentrative or non-concentrative, depending on the tissue.

Smyth (1961) found that all the amino acids that he tested, with the exception of dicarboxylic and basic amino acids, were concentrated in rat intestine. This was also shown by Wiseman (1955) with hamster intestine. Rosenberg (1962) showed that lysine, arginine, ornithine and cystine were concentrated in rat kidney cortex slices in a system where arginine, ornithine and lysine competed with each other but not with cystine.

The uptake of amino acids is concentrative in Ehrlich's ascites carcinoma cells; 17 amino acids have been shown to be concentrated by different amounts (Christensen and Riggs, 1952; Christensen et al. 1952; Christensen, 1961a; Oxender and Christensen, 1963). The prevalent theory of amino acid uptake in animal cells may be summarised as follows.

Substances entering a cell may do so in several different ways. Larger particles may be engulfed in vacuoles of the cell membrane by phagocytosis and smaller particles and liquid droplets by pinocytosis, a similar process to phagocytosis involving smaller areas of the membrane per vacuole (Holter, 1959). The fate of material engulfed in this way is not known precisely and it is uncertain whether breakdown of the membrane is required before the contents become freely available to the cell sap. It is possible that amino acids may enter in this manner but

the necessary evidence is lacking.

Amino acids are probably required to pass through intact cell membrane during uptake. The permeability of the membrane to metabolites may be described briefly as follows. Where the metabolite enters down a concentration gradient uptake may occur by simple diffusion. This is limited by the relationship of the charge of the metabolite to the charge of the membrane, and the diffusion of polar substances will be further restricted by their low solubility in the lipid component of the membrane.

Diffusion may be assisted by sites in the membrane adapted to the uptake of specific metabolites. This is known as facilitated diffusion or mediated transport, and may involve a specific carrier mechanism in the membrane. The entry of a metabolite by facilitated diffusion may be accompanied by the efflux of an intracellular component. This is known as exchange diffusion, and features in the uptake of hexoses (Lefevre, 1961).

Where substances are transported against a concentration gradient, the process is known as active transport and is energy requiring. The following hypothesis, suggesting a receptor site or a carrier molecule, has been proposed (Riggs et al. 1954; Christensen, 1961; Oxender and Christensen, 1963) based on data from Ehrlich's ascites cells.

The active carrier is a single molecule, or molecular relay, which is physically or chemically modified at one side of the membrane to accept the substance to be transported and at the other to release it again. In a similar fashion to exchange diffusion, the entry of one molecule may be accompanied by the exit of a different molecule or ion. Potassium ions

may exchange with amino acids in this manner (Christensen et al. 1958a, b,c; Riggs et al. 1958; Quastel, 1961). By such a mechanism the uphill movement of molecules is a property of the differing specificity of the groups at the inside and outside of the membrane.

The accumulation of phenylalanine, threonine, isoleucine, valine, proline and methionine in yeast is inhibited by azide and dinitrophenol (Halvorson and Cowie, 1961). Similar inhibition has been noted in some mammalian systems and may be due to removal of intracellular potassium ions by binding or to inhibition of the sodium potassium exchange pump mechanism (Christensen, 1963a). Respiration is affected by inhibitors such as iodoacetate, azide, and dinitrophenol (Webb, 1963), and abolition or reduction of active transport in their presence may reflect a reduction in the supply of ATP. Although the transport mechanism is energy dependent it can utilise glycolysis alone in ascites cells in the absence of aerobic stages (Christensen, 1963a).

Separate transport mechanisms have been detected in ascites cells for acidic, basic, and neutral amino acids (Hokin and Hokin, 1963; Christensen, 1960). Competition may exist between different amino acids within these groups (Scholefield, 1961); e.g. lysine, arginine, and ornithine compete with each other for entry but do not compete with cystine, alanine, phenylalanine, histidine and glycine (Rosenberg et al. 1962).

Although non-specific within these groups, the transport system is sensitive to certain alterations in the configuration of the entering

molecule. A high degree of concentration has been reported for diamino acids in ascites cells (Christensen et al. 1952; Riggs et al. 1952, 1954), sufficient to displace most of the intracellular potassium in one instance (Riggs et al. 1952). Substitution of amino or carboxyl groups (Paine and Heinz, 1960; Halvorson and Cenis, 1961) renders the transport mechanism insensitive as does the presence of a charged group in the side chain of the amino acid (Christensen et al. 1960). It has been suggested that a certain optimal separation of the amino groups favours the formation of a cyclic linkage with the carrier molecule(s) (Riggs et al. 1954; Hokin and Hokin, 1963), perhaps also involving the carboxyl group (Paine and Heinz, 1960; Halvorson and Cenis, 1961). The presence of a non-polar side chain may aid solubility in the lipid component of the membrane (Hokin and Hokin, 1963).

The uptake of α-aminoisobutyric acid (AIB) is increased in rat liver by hydrocortisone and in rat uterus by oestradiol (Nemi et al. 1957) and Kostyo et al. (1959) has shown an increased uptake of the same amino acid in isolated rat diaphragm by adding pituitary growth hormone. Using a similar system to Kostyo, Fritz and Knobil (1963) were able to double AIB uptake with insulin.

The studies that have been made of the uptake of amino acids in cultured cells indicate that active concentration mechanisms are operating for the most of the nutritionally essential amino acids, with minor differences between cell strains (Pisz and Eagle, 1953; Eagle, 1959; Eagle et al. 1961).

As the present work involves the study of the effect of arginine

on arginase activity, the nature of the process governing the uptake of arginine may be of considerable importance.

Uptake of arginine

Christensen et al. (1958) reported a 1.3-2.8-fold concentration of arginine in Ehrlich's ascites cells, although Johnstone (1959) did not obtain concentration of arginine in these cells. Results from intestinal absorption in rat and hamster imply that there is no active uptake for the basic amino acids lysine and ornithine (Wiseman, 1955; Smyth, 1961). Concentration of lysine, arginine and cystine has been reported in rat kidney cortex slices (Rosenberg et al. 1962) in a competitive system where arginine, lysine and ornithine compete for an active transport mechanism not shared by alanine, histidine, and glycine.

In general basic amino acids seem to have their own active uptake process, though this does not appear to function in all tissues. If arginine uptake in particular is non-concentrative in HeLa cells, then this might affect the success of attempts to induce arginase with substrate. For this reason a set of experiments was performed to examine the nature of arginase uptake in the cultured HeLa cells used in the present series of experiments on induction.

1.5 Objects of the present study

A preliminary investigation was designed to establish whether cultured cells are "dedifferentiated" or deadapted; i.e. whether the low activity of many enzymes in cultured cells is due to irreversible changes

compatible with a reversal of differentiation or to the absence of inducers in the cell culture environment. A survey of the enzyme activities of several cell strains was carried out to reveal characteristic stable differences between strains.

The presence of reversible changes in enzyme activity indicated that adaptive regulation as found in bacteria might exist in these cells. Arginase was selected as a suitable enzyme for further studies on enzyme induction because of the variations in arginase activity observed in culture and the large differences between the activity of arginase *in vitro* and *in vivo*. The demonstration of adaptation in this enzyme would provide evidence of deadaptation in cultured cells as opposed to "dedifferentiation". Substrate induction of arginase in culture provides a suitable system for comparing animal cell adaptation with bacterial adaptation.

The difficulty in inducing increases in activity of arginase with substrate suggested that the permeability of the cell membrane might influence induction by the restriction of the entry of substrate. Hence, experiments were undertaken to examine the mechanism of arginine uptake in cultured cells.

Enzyme adaptation in bacteria, as described by Jacob and Monod (1961) requires a labile messenger RNA to carry information from the genes to the site of protein synthesis. Adaptation in animal cells would be expected to involve a similar fraction of RNA. Some studies were therefore made of the rapidly labelled RNA of cultured cells to determine if this fraction could perform the function of messenger in animal cells.

M A T E R I A L S and M E T H O D S

2. Materials and Methods

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2.1. Cell culture

The cell-strains and culture conditions used are outlined in Table 2.1. Strains HBH, HeLa, Y-5, L2 and 013 (and other clones of strain BHK 21) were grown as monolayers in flat sided glass bottles, according to the following routine. Cells were inoculated at 10^5/ml. in 10 ml. medium (4 oz. bottles), 25 ml. medium (16 oz. bottles) or 50 ml. medium (Roux flasks). The media used are detailed in Table 2.1, and in the Appendix. The inoculated cells usually adhered to the glass within 12 hours. The culture medium was changed after 3-4 days. After 7 days the medium was removed and 5-10 ml., 0.25% trypsin (Difco 1:250) was added. The trypsin was removed after 1 minute and the cell sheet was incubated at 37°C with the remaining film of trypsin for 10 minutes. The cells were then resuspended in the appropriate medium for the strain; 0.5 ml. of the suspension was diluted in 24.5 ml. counting fluid (see Appendix) and was counted with an electronic cell counter (Coulter Counter, Model D, Coulter Electronics Ltd.). Fresh culture vessels were then reinoculated at 10^5 cells/ml.

The technique of counting cells by this method is subject to some error. There is no guarantee that all the cells are viable. Also clumped cells give a erroneous reading; clumps are either recorded as one cell or the size discrimination of the machine prevents the clump from being counted. Consequently, care must be taken (1) to ensure that suspensions of cells for counting are monodisperse, and (2) to ensure that all cells are viable.

Table 2.1

Cell strains and culture conditions used in present studies

Cell Strain	Origin	Culture Medium	% Supplement C.S.	H.S.	B.P.	Tr.
HeLa (for survey)	Adult human cervical carcinoma (Gey et al. 1959)	Waymouth MB752/1 (1959)	5	2	-	-
(for all other expts.)	"	Eagle* (1955)	5	2	-	-
HLM	Foetal human liver (Leslie et al. 1956)	Waymouth MB752/1 (1959)	5	2	-	-
L	Adult mouse subcutaneous fibroblasts, Clone 929 (Sanford et al. 1949) Recloned P2.	Waymouth MB752/1 (1959)	5	-	-	-
Y-5	Adult Chinese hamster fibroblasts, Clone 10-1 Strain 16bFAF7 (Yerganian & Leonard, 1961)	Eagle* (1955)	10	-	0.1	-
		After subsequent cloning	5	-	-	-
C13 and other transformed and untransformed clones.	Adult golden hamster fibroblasts, HHK21; (Stoker, 1962)	Eagle* (1955)	10	-	-	0.1
L5178Y	Mouse lymphoma ascites tumour (Fisher, 1958)	Fisher (1958)	10	-	-	-
L5178Y	"	Also maintained as ascites tumour in DBA mice and F1 crosses.				
Landschutz	Mouse ascites tumour	Eagle* (1955)	10	-	-	0.1
"	"	Also maintained as ascites tumour in Porton white mice.				
L "S"	As L strain; selected to grow in suspension. (Paul & Struthers, 1960 unpublished)	Eagle* (1955)	5	-	-	-

C.S. - calf serum; H.S. - human serum; B.P. - bactopeptone (amino acid hydrolysate); Tr. - tryptose (amino acid hydrolysate). Media were made up in balanced salt solution (see Appendix).

* The concentrations of amino acids in Eagle's medium (1955) were raised 10 times in all cultures except Y-5 cells of enzyme survey.

Clumping of cells can be detected by examining a drop of cell suspension under a low power microscope.

The exclusion of some viability stains may be regarded as an index of viability (Cameron, 1950). Naphthalene black has been found to be particularly suitable for this purpose in this laboratory. If one drop of 0.1% naphthalene black is added to a drop of cell suspension on a slide and examined under a microscope, the proportion of unstained cells gives the viability of the culture.

Enzyme assays were accompanied by DNA phosphorus and protein nitrogen determinations to eliminate errors incurred in cell counting.

Maintenance of special lines - Y-6 Chinese hamster fibroblasts

The Chinese hamster strains used in the present studies were derived from strain 16b/AF7 of Yerganian (1961, unpublished). The original strain was maintained in this laboratory in Eagle's medium plus 10% calf serum and 0.1% bactopeptone (Ruddle, 1961, unpublished). Ruddle developed a strain of these cells able to grow in the absence of bactopeptone. During maintenance for the present studies, the serum concentration was reduced to 5%, and the standard cell inoculum during culture was reduced to 10^4/ml.

These cells were cloned (i.e. strains were derived from single cells) by Ruddle in the presence of irradiated 4-day mouse embryo cells, and were cloned for the present work in Eagle's medium plus 5% calf serum alone (see below).

The purpose of maintenance in low supplement concentrations, and of cloning in the absence of mouse feeder cells (irradiated embryonic cells),

was to develop a strain of cells able to be cloned with high efficiency in almost completely defined medium. Such a strain was required for selection experiments, such as those on analogue resistance reported in a later section.

The clone Y-5-10-1-11 (Y-5-11) was one of several clones of the Y-5-10-1 strain, grown without mouse feeder cells, and was selected because of its rapid growth and healthy appearance. (Hyaline cytoplasm and homogeneity of the sizes of the nuclei were used as criteria of healthy cells). This clone was used for further experiments and for studies of resistance to metabolic analogues. The medium used for the maintenance of these cells during studies of resistance is detailed in Table 2.2. in Appendix.

Human cervical carcinoma grown in tris-citrate buffered medium

Hela cells used in permeability experiments were adapted to grow in tris-citrate-buffered medium (see Appendix). The use of this medium enabled more careful control of pH even when cultures were left unstoppered, as was necessary while making serial additions during experiments. Two transfers were required (trypsinisation and reinoculation) before this strain became established.

Landschutz ascites cell cultures in tris-citrate buffered medium (Postlethwaite and Macpherson, 1965)

Ascites cells from Porton white mice, obtained from Postlethwaite in 1964 were grown as suspension cultures without agitation in Eagle's medium plus 10% calf serum and 0.1% tryptose. (This strain had previously

been cultured in this medium by Postlethwaite and Macpherson, 1958).

As these cells produced large quantities of acid in cell culture they were transferred to tris-citrate-buffered medium. Adaptation to this medium occurred readily.

Suspension cultures

The L "S" strain (Paul and Struthers, 1960, unpublished) was maintained in suspension in Roux flasks without agitation. Fresh medium was added twice per week, and the population was diluted to 10^6 cells/ml. medium once per week. Landschutz suspension cultures were maintained in Roux flasks and shallow 1,000 ml. conical flasks. L "S" cells gave a twentyfold yield per week and Landschutz cells fifteenfold.

Cloning

The Chinese hamster cell strain was cloned several times by the dilution technique modified from Puck et al. (1956).

The cells were trypsinised by adding 0.25% trypsin (see Appendix) for 2 minutes, then withdrawing it and leaving the residue of trypsin to act for 20 minutes at 37°C. The I-5 cells required a long time in trypsin to obtain a single cell suspension, upon which the validity of the method depended. The cells were then resuspended in Eagle's medium plus 5% calf serum. The suspension was diluted to give 1,000 cells in 0.1 ml. which was then added to a 5 cm. plastic petri dish (Falcon Plastics Ltd.), containing 5 ml. medium (Eagle's plus 5% serum). A drop of 6%

sodium bicarbonate was added and the culture placed in a CO_2 incubator. Half the medium was replaced weekly until the cultures were about one month old. Surviving clones were counted and expressed as a percentage of the inoculum (cloning efficiency).

At this stage surviving clones contained from a few hundred to a few thousand cells; the largest and consequently the fastest growing, were marked and trypsinised. In order to trypsinise isolated clones without contamination from neighbouring cells, stainless steel rings were placed round the clones and sealed at the base with silicone grease. These formed walls and a few drops of trypsin were placed in each. After two minutes the trypsin was carefully removed and the cultures incubated with the dregs of the trypsin for about 10 minutes. (A single cell suspension is not required, hence a shorter exposure to trypsin is adequate). The cells in each well were then suspended separately and the suspensions inoculated into 2 ml. medium in test-tubes. The clones were grown up in these tubes until ready for transfer to larger vessels. When established, the cloned lines were either retained or discarded according to their growth rate and chromosome pattern. The process was repeated several times in an attempt to obtain a uniform karyotype and a high cloning efficiency.

Freeze-storage

Cloned cell strains and other valuable lines were stored under liquid nitrogen. The principle of the procedure used depends on the findings (Porterfield and Ashwood-Smith, 1962; Feraidsky and Richards,

1963) that the highest viability may be retained when cells are cooled at a rate of 1°C./minute in the presence of a preservative such as glycerol or dimethyl sulphoxide. Water may be lost from the cell during freezing and the ensuing osmotic effects and high salt concentration may cause structural damage and may denature some macromolecules. Glycerol and dimethyl sulphoxide are hydrophilic and may serve to retain water inside the cell. They may also help to prevent the generation of intracellular ice crystals which would damage the cell (Meryman, 1962).

When the protective properties of glycerol and dimethyl sulphoxide were compared, dimethyl sulphoxide gave a higher proportion of surviving cells (Strain L, Clone 929) than glycerol. Because of this, and a recommendation in the literature (Porterfield and Ashwood-Smith, 1962), dimethyl sulphoxide was used in all subsequent freeze-storing.

A cooling rate of 1°C./minute was obtained by selecting a suitable expanded polystyrene box (with a wall thickness of about 1"), placing the ampoules to be frozen inside, and leaving the box at -70°C. for two hours. A thermocouple enclosed within the box showed that the ampoules cooled at approximately 1°/minute (Fig. 2.1).

Cells to be frozen were cultured to give sufficient cells for 2-3 x 10^6 per ampoule of Y-Bs and 5-10 x 10^6 per ampoule of any other type of cell. The cells were collected by trypsinisation and resuspended in growth medium plus 10% dimethyl sulphoxide. Between addition of dimethyl sulphoxide and transfer to -70°C. the cells were kept on ice to minimise the toxic effect of the preservative. 2 ml. of cell suspension

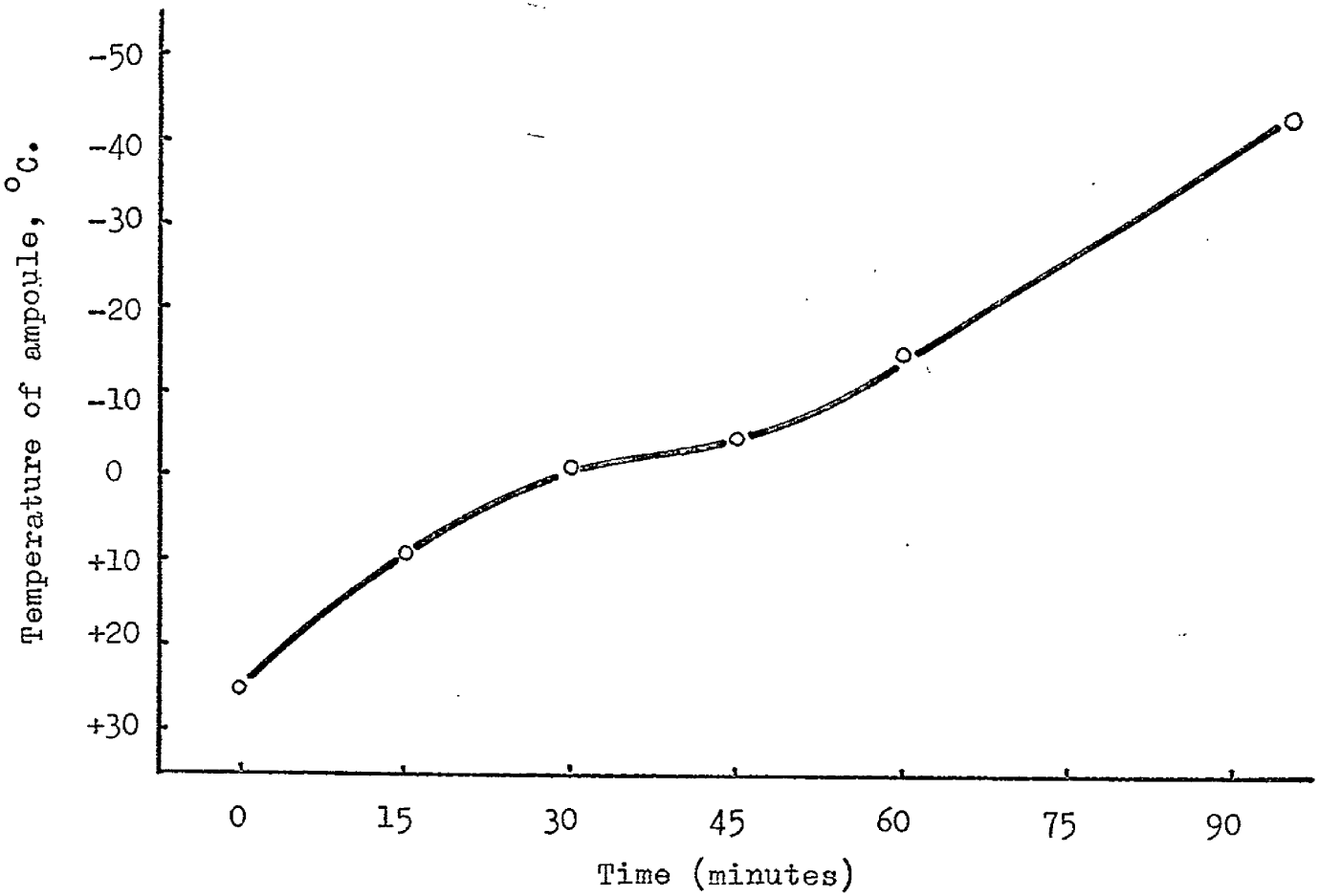
Fig. 2.1

Determination of cooling rate during freezing of cultured cells.

Twelve 2 ml ampoules, containing a cell suspension in medium plus 10% glycerol were packed in a ribbed polystyrene box and placed at -70°C. The temperature drop was recorded by enclosing a thermocouple with the ampoules.

This type of container, with an average wall thickness of 1", gave a cooling rate of approximately 1°C/minute.

Fig. 2.1


Temperature of ampoule, °C.
-50
-40
-30
-20
-10
0
+10
+20
+30
0
15
30
45
60
75
90
Time (minutes)

were inoculated into each ampoule, the ampoule sealed, and placed in an insulated box at -70°C.

The box was left at -70°C for 2 hours whereupon the ampoules were submerged in liquid nitrogen in a Linde liquid nitrogen refrigerator (LNR-25-B). After a day or two one ampoule was thawed as a test (the cells were thawed rapidly in a 37°C water bath) and the cells inoculated into a 4 oz. bottle. After thawing, the dregs of the cells in the ampoule were stained with a viability stain such as naphthalene black and the unstained cells scored as a proportion of the total giving an estimate of the percentage viability.

Viability after freezing can be improved by washing the thawed cells in balanced salt solution before inoculation into medium. L "S" cells do not survive unless this precaution is taken.

2.2. Chromosome preparations by the air-drying technique (Rothfels and Siminovich, 1958)

Cells for chromosome preparations were grown in 3 cm. plastic petri dishes (Falcon Plastics Ltd.) containing a 7/8" square coverslip, with 2 ml. sodium bicarbonate buffered medium, and incubated in a CO_2 water-jacketed incubator. 10^6 cells were inoculated into each dish and incubated for 48 hours by which time a thin sheet of cells had formed; the cells were touching in some cases at their extremities but did not form a confluent sheet. This permitted maximum spreading and coincided with the period of maximum cell division.

0.004% colchicine (Hopkins and Williams Ltd.) was added for the final five hours of incubation to arrest mitoses in metaphase. The coverslip was then removed from the petri dish and placed in 0.95% (hypotonic) sodium citrate (25 minutes for Y-5 cells, 10 minutes for L cells). Hypotonic citrate causes the cells to swell, disperses the chromosomes, and helps to counteract condensation of the chromosomes induced by colchicine. The citrate was then gradually replaced by ice-cold acetic acid alcohol fixative (1 part glacial acetic acid to 3 parts absolute alcohol). After about half the citrate had been replaced the coverslip was transferred to fresh ice-cold acetic alcohol for 10 minutes. The coverslip was then removed from the fixative and allowed to dry in air; this has the effect of reducing all the chromosomes to the same plane.

The air-dried coverslip was stained in 1% natural orcein (George Gurr) in 50% acetic acid for 2 minutes, rinsed briefly in two washes

methoxyethanol (May and Baker Ltd.) and two washes euparol essence (Flatters and Garnet Ltd.) and mounted in euparol (Flatters and Garnet Ltd.). The preparation was examined under a 100X phase contrast objective of a Zeiss WL microscope.

The chromosomes of 20-50 cells were counted to give the modal distribution, and aberrant chromosomes were recorded. Photographs were taken with a Leica camera on 35 mm. Ilford Pan F fine grain film.

Preparation of ideograms

Cells which showed a good spreading of chromosomes and an intact cell membrane were photographed by phase contrast microscopy with a 100X oil-immersion objective. The negative was enlarged to give a 10" x 8" print on high contrast paper, and the shapes of the chromosomes were cut out. These were arranged in order of size in two groups: (1) those with the centromere at the middle of the chromosome (metacentric) and (2) those with the centromere not at the middle, including those with the centromere near (but not at) the middle (sub-metacentric), those with the centromere near one end (sub-telocentric) and those with the centromere at one end (telocentric). The chromosomes were numbered according to Ford and Yerganian (1958).

A synopsis was prepared from the average occurrence of each chromosome in ten ideograms; only those occurring in more than five ideograms were included in the synopsis.

2.3. Collection and passage of ascites tumour cells

Two ascites tumours were used in the present work. These were the L5178Y mouse lymphoma, which was grown in DBA mice or DBA/F1 crosses, and the Landschutz tumour grown in Porton white mice. L5178Y ascites tumours were grown for about seven days, and Landschutz tumours for about five days, before passage. During passage, tumour was withdrawn aseptically from the peritoneum of an anaesthetised mouse into a sterile plastic syringe, and 0.2 - 0.5 ml. aliquots were reinoculated directly into other mice. When non-sterile tumour was required for analysis, mice were anaesthetised, killed by breaking the neck, and the skin stripped from the trunk. Tumour was extracted by a Pasteur pipette through a small hole in the body wall.

2.4. Enzyme assays, total nitrogen, and DNA phosphorus determinations

Preparation of cell extracts for enzyme assays

In preliminary studies extracts were prepared from L5178Y ascites cells by three different methods and assayed for the twelve enzymes of the subsequent survey (Table 2.2). L5178Y cells were suspended in 0.85% NaCl at a concentration of 2 x 10^8 cells per ml. Half of this suspension was frozen and thawed rapidly, three times, by alternate immersion in solid carbon dioxide in absolute ethanol and in a 37°C. water bath. The other half of this suspension was placed in a small bore tube on ice under a modified Mullard drill and ultrasonicated for 2 minutes (50 w., 20 kc./sec.).

A similar suspension was prepared in 2% sodium deoxycholate in 0.85% NaCl, and frozen and thawed three times. Deoxycholate is a detergent, and it was used to facilitate extraction of some particulate-bound, or membrane-bound enzymes.

Each suspension was centrifuged at 2,000 g for one hour at 2°C., and the supernatants used for enzyme estimations. Deoxycholate preparations were also used without prior centrifugation.

The results of enzyme estimations of extracts prepared as above are displayed in Table 2.2. Repeated freezing and thawing in 0.85% NaCl alone proved most satisfactory for the extraction of acid phosphatase, arginase, thiosulphate sulphur-transferase, β-glucuronidase, glucose-6-phosphatase, cathepsin-C, glucose-6-phosphate dehydrogenase, and arylesterase. Disruption of cells by sonication gave essentially similar results, although

Table 2.2

Preparation of extracts for enzyme estimations

Table 2.2

Preparation of extracts for enzyme estimations

	U	FT	C	NC
Aspartate Amino Transferase	1.0	1.0	2.1	2.2
Alkaline Phosphatase	2.1	1.0	5.5	6.9
Acid Phosphatase	1.0	1.0	0.3	4.1
D-Glutamyl Transferase	0.8	1.0	0.3	0.3
G-6-P Dehydrogenase	0.8	1.0	-	0.3
Arginase (Mouse Liver)	1.0	1.0	-	1.2
(L5178)	-	1.0	-	0
Arylesterase	1.1	1.0	0.4	0.6
Thiosulphate Sulphur-Transferase (Mouse Liver)	1.1	1.0	-	0.8
Cathepsin-C	0.6	1.0	0.4	0.4
β-Glucuronidase	0.4	1.0	0.9	0.9
Lactic Dehydrogenase (Landschutz)	1.0	1.0	-	1.0
Glucose-6-Phosphatase (HeLa)	-	1.0	-	0.9

U = ultrasonicated, 50 w., 20 kc./sec. for 2 mins.
FT = frozen and thawed 3X in 0.85% NaCl.
C = frozen and thawed 3X in 0.85% NaCl with 2% sodium deoxycholate.
NC = as C but not centrifuged. (All extracts except NC were centrifuged at 2,000 g. for one hour at 1°C.)

Extracts were prepared from L5178Y ascites tumour cells, except where otherwise stated.

Enzyme activities obtained by different preparative procedures are quoted as a ratio of the activity in frozen and thawed extracts.

some activity of cathepsin-C and β-glucuronidase appears to be lost during sonication. Sonication also gives an extract with a higher alkaline phosphatase activity.

The presence of deoxycholate during extraction yields higher alkaline phosphatase and aspartate amino transferase activity, and this suggests that these enzymes may be membrane- or particulate-bound. A higher acid phosphatase activity was also obtained with the uncentrifuged deoxycholate extract. However, this was not apparent in the centrifuged extract. This suggests that deoxycholate does not render acid phosphatase more soluble in the extract, and the higher activity in the uncentrifuged extract was probably due to retention of particle-bound acid phosphatase lost by centrifugation in the other extracts. Acid phosphatase is found in lysosomes, and it would appear that deoxycholate does not aid its separation from them.

Aspartate amino transferase and alkaline phosphatase were extracted by freezing and thawing in deoxycholate, and the remaining enzymes by freezing and thawing in 0.35% NaCl alone, in all subsequent experiments. The more labile enzymes, lactate dehydrogenase, glucose-6-phosphatase, and acid phosphatase, were assayed in fresh extracts; otherwise the extracts were stored at -20°C. until required.

Enzyme assays

All determinations were by spectrophotometric methods, and the Unicam SP600 spectrophotometer was used in all cases, except where otherwise stated.

Non-specific absorption due to reagent and extract was estimated by preparing blank determinations, and this was subtracted from the test readings. Blanks were either prepared by replacing the enzyme with deionised water ("reagent blank"), or by adding the reagent designed to terminate the enzyme reaction before adding either enzyme or substrate ("whole blank").

British Drug Houses (B.D.H.) "Analar" grade reagents were used, except where otherwise stated.

Alkaline phosphatase

Alkaline phosphatase activity was estimated by measuring p-nitrophenol released from p-nitrophenyl phosphate (Bessey et al. 1946).

8.6 ml p-nitrophenyl phosphate (L. Light & Co.) was made up in 0.05 M glycine buffer at pH 10.5, and was prepared fresh for each assay.

0.1 ml. of extract was added to 1 ml. buffered substrate at 37°C. and incubated for 30 minutes. The reaction was terminated by addition of 10 ml. 0.02 N NaOH. The absorption of liberated p-nitrophenol was measured at 410 mμ against a reagent blank.

Free p-nitrophenol was used as a standard and was diluted (1:200) from a deep frozen (-30°C.) stock (0.01 M) for each assay.

Enzyme units are expressed as μ moles phosphate released per minute.

The approximate range of the reactions was from 0.1 - 1 μ moles phosphate per ml. reaction mixture.

Acid phosphatase

Acid phosphatase activity was estimated in a similar manner to alkaline phosphatase; in this case the substrate was dissolved in 0.05 M acetate buffer at pH 4.9. The range of the reactions was similar.

Arylesterase

Arylesterase activity was estimated by the condensation of tetrazotised o.dianisidine with β-naphthol released from β-naphthyl acetate (Hachlas and Seligman, 1949).

The naphthyl acetate substrate was prepared by dissolving 5 mg. of β-naphthyl acetate (L. Light & Co.) in 1 ml. acetone, and adding this to 100 ml. 0.06 M phosphate buffer at pH 6.5, to give a final substrate concentration of 0.025 mM. Constant swirling is necessary to avoid precipitation of naphthyl acetate when the acetone solution is added to the buffer. The substrate is unstable and was prepared fresh for each assay.

O-dianisidine Fast Blue B (G. Gurr Ltd.) was made up at 4 mg./ml. to deionised water, and freshly prepared for each assay.

0.1 ml. extract was added to 3 ml. substrate solution at 22°C. After 30 minutes, 0.5 ml. of the Fast Blue B solution was added and the incubation continued for 10 minutes. The enzyme reaction was then terminated by the addition of 0.5 ml. 2M trichloracetic acid. The pink colour of the reaction mixture was extracted with ethyl acetate, and the ethyl acetate solution was cleared of sediment by spinning at 250 g for 10 minutes. The absorption of the supernatant was measured at 540 mμ

against a reagent blank.

Enzyme units are expressed as μMoles acetate released per minute, and were calculated from the absorption of a β-naphthol standard.

The range of the method as used was 0.1 - 1 μMoles acetate/ml. reaction mixture.

β-glucuronidase

β-glucuronidase activity was determined by estimating phenolphthalein released from phenolphthalein glucuronide (Talalay et al. 1946).

5 ml substrate was prepared by diluting phenolphthalein glucuronicle (L. Light & Co.) from a 1.09 mg./ml. stock with an equal quantity of 0.1 M phthalate:NaOH buffer at pH 4.6.

0.1 ml. enzyme extract was added to 1 ml. buffered substrate at 37°C. The reaction was terminated after 30 minutes with 5 ml. 0.2 M glycine buffer at pH 10.4. The absorption of the pink coloured solution was measured at 540 mμ against a reagent blank.

Enzyme units are expressed as μMoles glucuronide released per minute, and are calculated from the phenolphthalein liberated in the reactions compared with a phenolphthalein standard.

The range of the method as used was from 0.01 μMoles - 0.5 μMoles glucuronide in the reaction mixture.

Glucose-6-phosphatase

Glucose-6-phosphatase activity was estimated by utilising glucose oxidase to measure glucose (Huggett and Nixon, 1957) liberated from

glucose-6-phosphate (Campbell, 1962).

0.1 M substrate was prepared by dissolving 30.4 mg. in 1 ml. 0.1 M citrate:NaOH buffer at pH 6.5. The liberated glucose was estimated with a Boehringer glucose test pack. This consisted of a glucose reagent and a standard. The glucose reagent was 67 µg./ml. o-dianisidine-HCl, 250 µg./ml. glucose oxidase, and 40 µg./ml. peroxidase in 0.12 M phosphate buffer at pH 7.0. The glucose standard was 91 µg./ml.

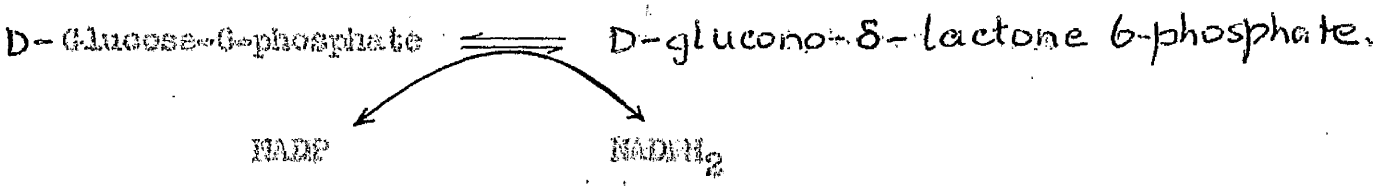
0.1 ml. enzyme extract was added to 0.1 ml. of the substrate solution at 37°C. The reaction was terminated after 90 minutes with 0.25 ml. 0.73 N perchloric acid. The reaction mixture was centrifuged at 2,000 g for 30 minutes, and 0.2 ml. of the supernatant was added to the glucose reagent at 37°C. After 20 minutes the glucose reaction was terminated by placing the sample tubes on ice. The absorption of the yellow colour was measured at 436 mµ against a whole blank. The absorption of the glucose standard was measured against a reagent blank of the glucose reaction.

Enzyme units are expressed as µM^oles glucose produced per minute.

The range of the method as used was from 0.01 - 0.1 µM^oles glucose per ml. of the first reaction mixture.

Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase activity was determined by the increase in ultraviolet absorption of NADP in the reaction



(Glock and McLean, 1955).

The reaction mixture consisted of 0.3 mM glucose-6-phosphate (B.D.H.), 4 μM NADP (Sigma), 12 mM $MgCl_2$, in 0.5 M tris-maleate:NaOH buffer at pH 7.6.

2.1 ml. buffer, 0.1 ml. extract, 0.1 ml. NADP, and 0.1 ml. $MgCl_2$, were added to a cuvette and allowed to warm to 37°C. in the heated cuvette compartment of a Unispek spectrophotometer. The machine was then set to zero and the glucose-6-phosphate added. The absorption at 340 mμ was measured at 15 second intervals for three minutes, or at 30 second intervals for five minutes. If the reaction was linear, the results were accepted and the run was duplicated. Linearity was generally achieved by altering the enzyme concentration.

One enzyme unit was expressed as a change in absorption of 0.100/min.

D-glutamyl transferase

D-glutamyl transferase was measured by the formation of glutamyl hydroxamate (Delfare, 1953).

The incubation mixture contained 0.4 ml. 0.5 M acetate buffer at pH 5.6, 0.1 ml. 0.25 M sodium arsenate, 0.2 ml. 200mM glutamine (L. Light & Co.), 0.1 ml. 1 M hydroxylamine-HCl, 0.1 ml. mM ADP (Sigma), and 0.1 ml. 16.5 mM $MnCl_2$. This gave a final concentration of 40 mM glutamine in 0.2 M acetate buffer.

The reaction was started by adding 0.1 ml. enzyme extract to the substrate solution at 37°C. After 90 minutes 0.5 ml. 0.6 M $FeCl_3$ was added, the solution centrifuged for 15 minutes at 2,000 g and the absorption of the yellow supernatant solution was measured at 540 mμ. against a whole blank.

Enzyme units are expressed as μM glutamyl hydroxamate formed per minute calculated from the absorption of a standard solution of glutamyl hydroxamate (1 mM).

The range of the method as used was from 0.01 - 0.5 μM/ml. of reaction mixture.

Thiosulphate sulphurtransferase

Thiosulphate sulphurtransferase was estimated by determination of the production of thiocyanate (Cosby and Sumner, 1946).

The substrate was 0.113 M KCN, 0.1 M $Na_2S_2O_3$, in 0.5 M phosphate buffer at pH 7.0.

0.1 ml. of enzyme extract was added to 0.4 ml. buffered substrate at 30°C. and the reaction was stopped after 10 minutes with 0.5 ml. $Fe(NO_3)_3$. This produced a violet colour which faded in about 15 minutes. The absorption of the remaining yellow colour was measured at 490 mμ. against a whole blank.

Enzyme units are expressed as μM thiocyanate produced per minute.

The range of the method as used was 0.1 - 1 μM thiocyanate per ml. reaction mixture.

Cathepsin-C

Cathepsin-C activity was measured by estimating, by means of the Folin Ciocalteau reagent (B.D.H.), the aromatic acids released from haemoglobin (Anson, 1937).

Haemoglobin was extracted from ox blood erythrocytes by osmotic shock, dialysed overnight against deionised water, and the concentration adjusted to 2.5 %. 4 ml. of this solution were added to 1 ml. of a solution 1.35 M to acetic acid and 0.02 M to $(NH_4)_2SO_4$. This prepared substrate was used immediately.

0.1 ml. enzyme extract was pipetted into 0.5 ml. substrate at pH 5.7, and 37°C. and incubated for 60 minutes. 1 ml. 0.3 N trichloracetic acid was added and the solution centrifuged at 2,000 g for 20 minutes. 3 ml. 0.5 N NaOH were added to 1 ml. supernatant. 1 ml. Folin's reagent was then added and the tube left for 10 minutes for the colour to develop. The absorption was measured at 750 mμ against a whole blank. A solution of 0.16 mM. tyrosine was used as a standard.

Enzyme units are μM tyrosine released per minute, and the range of the method as used was from 0.05 - 1 μM tyrosine/ml. of reaction mixture.

Aspartate aminotransferase

Aspartate aminotransferase activity was measured by estimating the formation of oxaloacetic acid (Wroblewski and Cabaud, 1957).

The substrate solution contained 40 mM dl-aspartic acid (B.D.H.) and 0.4 mM α-keto-glutaric acid (B.D.H.) buffered to pH 7.4 with 0.1 M

phosphate. 1 mM dinitrophenyl hydrazine in 0.1 N HCl was used to terminate the reaction.

0.1 ml. of enzyme extract was added to 0.5 ml. of the substrate solution at 40°C. 0.5 ml. dinitrophenyl hydrazine was added after 60 minutes, followed by 5 ml. 0.4 N NaOH after a further 10 minutes. The solution was allowed to stand for 30 minutes and then the absorption was measured at 505 mμ against a whole blank.

Enzyme units are expressed as μM°les oxalacetate produced per minute, and were calculated from the absorption of a standard pyruvate solution.

The range of the method as used was from 0.2 - 3 μM°les oxalacetate in the reaction mixture.

Lactate dehydrogenase

Lactate dehydrogenase was measured by estimating the disappearance of pyruvic acid (Cabaud and Wroblewski, 1958).

The substrate solution was 2.3 mM pyruvic acid (L. Light & Co.) buffered to pH 7.3 by 0.23 M phosphate, and contained 1 mg./ml. NADH. 5 mM dinitrophenyl hydrazine was made up in 2N HCl.

0.1 ml. enzyme extract was added to 1 ml. of buffered substrate at 37°C. After 30 minutes 1 ml. of dinitrophenyl hydrazine was added, and, 20 minutes later, 10 ml. 0.4 N NaOH were added. 10 minutes were allowed for colour development, and the absorption of the brown solution was measured at 550 mμ. The absorption of a whole blank and a pyruvate standard were also measured. The spectrophotometer was set to zero with deionised water.

Enzyme units are expressed as μM pyruvate hydrolysed per minute. The range of the method as used was from 1 - 5 μM/ml. reaction mixture.

Arginase

Arginase activity was determined by estimating the urea (Archibald, 1945) produced from arginine (Greenberg, 1955). Two methods were used, employing the same technique.

Method 1

The substrate solution was 0.05 M arginine at pH 9.5. The reagents for urea determination were sulphuric acid:phosphoric acid:water (1:3:1), and 4 mg.% α-isonitrosopropriophenone (Hopkins & Williams Ltd.) in absolute ethanol.

0.08 ml. enzyme extract was diluted to 0.2 ml. with 5 mM $MnSO_4$, and this was incubated for three hours at 37°C. 0.1 ml. arginine was then added and the incubation lasted 15 minutes at 25°C. The reaction was terminated with 15% acetic acid, and the supernatant, after centrifuging at 2,000 g for 20 minutes, was decanted into fresh tubes. 0.5 ml. sulphuric acid:phosphoric acid:water was added followed by 0.04 ml. α-isonitrosopropriophenone. This was boiled for one hour in the dark and the absorption of the resultant pink colour was measured at 540 mμ against a whole blank. The absorption of a urea standard was measured against a reagent blank of the urea reaction.

Arginase activity proved to be fairly low in most cells studied, with one or two exceptions, so the technique was modified with the aid of the Beckman/Spinco micro-analytical apparatus, to produce a higher sensitivity.

Method 2

This micro-analytical system employs 0.5 ml. capacity micro-centrifuge tubes, and a wide range of dispensing micro-pipettes delivering a measured volume. A micro-centrifuge and a tube-shaker are supplied, and the coloured test solutions may be read in a micro-spectrophotometer, capacity about 0.1 ml.

The reagents were modified slightly. The substrate was 0.125 M arginine. 0.1 M glycine:NaOH buffer and 2.5 mM $MnCl_2$ were made up separately and mixed, 7 parts buffer to 1 part $MnCl_2$, just before the assay (manganese is precipitated if this solution is stored).

The procedure was as follows:-

Sampling. 2×10^6 - 2×10^6 cultured cells were required for arginase assays by the micro method. Monolayer cultures were harvested by scraping with a rubber-coated glass rod, or by treatment with 0.25% trypsin. The cells were suspended in 10 ml. ice-cold Hanks' balanced salt solution (lacking phenol red); trypsinised samples were counted. Each sample was divided equally between two 10 ml. centrifuge tubes (the cells of one to be used as the test and the cells of the other to be used as a blank) and centrifuged at 900 g for 5 minutes. The pellets were resuspended in balanced salt solution (minus phenol red) and transferred to plastic microcentrifuge tubes. The plastic tubes were centrifuged for 15 seconds in the Beckman Microcentrifuge and the supernatant discarded. Sample tubes were stored at -70°C. until required for assay.

For arginase assay, 80 µl. buffered $MnCl_2$ was dispensed into each tube. The cell pellet was disrupted with a platinum wire, and the suspension was frozen and thawed three times. 10 µl. arginine were added to the tubes at 37°C., and, after 30 minutes, the reaction was terminated with 20 µl. 9 N perchloric acid. (Perchloric acid was added to the blanks before arginine). The precipitated protein was centrifuged for 2 minutes in the micro-centrifuge, and the supernatants transferred to micro-boiling tubes. 60 µl. sulphuric acid:phosphoric acid:water and 5 µl. α-isonitrosopropriophenone were added to the boiling tubes and they were placed in a light tight boiling water bath for one hour. The absorption of the resultant pink colour was measured in the microspectrophotometer at 540 mµ against a whole blank.

A urea standard was measured as above. Enzyme units are expressed as µM urea produced per minute.

The range of the method as used was from 0.1 - 1.0 µMole urea per ml. of reaction mixture. The micro-procedure allowed measurement of 0.01 - 0.1 µMole urea.

Total nitrogen

Total nitrogen was measured in extracts by Nessler ammonia determination of a sulphuric acid digest (Paul, 1958).

Digestion was carried out in 1% SeO_2 in 5 N H_2SO_4. Nessler's reagent was made up according to Paul (1958). 3.5 gm. gum acacia in 750 ml. water were added to a solution of 4 gm. potassium iodide and 4 gm. mercuric iodide in 25 ml. of water, and the volume was adjusted

to 1 litre.

0.2 ml. of the digestion mixture was pipetted into a 10 ml. freeze-dry ampoule. This type of tube was used, in conjunction with a glass rod to minimise loss of material by spurting. 0.1 ml. cell extract was added and the tube was placed on an electric element at such a temperature that the sulphuric acid was just on the point of boiling. (The mixture boiled rapidly at first until water was driven off, but after this boiling continued at a very slow rate). The tube was shielded with aluminium foil to avoid condensation on the stem of the tube during the early stages, and to maintain a high even temperature with the element at a low setting, producing even boiling without spurting.

The tubes used were marked to 10 ml., and the digest was diluted to this mark with distilled water. 2 ml. Nessler's reagent, and 3 ml. 2 N NaOH were added to 2 ml. of the diluted digest, and 15 minutes was allowed for colour development. The absorption of the yellow solution was then measured at 490 mμ.

The blank contained 0.1 ml. of the saline used for preparing the cell extract, in place of cell extract during digestion.

A range of 0.25 μg. - 7.5 μg. nitrogen was measured by this method.

The protein nitrogen of perchloric acid precipitates from arginase assays by the micro-method was also estimated. The precipitate was digested in 100 μl. of 2N NaOH at 100°C. for 30 minutes. 100 μl. were then withdrawn (the total volume in the tube equals the volume added

plus the volume of the precipitate) digested in H_2SO_4:SeO_2, and the protein nitrogen estimated by Nesslerisation as described above.

DNA-phosphorus estimation

DNA estimations of perchloric acid precipitates from arginase assays were performed by indole estimation of the DNA-phosphorus (Cerlotti, 1955).

The indole reagent was prepared by dissolving 0.4 gm. indole in 1 ml. absolute alcohol, and adding this solution to 1 litre of deionised water with constant swirling. Chloroform used for extraction after boiling was May & Baker laboratory reagent grade.

0.2 ml. 1 N perchloric acid was added to the precipitate from arginase estimations, and the pellet was suspended with a platinum wire. The tube was incubated at 70°C. for 45 minutes with repeated shaking. After 2 minutes centrifugation on the micro-centrifuge, 0.1 ml. of the supernatant was transferred to a fresh tube. 40 µl. concentrated HCl and 50 µl. indole reagent were added and the tube was placed in a boiling water bath for 10 minutes. The solution was then shaken three times with 150 µl. of chloroform, and the absorption of the residual yellow of the aqueous phase was measured at 490 mµ on the Beckman micro-spectrophotometer.

A standard was prepared from pure DNA.

The DNA of cultures used in permeability experiments was also estimated by the indole method. After extraction of acid soluble material, the cells were treated with 2 N perchloric acid at 70°C. for

10 minutes. The resultant extract was incubated at 100°C. for 20 minutes with 1 ml. concentrated HCl and 1 ml. 0.04% indole. After this the solution was shaken three times with chloroform. The absorption was measured at 490 mμ on the Beckman DB spectrophotometer.

The approximate range of these DNA-phosphorus determinations was from 0.5 - 5.0 μg. of DNA phosphorus.

Histochemical enzyme determinations on colonies

Colonies were grown in 9 cm. petri dishes from a monodisperse inoculum of 2,000 - 4,000 cells. Enzyme determinations were done on unfixed cells by the agar overlay method of Maio and DeCarli (1965).

Alkaline phosphatase

3% agar (Difco) was melted by heating under pressure (15 lbs./sq.in.) for 15 minutes. It was then cooled to 45°C. and an equal quantity of 0.2% paranitrophenyl phosphate (L. Light & Co.) in 0.1 M, pH 9.4, tris buffer added to it. 10 ml. of the agar mixture was then added to each petri and allowed to set.

After 10 minutes at 37°C. alkaline phosphatase activity could be detected over colonies with high activity by the appearance of a yellow colour.

β-glucuronidase

An equal quantity of 0.3% phenolphthalein glucuronide (L. Light & Co.) in 0.1 M phthalate buffer at pH 4.5 was mixed with 3% agar as above. 10 ml. were added to each petri dish and allowed to set. After one

hour 0.2 M NaOH was added to the plates and the occurrence of pink colour noted over colonies.

Glucose-6-phosphatase

Colonies were incubated at 37°C. in a solution containing 1 mg./ml. NADP (Sigma), 0.1 M glucose-6-phosphate (B.D.H.) and 50 µg./ml. iodonitrotetrazolium (B.D.H.) in tris-citrate balanced salt solution (see Appendix) at pH 7.5. The appearance of a faint pink colour indicated glucose-6-phosphatase activity (this required 24 hours in Y-5 cells).

2.5. Detection and characterisation of RNA

Autoradiography

The stripping film technique modified after Pelc (1956), was used to determine the activity and location of ^{3}H-labelled RNA in L "S" cells. The cells were labelled by incubation with ^{3}H-uridine (2 mc/μ Mole) in rotating suspension cultures (at ca. 50 revs./hour). In some experiments samples were withdrawn at intervals, immediately washed in ice-cold balanced salt solution, and fixed in ice-cold acetic-alcohol (one part glacial acetic acid to three parts ethanol). A drop of each fixed cell suspension was placed on separate slides and allowed to spread and dry (Method 1).

In other experiments cells were washed in cold balanced salt solution, and fixed in cold 70% ethanol. They were transferred to absolute ethanol, ethanol-xylene (1:1), xylene, xylene-ester wax (British Drug Houses) (1:1), and finally embedded in pure ester wax. The wax was centrifuged while still hot, and then allowed to set in the centrifuge tube. The block was removed and sectioned at 3 μ on a Leitz rotating microtome. Sections were mounted on gelatin-chrome alum treated slides (Method 2).

The slides bearing cells or sections were then treated with ice-cold ether-alcohol (3:1), or chloroform, to remove any traces of labelled material incorporated into lipid components of the cells. Test slides were placed in 0.1 M, pH 5.3 acetate buffer for five minutes, and control slides in 0.1 mg./ml. RNAse (Seravac, chromatographically prepared) in the same buffer. RNA is hydrolysed in 0.1 M pH 5.3 acetate buffer

if incubation is carried out for longer than five minutes (Fig. 2.2). It was for this reason that incubation of controls was reduced to five minutes in a high concentration of RNAse. The RNAse reaction was terminated by ice-cold 10% trichloracetic acid which also served to remove the acid soluble precursors of RNA.

The slides were then washed thoroughly in ice-cold distilled water (10 minutes, 5 changes) and dried.

The application of stripping film (Kodak AR 10) was carried out in the dark with a red safelight. The emulsion was either peeled off directly from plates marked into rectangles with a scalpel, or lifted from plates which had been marked and stored in a desiccator in the darkroom overnight. The emulsion peels off spontaneously from plates in very low humidity. The emulsion rectangles were floated on water and draped round the slides to cover the area bearing the cells. All slides were pretreated with 0.5% gelatin and 0.05% chrome alum to help the emulsion to adhere.

The slides were dried and packed in light tight boxes with silica gel and stored at 4°C. for two weeks. After this time they were developed and fixed in ice-cold, fresh D19b developer (Kodak Ltd.) and Amfix (May & Baker Ltd.) washed in iced water, and dried.

Coverslips were mounted in a drop of stain (methyl green pyronin - see below) and the preparations examined with a phase contrast 100X objective. Permanent preparations were made by mounting a coverslip in DPX resin on prestained specimens.

Fig. 2.2

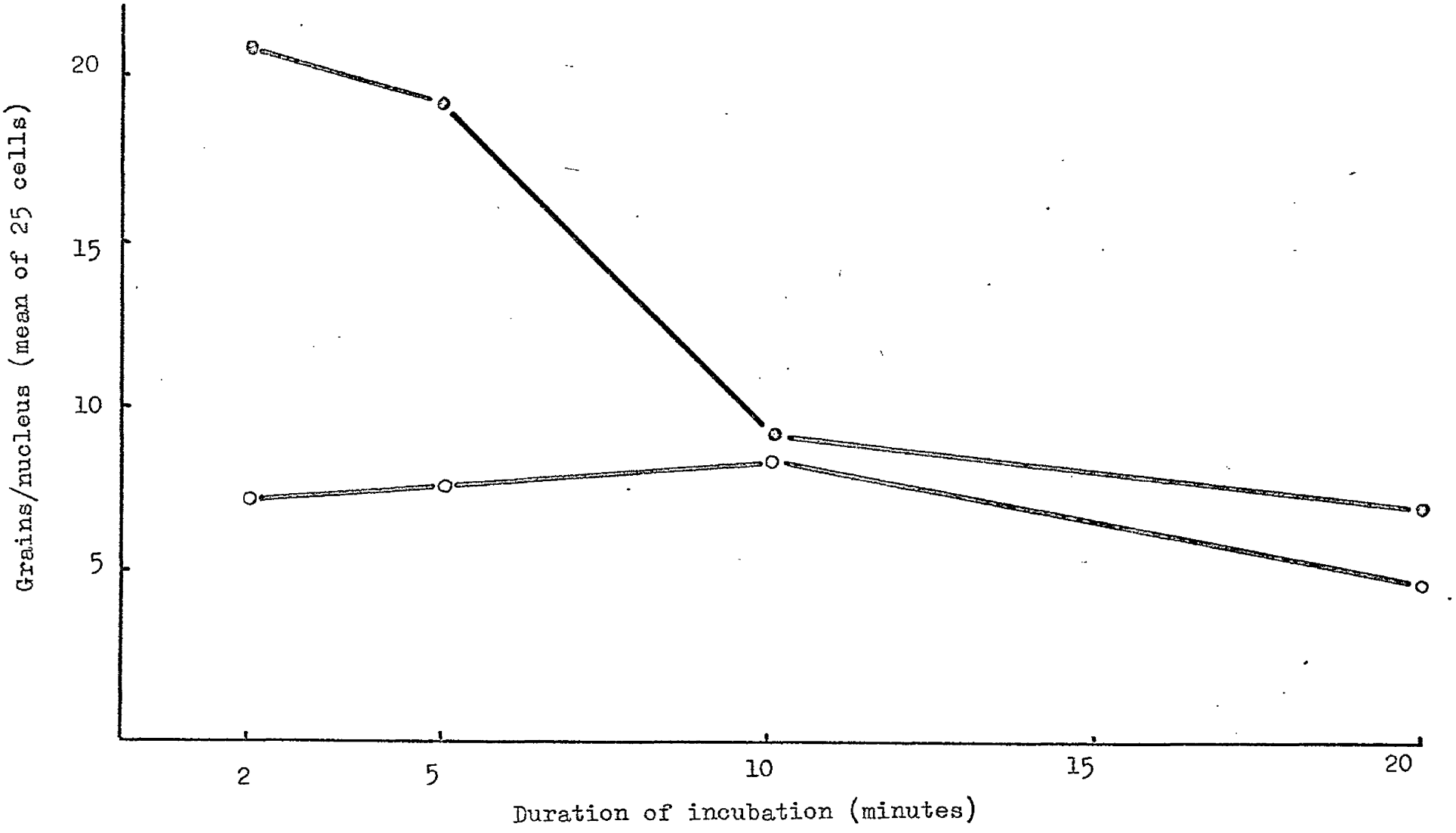
Loss of radioactivity during incubation of labelled L"S" cells in 0.1M acetate buffer at pH 5.3.

L"S" cells were labelled for 60 minutes with ^{3}H-uridine. Unfixed smears were prepared, and incubated with 0.1 mg/ml RNAse in 0.1M acetate buffer at pH 5.3 and in buffer alone. Slides were removed from the incubations at intervals, placed in ice-cold 10% TCA for 10 minutes, washed in ice-cold deionised water, and dried. Autoradiographs were prepared, and the nuclear grains counted.

o—o RNAse treated.

●—● Incubated in buffer only.

Fig. 2.2


Grains/nucleus (mean of 25 cells)
20
15
10
5
2
5
10
15
20
Duration of incubation (minutes)

The preparations were analysed by counting the silver grains occurring over specific regions of the cells. The grains over 50 cells were counted on each slide, and two slides were prepared from each sample. Only cells with complete cell membranes were counted.

Cytology of actinomycin-treated cells

Routine staining of RNA autoradiographs was performed with 0.065% methyl green, 0.165% pyronin G in 0.1 M, pH 4.8 acetate buffer. Slides were stained on ice (to prevent loss of adhesion of the autoradiographic emulsion) for 20 - 30 minutes, rinsed briefly in running tap water and dried. Extra-thin guage coverslips were mounted in DPX resin.

Slides of L "S" cells used for cytological description after prolonged treatment with actinomycin D were stained with methyl green and pyronin by the method of Gurr (1958). 0.15 gm. of methyl green (E. Gurr) was washed with chloroform and dried. It was then dissolved with 0.25 gm. Pyronin-Y (E. Gurr) in 97.5 ml. 0.02 M, pH 4.7 acetate buffer and 2.5 ml. 95% ethanol. Slides were stained for 20 minutes, washed rapidly in deionised water and differentiated in 95% ethanol for 5 minutes. After dehydration in absolute ethanol the preparations were cleared in toluene and mounted in DPX resin.

Slides from this experiment were also stained with haemalum and eosin for general morphological observations.

Preparations for fluorescent studies were fixed in acetic alcohol as before, and washed in dilute ethanol and in 0.1 M acetate buffer at

pH 5.6. The slides were stained for 15 minutes in 0.005% acridine orange in 0.1 M acetate buffer at pH 5.6 and mounted in 50% glycerine jelly in the same acetate buffer. They were examined on a Zeiss WL microscope with HBO 200 high pressure mercury lamp illumination with Zeiss BG 12 and UG 5 exciter and a Zeiss No. 47 barrier filter.

Separation of RNA in sucrose density gradients (After Britten and Roberts, 1960)

Reagents

Bentonite, an RNAse inhibitor used throughout this procedure, was prepared as follows: 8 gms. of crude bentonite (B.D.H.) was suspended in 100 ml. of deionised water. The suspension was homogenised for one minute and then centrifuged at 900 g for 10 minutes. The supernatant and fluid sediment were collected and recentrifuged at 25,000 g for 20 minutes. The resultant sediment was resuspended in 0.1 M versene (ethylene-diamine-tetraacetic acid, B.D.H.) at pH 7 and homogenised briefly. The suspension was left for 48 hours at 25°C. and thencentrifuged at 25,000 g for 20 minutes.

The final sediment contained a dark grey layer at the bottom of the tube and a lighter grey layer sedimenting above it. The dark grey material was discarded and the lighter grey band was resuspended in 30 ml. 0.01 M, pH 6, acetate buffer. One ml. was dried and weighed, and the concentration of the remainder adjusted to 6%.

The extraction of RNA utilised 90% phenol, 0.5% duponal (dodecyl sulphate, L. Light & Co.), and a 0.01 M acetate buffer at pH 5.2

containing 0.05 M NaCl and 1 mM $MgCl_2$ (buffer A). 0.01 M acetate buffer at pH 5.2 containing 0.05 M NaCl and 0.02 M versene (ethylene-diamine-tetraacetic acid, E.D.H.) was also used (buffer B). 3% and 25% sucrose solutions were made up in either buffer A or buffer B.

Preparation of sucrose gradients

Sucrose gradients were first prepared by using a mixing device (Fig. 2.5) but more satisfactory results were obtained by simply pipetting 2.25 ml. 25% sucrose in buffer A into the bottom of the tube and carefully layering 2.25 ml. 3% sucrose in buffer A on top of it. This was allowed to diffuse either for 24 hours at 4°C. or 12 hours at room temperature. The gradient tends to be sigmoid rather than linear but is reproducible (Fig. 2.4). Gradients prepared in the mixer were more linear but non-reproducible deviations from linearity were common (Fig. 2.5).

Preparation of RNA

RNA for sucrose density gradients was extracted with phenol and duponol (dodecyl sulphate) (Scherrer and Darnell, 1962) in the following manner. 50 - 100 million L "S" cells were suspended in 5 ml. buffer A. 1 ml. 0.6% duponol and 5 ml. 90% phenol were added to this and the whole shaken for 8 minutes. This was centrifuged at 900 g for 20 minutes and the aqueous phase removed. The phenol phase was re-extracted with 2 ml. buffer A, and the combined aqueous phases re-extracted with 1 ml. 90% phenol. The RNA in the aqueous phase was precipitated with

Fig. 2.3

Mixing device for the preparation of Sucrose density gradients.

The mixer was made from a perspex block. The sucrose reservoirs were designed to hold approximately 2.5 ml each. A slow stream of bubbles (about 5/sec.) was blown through the 25% sucrose to ensure mixing.

Gradients were prepared by opening valve 2, allowing a trickle of 25% sucrose to enter the centrifuge tube, and then immediately opening valve 1. 3% sucrose entered the 25% sucrose reservoir and gradually reduced its density as the level dropped. The sucrose solution, of gradually reducing density, was run into the centrifuge tube through a fine plastic tube, held just above the surface of the sucrose solution in the centrifuge tube.

Fig. 2.3

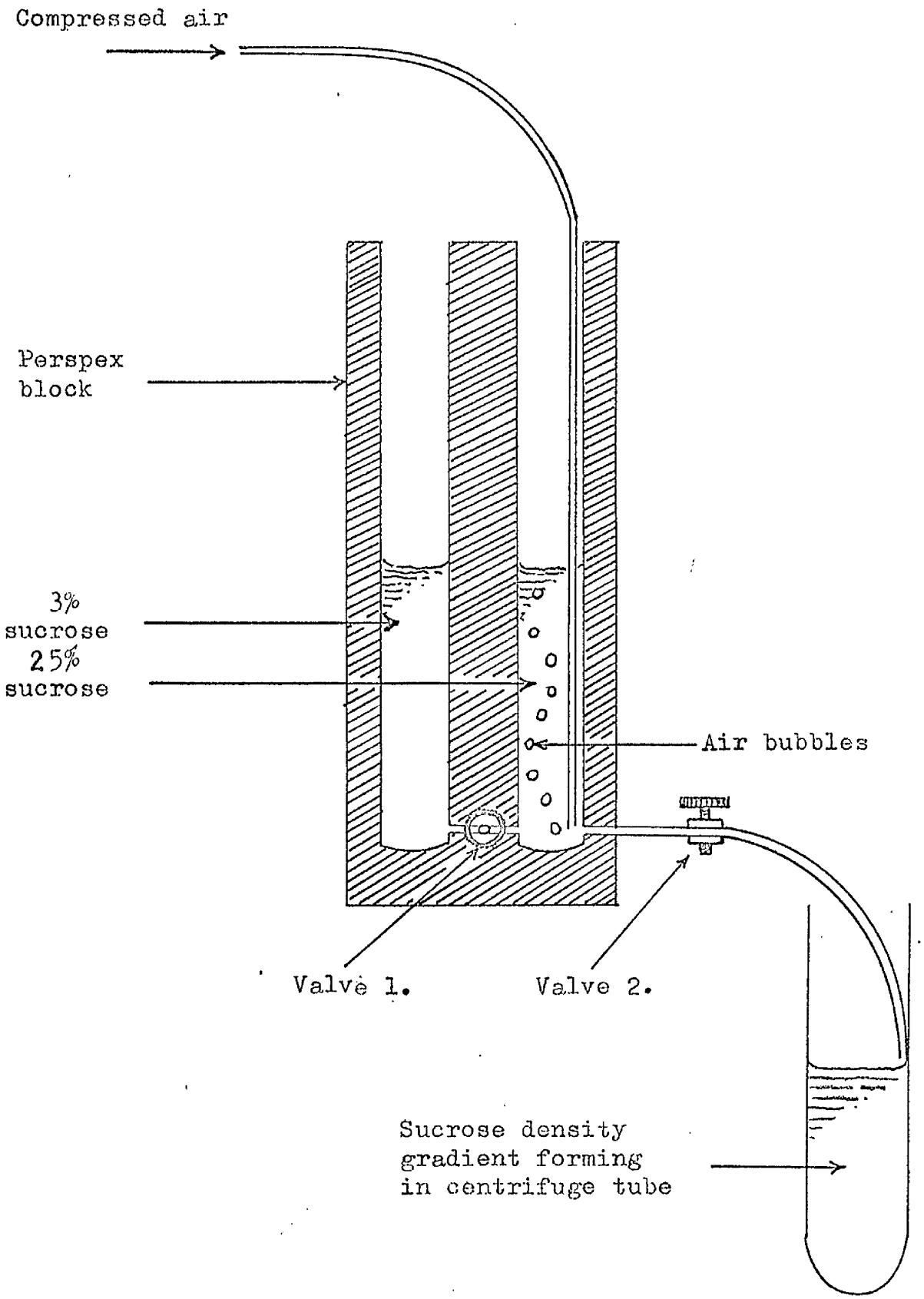

Compressed air
Perspex block
3% sucrose
25% sucrose
Air bubbles
Valve 1.
Valve 2.
Sucrose density gradient forming in centrifuge tube

Fig. 2.4

Sucrose gradients prepared by diffusion.

Sucrose gradients were prepared by layering 2.25 ml 3% sucrose in buffer A (see "Materials and Methods") on top of 2.25 ml 25% sucrose in buffer A. The gradients were allowed to stand for 12 hours, and then centrifuged at 44,000g with 0.5 ml of an RNA solution layered on the top.

The tubes were pierced and the sucrose concentration of the drop samples was measured with a refraction saccharimeter.

Fig. 2.4

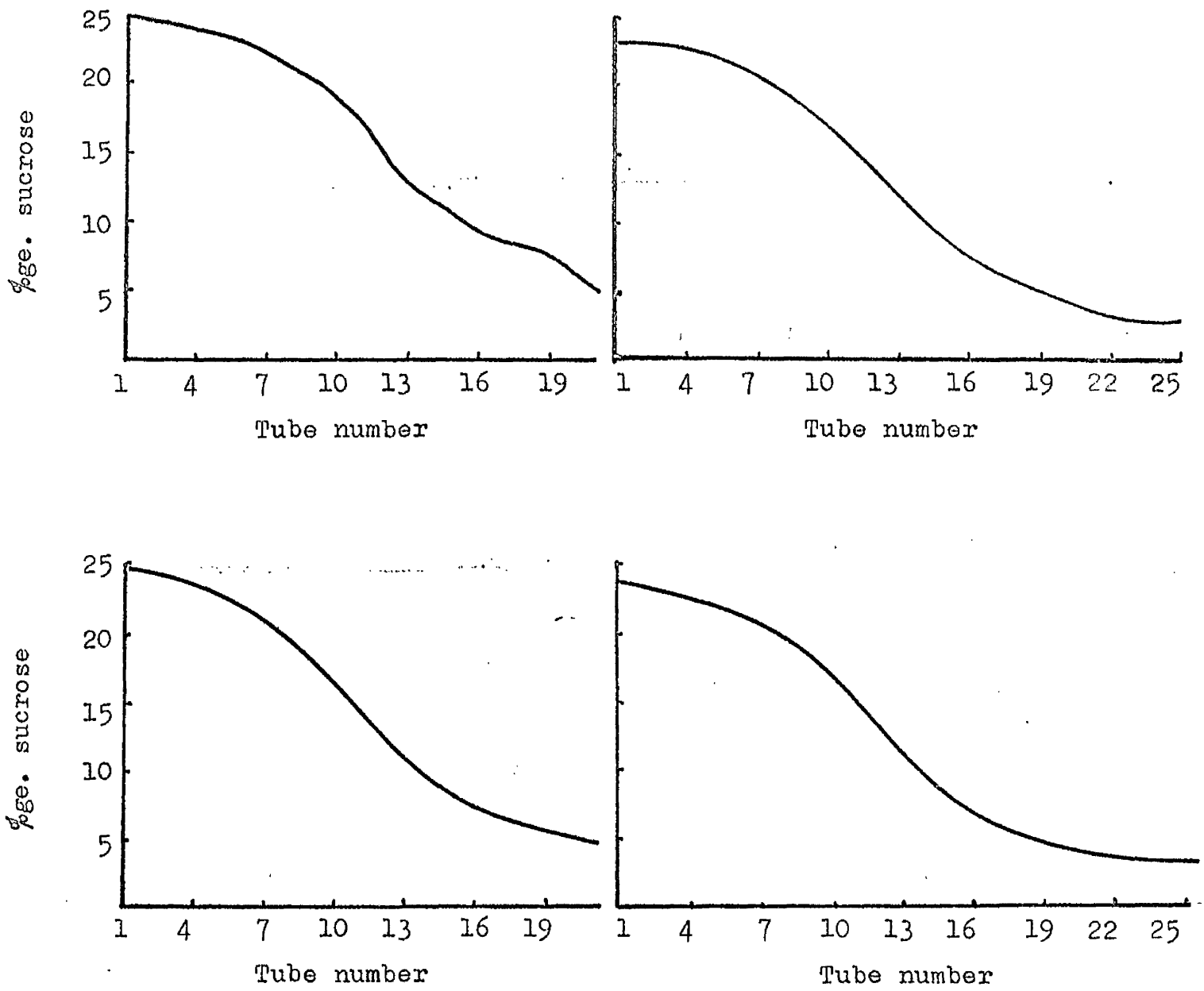
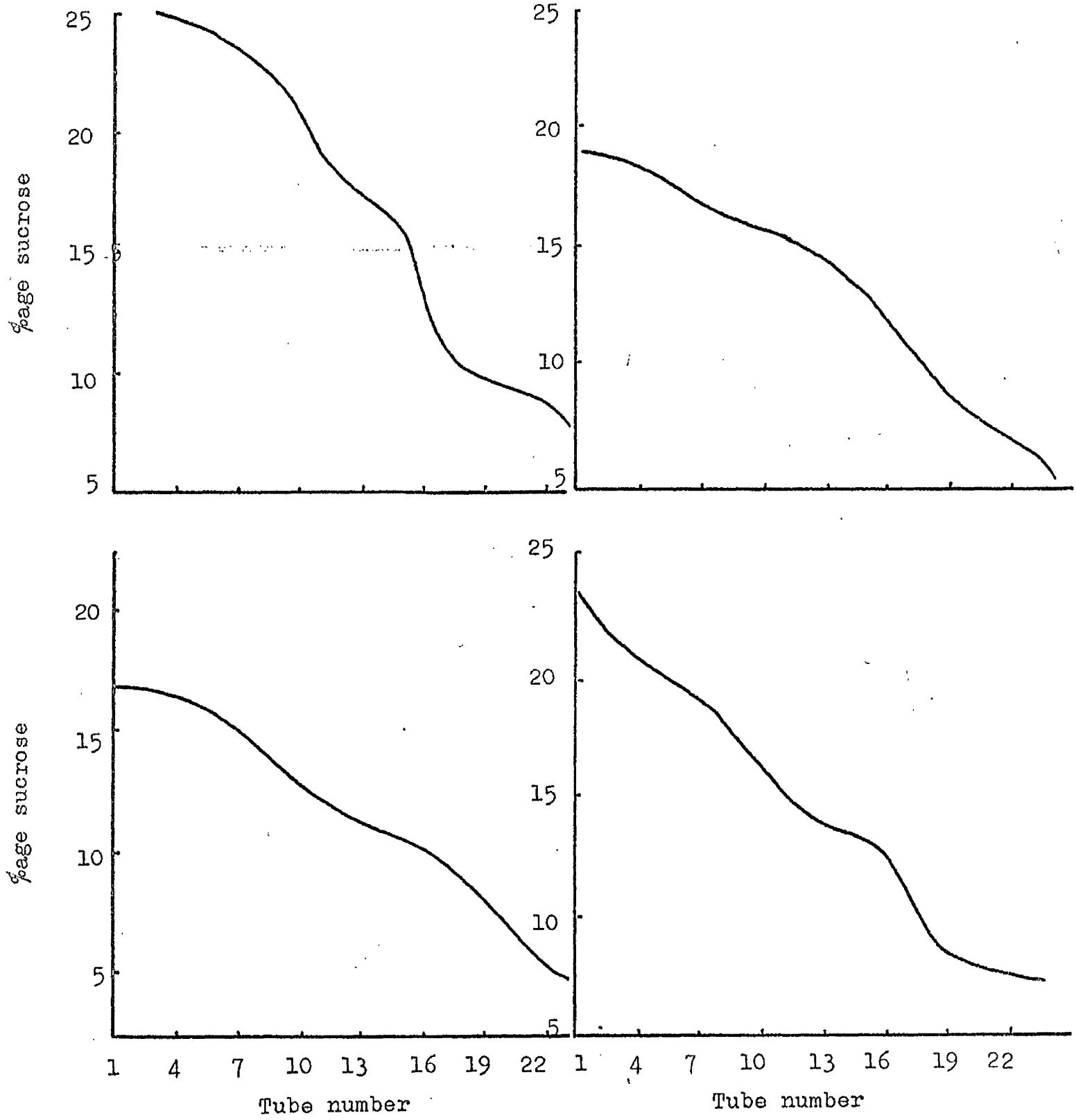

%ge. sucrose
25
20
15
10
5
1 4 7 10 13 16 19
Tube number
1 4 7 10 13 16 19 22 25
Tube number
%ge. sucrose
25
20
15
10
5
1 4 7 10 13 16 19
Tube number
1 4 7 10 13 16 19 22 25
Tube number

Fig. 2.5

Sucrose gradient prepared in mixer.

Sucrose gradients were prepared in the mixing device described in Fig. 2.3. They were centrifuged with an RNA supernatant and sucrose estimations of drop samples were performed as in Fig. 2.4.

Fig. 2.5



2 % NaAc and 2 volumes absolute ethanol (cold) and placed at -70°C. to flocculate. The flocculant precipitate was spun down at 900 g and the supernatant withdrawn. The precipitate was redissolved in buffer A and dialysed overnight against 7 litres of the same buffer, then for a further 5 hours against fresh buffer. Two drops of 6% bentonite were added at intervals.

The dialysate was precipitated with cold ethanol and redissolved in 1 ml. of buffer A. The ethanol was extracted with ether and the ether blown off with nitrogen. The solution was spun at 900 g for 10 minutes to remove bentonite and then 0.5 ml. of the supernatant was withdrawn and pipetted carefully on to the top of a 3% to 25% sucrose gradient. This was centrifuged in the SW 39 head of a Spinco model L centrifuge for 11½ hours at 44,000 g.

The spinco tubes were then pierced with a hypodermic needle and the drops collected; 10 - 20 per tube, depending on the drop size, to give a total of 25 - 30 tubes. 0.1 ml. of each drop sample was pipetted into fresh tubes and the remaining drop used for sucrose estimation in a refraction saccharimeter. The 0.1 ml. samples were diluted to 5 ml. and the ultraviolet absorption measured at 256 mμ in a Beckman DB spectrophotometer with an automatic sample changer.

1 ml. was transferred from every second sample (from every sample at peaks of ultraviolet absorption or at predicted peaks of activity) and mixed with 8 ml. NE572 "Scintsint" dioxane-based liquid scintillator (Nuclear Enterprises) in special vials. These vials were then fed automatically

into a Packard Tricarb scintillation counter and each one counted for 30 minutes or for a maximum of 10,000 counts.

As Montagnier and Sanders (1963) and Burness et al. (1963) have used versene in the preparation and centrifugation of RNA, it was prepared here in 0.02 M versene in 0.01 M, pH 5.2, acetate buffer containing 0.05 M NaCl (buffer B) and its sedimentation compared with RNA prepared in buffer A. (Versene is a chelating agent, and may influence the secondary configuration of RNA molecules and the degree of aggregation between molecules). The procedure was as before only buffer B was substituted for buffer A at all stages. Material prepared in buffer B was centrifuged on sucrose gradients made up in buffer A and sucrose gradients made up in buffer B. Similarly material prepared in buffer A was centrifuged on both types of sucrose gradient.

Fig. 2.6 shows that material prepared in versene yields only 25 - 30% of the RNA obtained by preparation in magnesium-buffer. In addition material prepared and centrifuged in versene tends to produce subdivided peaks of ultraviolet absorption in the gradient, although the presence of Mg^{++} in the gradient tends to reverse this. Consequently, the magnesium-buffer procedure was adopted.

Fig. 2.6

Sedimentation of RNA in sucrose gradients containing magnesium or versene.

RNA solutions, prepared from L"S" cells in buffer A and buffer B (see text) were layered on to 3-25% sucrose gradients in buffer A or buffer B. Buffer A contained 1mM $MgCl_2$ and no versene; buffer B contained 0.02M versene and no magnesium. After centrifugation at 44,000g for 11½ hours, the tubes were pierced and the optical density of the drop samples was determined at 260mμ.

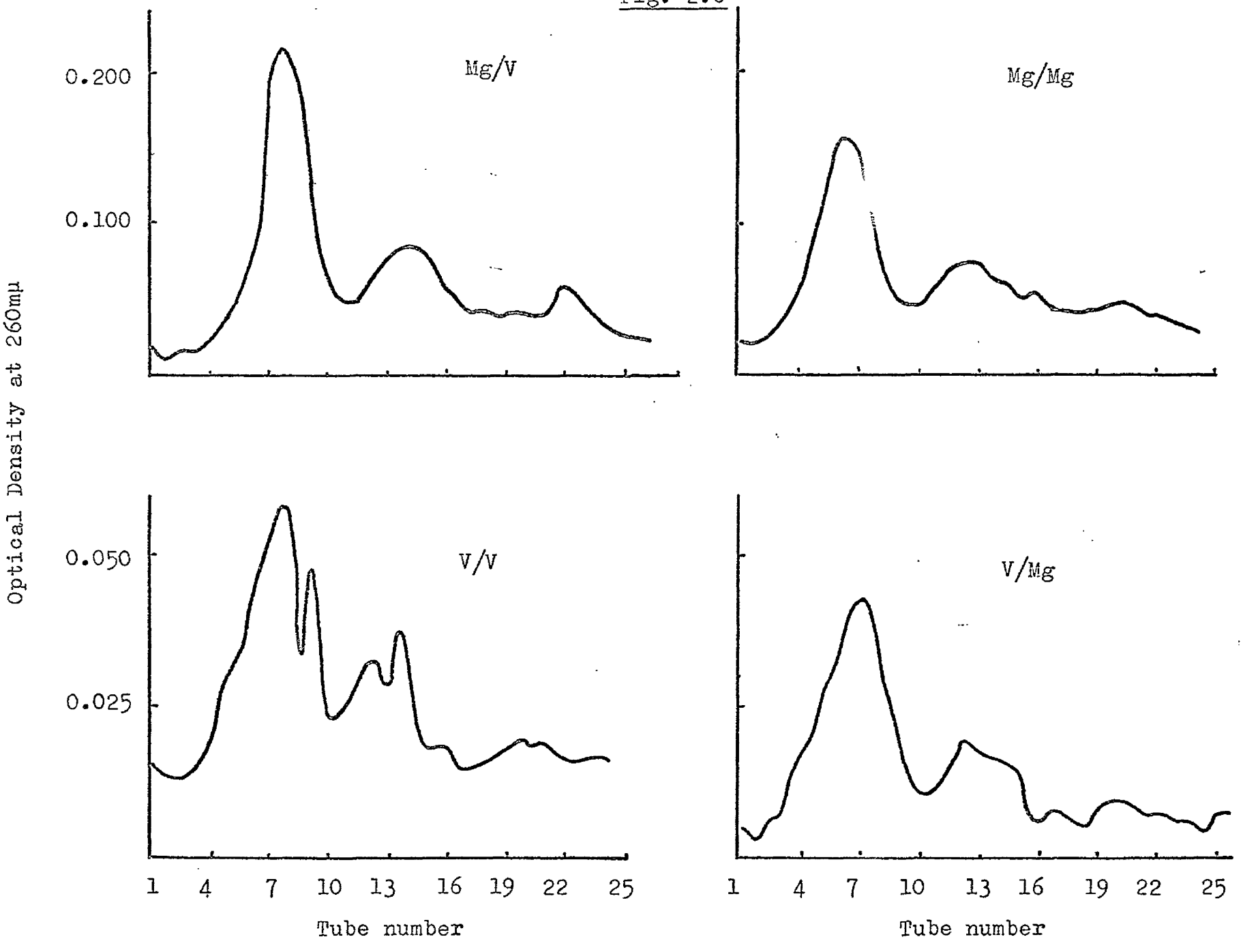
Mg/V - RNA prepared in magnesium/buffer (buffer A) and centrifuged on versene/buffer (buffer B)/sucrose.

Mg/Mg - RNA prepared in magnesium/buffer and centrifuged on magnesium/buffer/sucrose.

V/V - RNA prepared in versene/buffer and centrifuged on versene/buffer/sucrose.

V/Mg - RNA prepared in versene/buffer and centrifuged on magnesium/buffer/sucrose.

Fig. 2.6


Mg/V
Mg/Mg
V/V
V/Mg
0.200
0.100
0.050
0.025
Optical Density at 260mμ
1 4 7 10 13 16 19 22 25
Tube number
1 4 7 10 13 16 19 22 25
Tube number

2.6. Measurement of ^{3}H-arginine uptake

Final method adopted

Sufficient 25 ml. conical flasks to give three per experimental sample were inoculated with 0.5 - 1 x 10^6 HeLa cells in 5 ml. Eagle's medium plus 5% calf serum and 2% human serum in tris-citrate buffered balanced salt solution. These cultures were grown for 3 days. The supernatant medium was then replaced with 5 ml. tris-citrate buffered balanced salt solution containing 0.1 mM arginine and 1 mg./ml. glucose (preincubation medium), and the flasks incubated for a further 30 minutes at 37°C. in a shaking water-bath. The preincubation medium was then removed and 0.5 μc./ml. - 2.5 μc./ml. tritiated arginine (specific activity 5-25 mc/μM) in the same balanced salt solution was added for two minutes.

After removing the tracer, the flasks were washed five times in the course of 25 seconds with balanced salt solution containing 0.1 mM unlabelled arginine and no phenol red. After flicking off the last drop of balanced salt solution, 2 ml. ice-cold 0.2 N perchloric acid were added and the flasks placed on ice for 30 minutes. The perchloric acid extract was decanted into a test tube, and 0.5 ml. 1 N KOH added to it to precipitate the perchlorate which lowers the efficiency of counting the isotope.

The radioactivity of the acid soluble extracts was determined with a liquid scintillation counter by mixing 1 ml. test solution with 8 ml. NE572 "Scinstant" liquid dioxane-based scintillator (or 0.5 ml. test solution with 4 ml. NE 572) in special vials, which were fed automatically

into a Packard Tricarb or Nuclear Chicago liquid scintillation counter. The results were expressed as counts per minute at 100% counting efficiency. Counting efficiency was obtained from the ratio of the counts at two energy levels.

Finally, the amount of DNA in each sample was determined as follows. 2 ml. 2 N perchloric acid were added to the residue in each culture flask and they were then incubated at 70°C. in a shaking water-bath for 20 minutes. The perchloric acid extracts were decanted and DNA estimations carried out on each extract.

Justification of the assumptions of the method

The completeness of the acid soluble extraction was determined as follows: 25 ml. flasks containing HeLa cultures were incubated for one hour with 10 μc./ml. ^{3}H-arginine in tris-citrate balanced salt solution containing 100 μg./ml. glucose and 0.1 mM arginine. The cultures were then washed with ice-cold balanced salt solution and 2 ml. ice-cold 0.2 N perchloric acid were added. The flasks were left on ice for 30 minutes and then the perchloric acid was replaced with a fresh 2 ml. of ice-cold perchloric acid; this was repeated twice more. The second, third and fourth perchloric acid extractions were carried out for 10 minutes only.

0.5 ml. 1 N KCl was added to each perchloric acid extract, and after precipitation of potassium perchlorate, the radioactivity of the supernatant was determined. Table 2.3 shows that the cold perchloric acid extraction of the acid soluble pool is more than 95% complete with

Table 2.3

Extraction of acid-soluble ^{3}H-arginine from HeLa cells

	1st Extraction 0.2N PCA 30 min.	2nd 10'	3rd 10'	4th 10'
Sample A	394	15	5	10
Sample B	1022	60	13	16

500,000-1,000,000 HeLa cells were incubated with 10μc./ml. ^{3}H-arginine for one hour. They were then washed 4 times with cold balanced salt solution. The first acid soluble extraction was made with 2 ml. 0.2N perchloric acid for 30 minutes at 4°C. The second, third and fourth extractions lasted 10 minutes only. The figures represent counts per minute in the acid soluble extract.

the first extraction of 30 minutes.

The validity of the method also depends on the assumption that most of the labelled arginine is recovered during extraction with 0.2 N perchloric acid, and is not bound within the cell. The proportion of incorporation of ^{3}H-arginine into the acid soluble pool and into acid insoluble material is shown in Table 2.4. Four flasks of 5 x 10^{5} HeLa cells were grown for three days. After 30 minutes incubation in preincubation medium samples were labelled with 0.5 μc./ml. ^{3}H-arginine for 15 minutes and 2 hours. The cells were then washed 5 times with cold balanced salt solution, and the acid soluble material was extracted 0.2 N perchloric acid for 30 minutes on ice. The residues were then incubated with 0.2 N NaOH for 30 minutes in a 70°C. shaking water-bath. 0.5 ml. 1 N KOH was added to the perchloric acid extracts, and the NaOH soluble material was adjusted to neutrality with 1 N HCl.

The results (Table 2.4) showed that, during incubation with ^{3}H-arginine for less than 15 minutes, more than 90% of the tracer was recovered in the acid soluble extract. Longer incubation with tracer allowed greater incorporation into acid insoluble material and this reached about 30% of the total at 2 hours.

The rate of uptake was determined by incorporation for 2 minutes in most experiments. This was chosen as a time at which influx greatly exceeded efflux. This was determined by incubation of HeLa cells from 2 minutes to 2 hours, in 2.5 μc./ml. ^{3}H-arginine. Flasks of HeLa cells were grown up from 5 x 10^{5} inocula for three days.

Table 2.4

Proportion of ^{3}H-arginine incorporation into acid-soluble and insoluble material

Time of incubation with ^{3}H-arginine (0.5μc/ml)	Counts/minute in acid-soluble extract	Counts/minute in acid insoluble residue
15 mins.	13,800	946
	20,000	2,093
2 hours	23,100	8,820
	15,600	8,390

After cells were extracted with 2 ml ice cold 0.2N perchloric acid, the residue was dissolved in 2 ml 0.2N NaOH. Both sets of samples were neutralised and 0.5 ml was mixed with 4 ml NE572 scintillator in dioxane and counted.

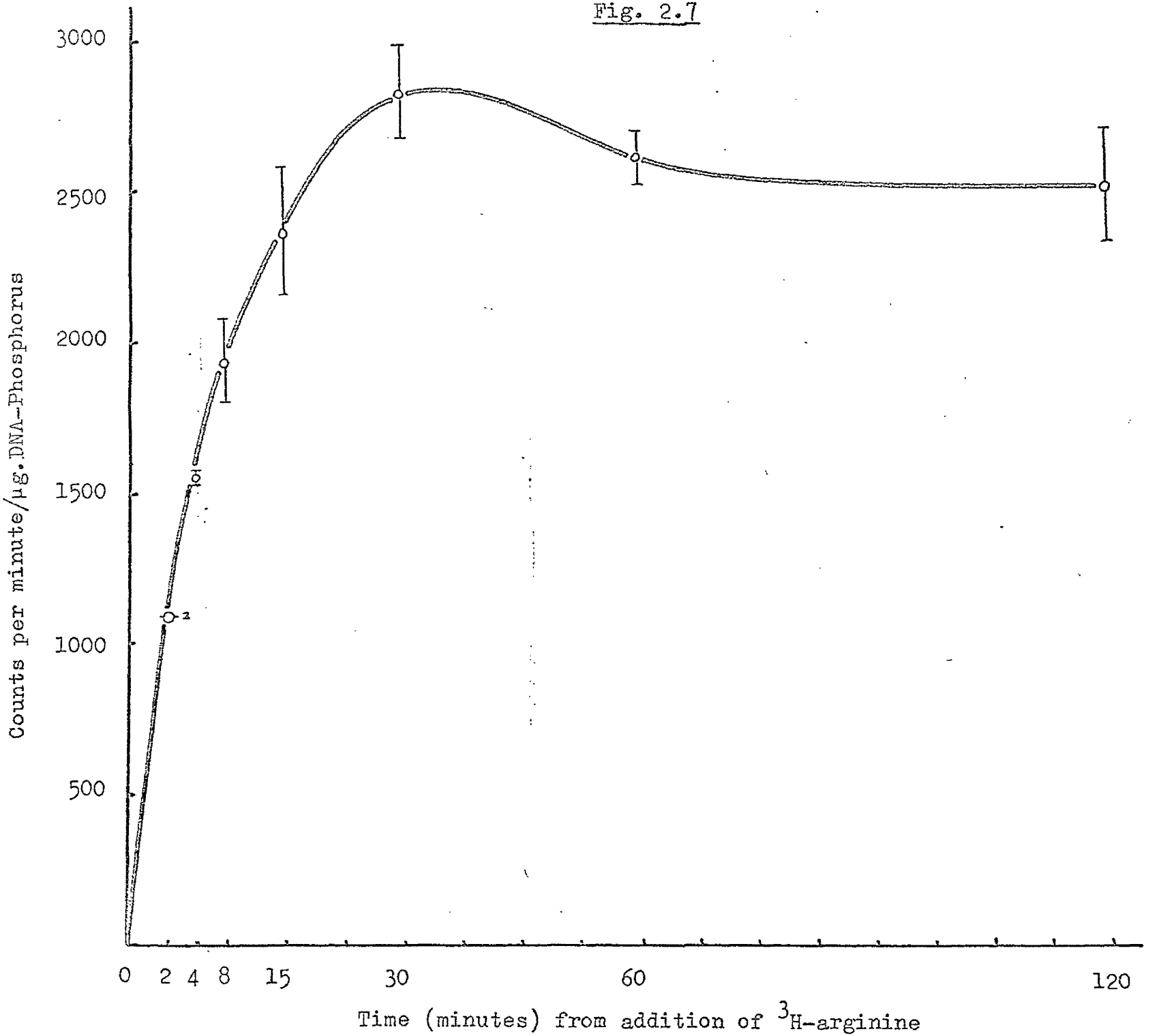
The cells were then incubated for 30 minutes in preincubation medium (see above). 2.5 µc./ml. ^{3}H-arginine were then added to the flasks and samples were taken for acid soluble extraction at times ranging from 2 minutes to 2 hours. The results are presented in Fig. 2.7. It appears from those results that equilibrium between influx and efflux was reached at 30 minutes, and hence at 2 minutes the efflux could be neglected for practical purposes.

Fig. 2.7

Uptake of ^{3}H-arginine in HeLa cells after incubation for different lengths of time.

3 day-old cultures of HeLa cells were incubated for 30 minutes in tris citrate BSS containing 0.1mM arginine and 1 mg/ml glucose and then incubated with 2.5 µc/ml ^{3}H-arginine. Samples were removed at intervals, washed and the acid soluble material was extracted in 0.2M perchloric acid. The DNA-phosphorus of the residues was then determined. The radioactivity of 1 ml of the acid soluble extract was measured on a Packard Tricarb liquid scintillation counter, and is expressed as counts per minute per µg.DNA-phosphorus.

Fig. 2.7


3000
2500
2000
1500
1000
500
Counts per minute/µg.DNA-Phosphorus
0
2
4
8
15
30
60
120
Time (minutes) from addition of ^{3}H-arginine

R E S U L T S

3. Results

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3.1. Spontaneous variation in cultured cells

Instability of the karyotype in culture

Y-5 Chinese hamster cells, grown in Eagle's medium (see Appendix) plus 5% calf serum, were cloned frequently in the same medium in an attempt to produce a strain of cells with a high cloning efficiency and a stable karyotype (chromosome complement) for use in experiments on drug resistance. However, this proved to be more difficult than had been anticipated.

Examination of the karyotype of these cells revealed that there was considerable variation in the modal number of chromosomes. Variation was also detected in the chromosomes between karyotypes with the same chromosome number. Spontaneous variation continued even after cell strains were cloned. When the strain was isolated by Yerganian in 1950 the chromosome count was 22 which is the diploid number found *in vivo*. During culture in this laboratory the strain was cloned several times and the chromosome number varied from 21 - 28 (Fig. 3.1). Five clones out of nine had 23 chromosomes; in two of these, Y-5-10-1-11(8) (Y-5-11) and Y-5-10-1-11-8-3 (Y-(6)3), there was considerable variation in the chromosomes between cells with the modal number (Fig. 3.2). Strain Y-(6)3, which had been cloned seven times in this laboratory, showed structural alterations particularly in the large metacentric chromosomes; both strain Y-5-11 and strain Y-(6)3 carried an extra sub-telocentric chromosome.

Strain Y-5-10-1 was grown in 10% calf serum and 0.5% bactopeptone,

Fig. 3.1

Modal distribution of chromosomes in clones of the X-5 Chinese hamster fibroblasts.

Clones were isolated by the dilution technique, and their chromosomes were examined by the air dry method. The chromosomes of 25, 50 or 100 cells were counted in each case. The modal distribution of 7-5-10-1 cells (Ruddle unpublished) was estimated from photographs of nine cells.

Fig. 3.1

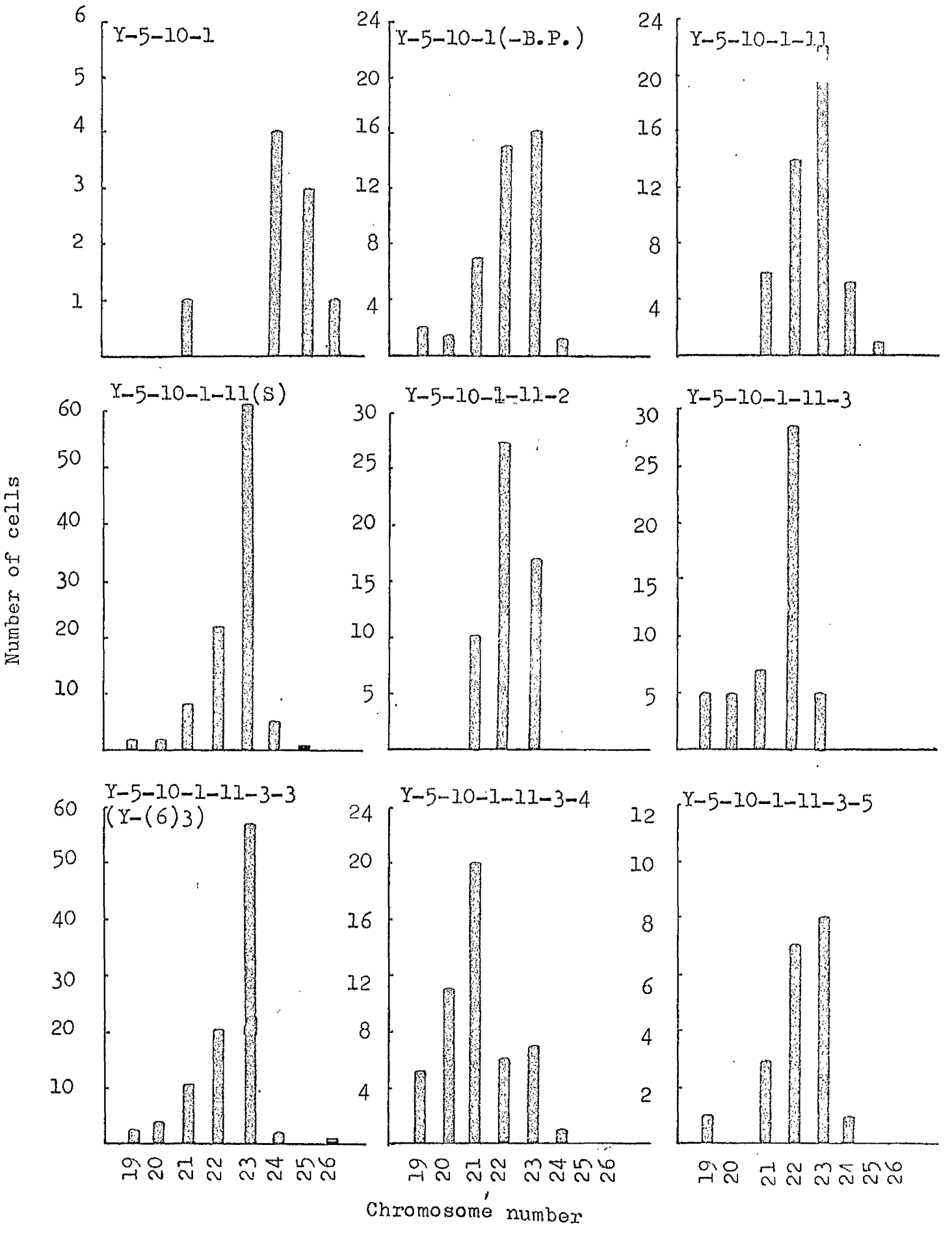

Y-5-10-1
Y-5-10-1(-B.P.)
Y-5-10-1-11
Y-5-10-1-11(S)
Y-5-10-1-11-2
Y-5-10-1-11-3
Y-5-10-1-11-3-3
(Y-(6)3)
Y-5-10-1-11-3-4
Y-5-10-1-11-3-5
Number of cells
Chromosome number
19 20 21 22 23 24 25 26

Fig. 3.2

Karyotypes of Chinese hamster fibroblasts.

Chromosome preparations were made by the air dry method, and stained with 1% orcein in 50% acetic acid. Photographs were taken and idiograms prepared. Each example (II, III & IV) is a synopsis of 10 idiograms.

I = clonal diploid (Yerganian, 1958)

II = X-5-10-1 clone (Biddle, 1961, unpubl.)

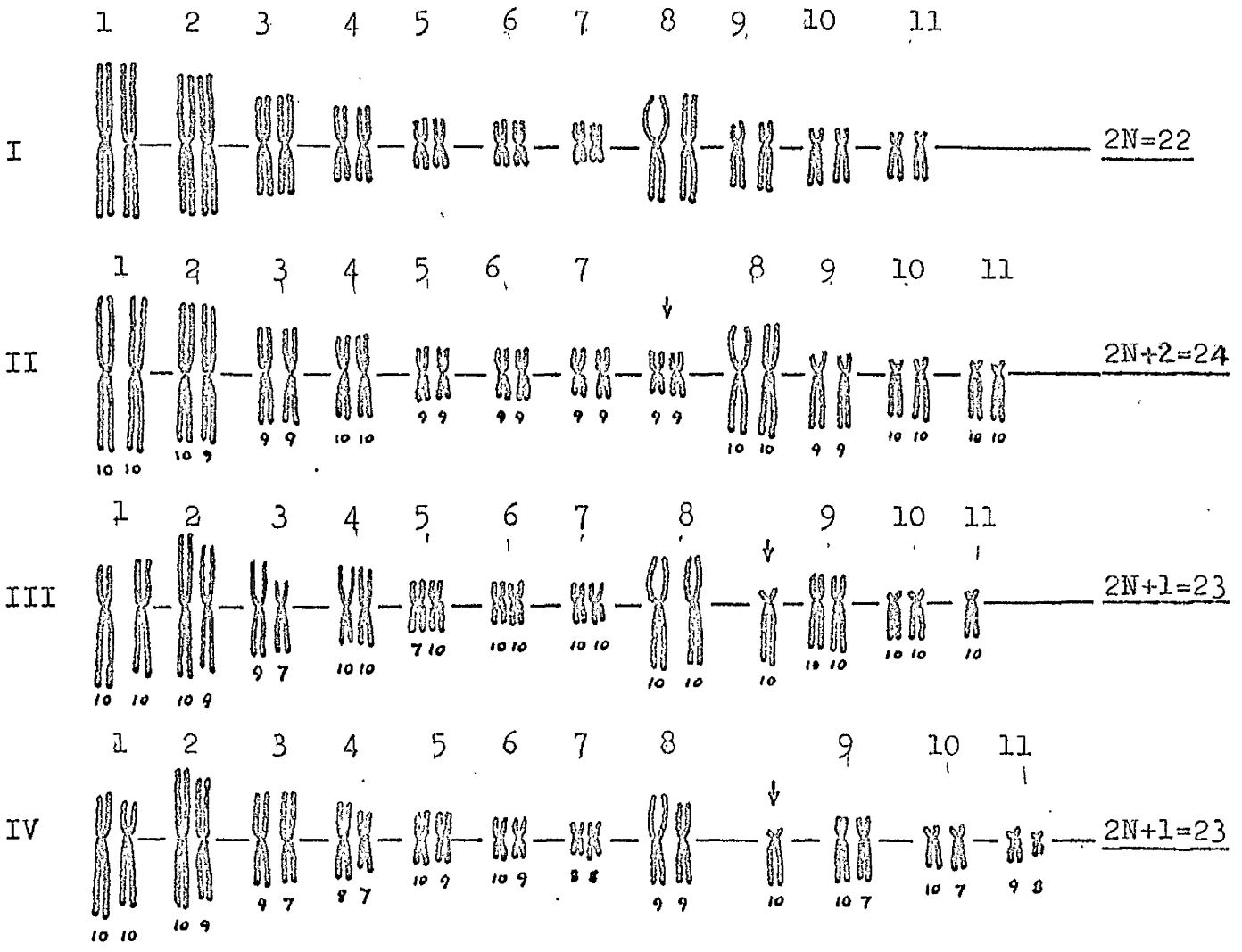
III = X-5-10-3-11 (X-5-11) clone

IV = X-5-10-1-12-3-3 (X-(6)3) clone.

Numbering (large numbers) is according to Yerganian (1958).

Small numbers refer to the frequency of occurrence of each chromosome in 10 idiograms.

Fig. 3.2



and was derived from one of a group of clones grown in a mouse feeder culture. The karyotype closely resembled the classic diploid although it contained an extra pair of metacentric chromosomes (Fig. 5.2). Further cloning of this strain was performed in the absence of feeder layers and in 5% serum with no bactopeptone; the cloned lines were maintained in this medium. The reduced supplement and stringent cloning conditions may have contributed to the instability in the karyotype.

The P2 clone of strain L mouse fibroblasts has a modal chromosome number of 56-57 although the mouse has 40-41 chromosomes. All the mouse chromosomes are telocentric but there are many metacentrics and sub-metacentrics in the P2 clone (Fig. 5.3). It is possible that many chromosomes of the P2 karyotype have arisen by centromeric fusion as is also suggested by the length of the centromeric regions of some of these chromosomes.

The evidence for the extensive changes in the karyotype of the P2 strain indicates that considerable alteration of the chromosomes may be involved in the adaptation of animal cells to culture. The variation of the karyotype in the Y-5 strain implies that a perpetual instability may be produced by certain culture conditions.

Enzyme activity of homologous clones

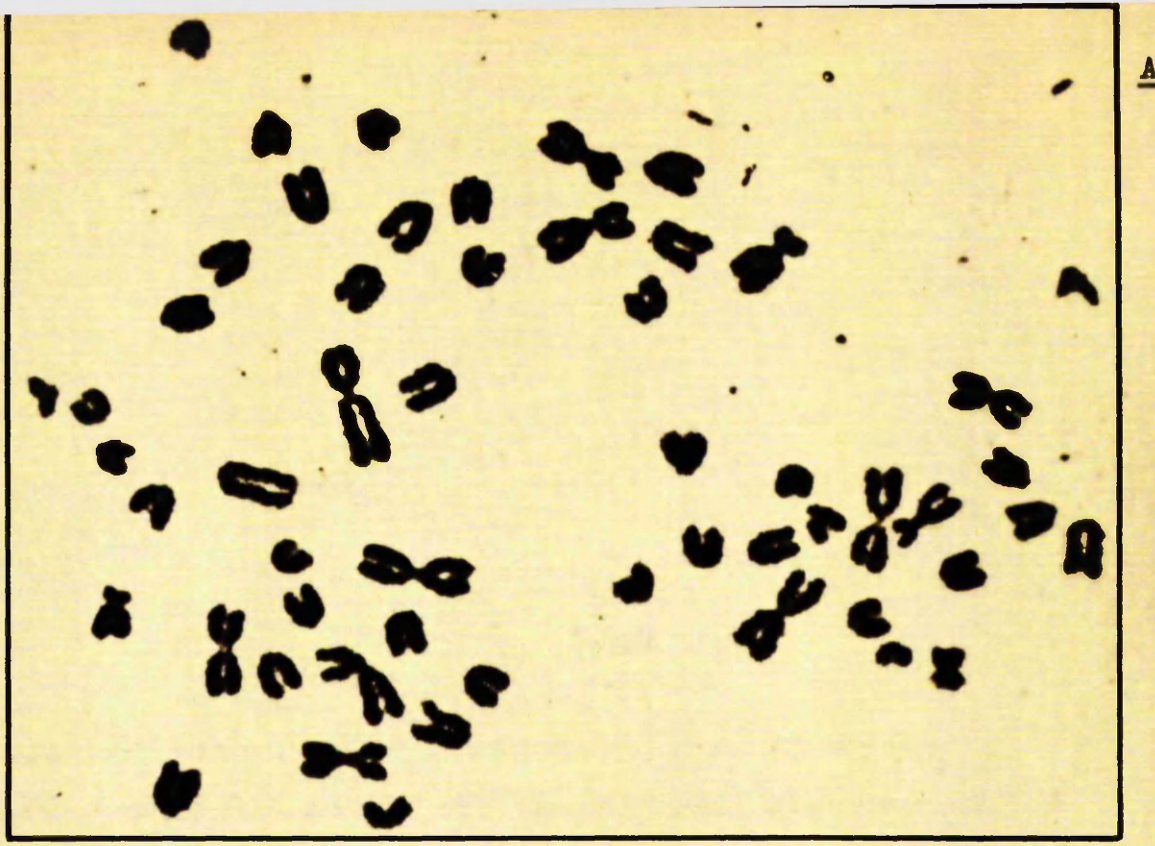
The instability of the karyotype implies a persistent heterogeneity of the cell population. A different example of heterogeneity, which may be related to chromosomal heterogeneity, has been observed in the results

Fig. 3.3

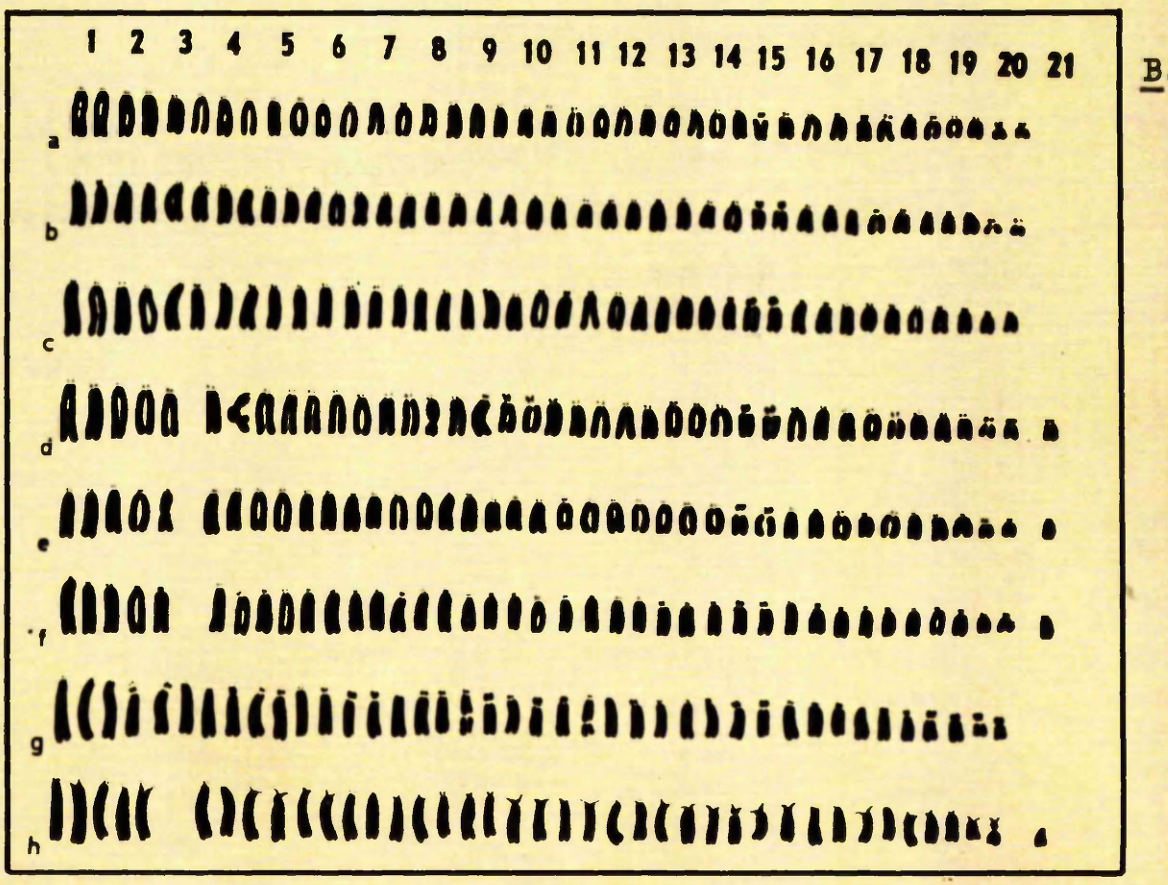
A P2 cells were grown on coverslips and chromosome preparations were made by the air-dry method. They were photographed under a 100X phase contrast objective. Total magnifications x2,400.

B Typical mouse karyotypes; a,d,g: Swiss mice; b,c,h: C3H; e,f: C57BL. a-c,g: female karyotypes; d-f,h: male karyotypes.

Magnifications x1,100.



A.



B.

Fig. 3.3

of estimation of the activities of a number of enzymes in colonies grown together in one vessel.

Hela cultures were established by inoculating 9 cm. petri dishes with 4,000 cells in a monodisperse suspension. These cells received fresh medium (Eagle's + 5% calf serum + 2% human serum in Tris-citrate buffered balanced salt solution) once per week, and after four weeks they had grown into about 300 colonies. 2,000 Y-5 cells, inoculated into 9 cm. petri dishes in Eagle's medium + 5% calf serum in tris-citrate buffered balanced salt solution were cultured as above and produced about 300 colonies. Cultures of both cell types were then stained without fixation by the agar overlay method described in "Materials and Methods".

The deficiencies in alkaline phosphatase, glucose-6-phosphate dehydrogenase, and β-glucuronidase observed in some colonies of Hela and Y-5-11 cells (Table 3.1) are compatible with the reports of Helo and De Carli (1963) who obtained evidence of similar heterogeneity in a strain of human epithelial cells.

The consideration of both chromosomal variation and the difference in enzyme activity between homologous clones strongly suggests that cell cultures are subject to continuous spontaneous variation.

Resistance to metabolic analogues

An attempt was made to isolate variant cell strains resistant to metabolic analogues in order to determine whether some variant strains might show specific chromosome markers which could be correlated with

Table 3.1

Enzyme Deficient Colonies

Cell Strain	Alkaline Phosphatase	β-Glucuronidase	Glucose-6-Phosphate Dehydrogenase	Esterase
Y-(7)2	-	40%	5 - 10%	-
HeLa	5%	5%	-	0

Colonies were cultured from inocula of 2,000 Y-(7)2 cells and 4,000 HeLa cells. Histochemical assays were performed on unfixed, 4-week old colonies by the agar overlay method.

Figures are percentages of colonies deficient in enzyme activity. They were estimated by counting the proportion of deficient colonies in randomly-selected fields.

Table 3.2

Resistance of Strain X-6-11 to Metabolic Analogues

Analogue	Concentration	Resistance
6-Chloropurine	77µg/ml	Fully Resistant Colonies after 12 weeks
5-Fluorouracil	65µg/ml	No Resistance
8-Azaguanine	100µg/ml	No Resistance
Allylglycine	100µg/ml	Complete Resistance No Selection
Allylglycine	1mg/ml	Little Apparent Selection
Allylglycine	10mg/ml	Partial Resistance*
Aminopterin	0.1µg/ml	Complete Resistance No Selection
Aminopterin	1µg/ml	No Resistance
Diaminopurine	100µg/ml	No Resistance

* See text.

Resistant strains grew up from chloropurine and allylglycine cultures, but only cells in chloropurine remained resistant to the analogue over a period of 6-12 months. Resistance was acquired gradually in both chloropurine- and allylglycine-treated cultures.

analogue resistance.

3-4 x 10^6 Y-5-11 cells were inoculated into each of fourteen 4 oz. bottles in 10 ml. of the special medium mentioned in the "Materials and Methods" section (Appendix). Metabolic analogues were added to pairs of cultures as in Table 3.2. The experimental medium was renewed once or twice per week, depending on the cell density.

After twelve weeks in the presence of analogue cells growing in chloropurine gave rise to a fully resistant strain, which remained resistant in the continued presence of the analogue. Resistance was acquired gradually, and selection was sufficiently severe on two occasions, at three weeks and twelve weeks from addition of analogue, to reduce the population to about 50 cells. All other cultures either showed no resistance or complete resistance. Cells treated with allylglycine continued to grow but never became fully resistant; they required rescue periods in normal medium at frequent intervals (every five to ten transfers).

The relatively frequent occurrence of alterations in individual chromosomes and in the modal number of chromosomes would have made correlation of analogue resistance with specific chromosome patterns very difficult. In addition, the gradual acquisition of resistance to metabolic analogues did not imply a single-step process which could have been correlated with a chromosomal change. Consequently no attempt was made to correlate resistance to metabolic analogues with chromosomal abnormalities.

The presence of variant cells resistant to metabolic analogues may also be used as evidence for the heterogeneity of the cultured cell population; continued selection in successive generations implies continued variation.

Fig. 3.4.

Morphology of cultured cell strains

(1) Epithelial cells.

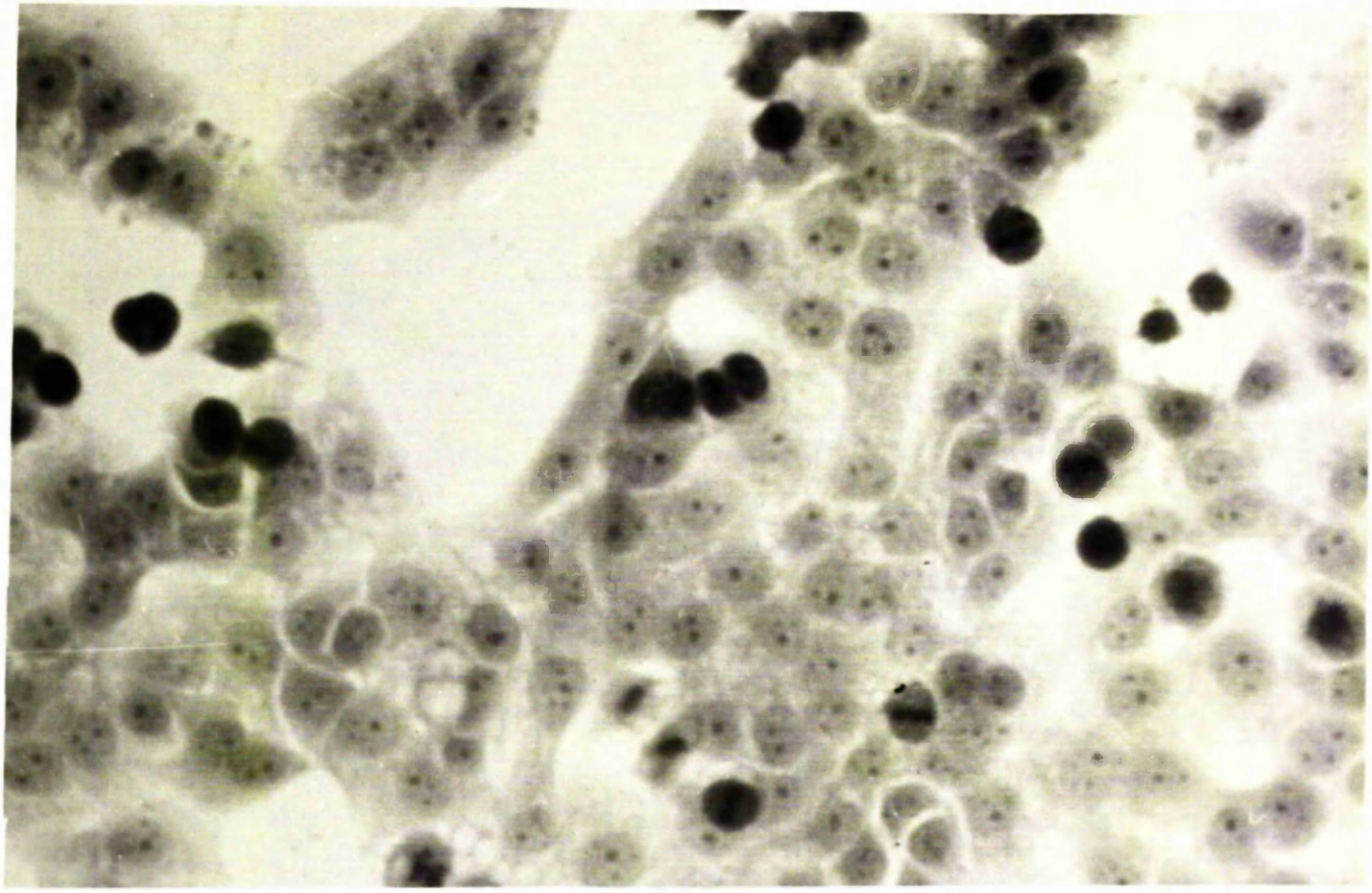
A HLL cells, grown on coverslips Stained haemalum and eosin.

B Hela cells, as above.

Both photographed under x40 phase contrast objective. Total magnifications x600.

Fig. 3.4

A.



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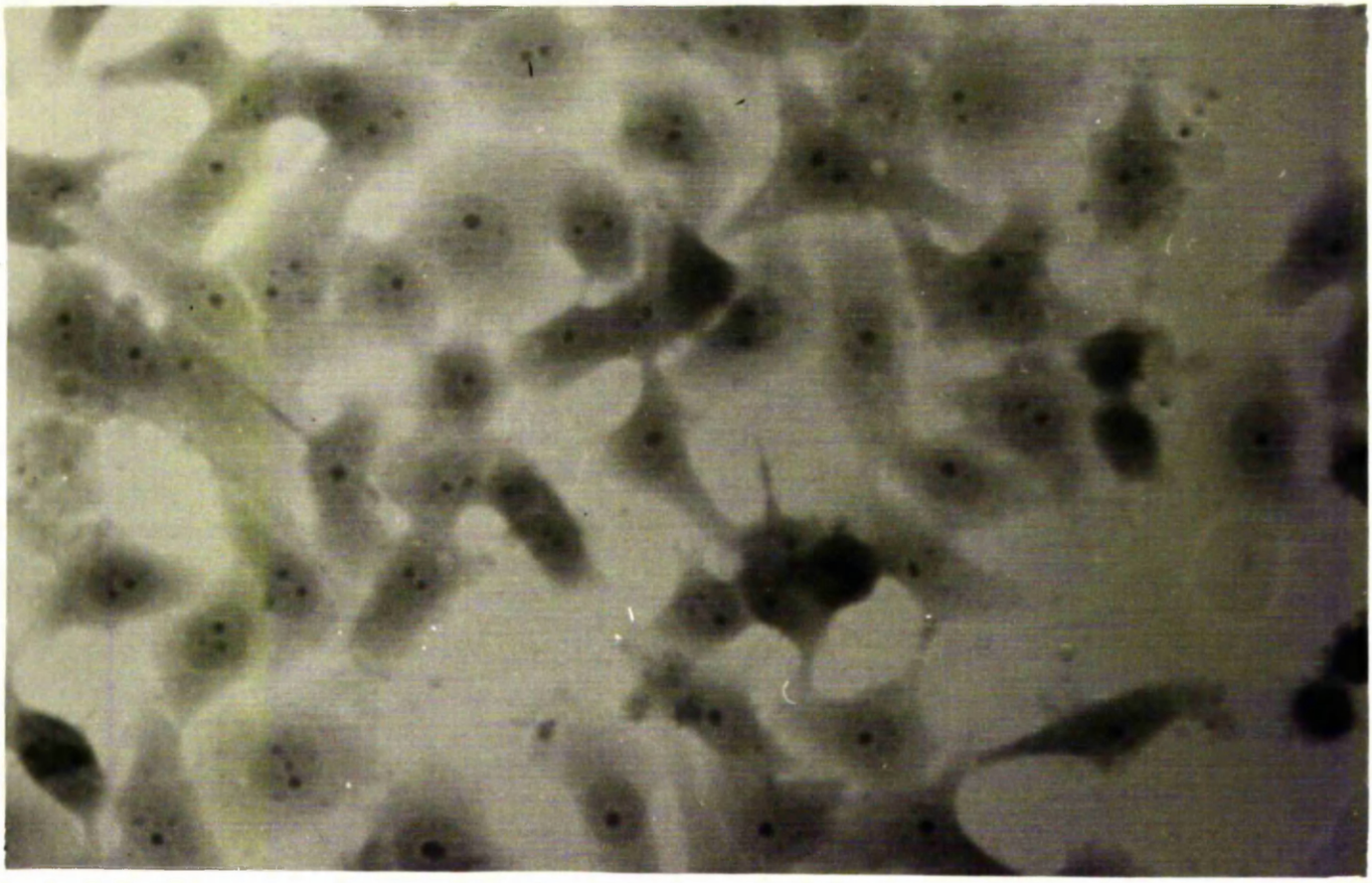


Fig. 3.5.

Morphology of cultured cell strains

(ii) Fibroblastic cells.

A L-5 cells.

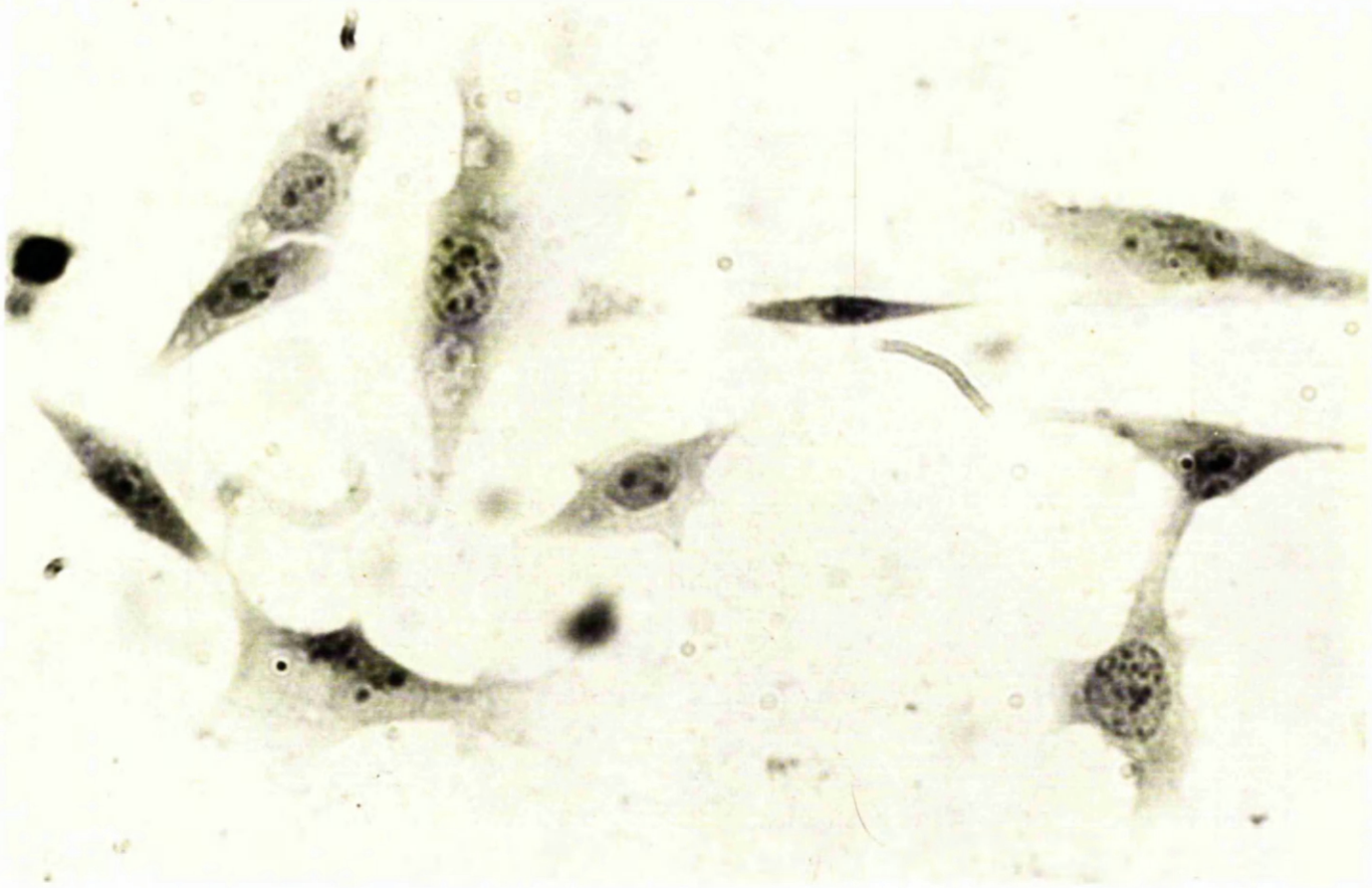
B Cl3A cells.

Stained haematoxylin and eosin.

Total magnification x600.

Fig. 3.5

A.



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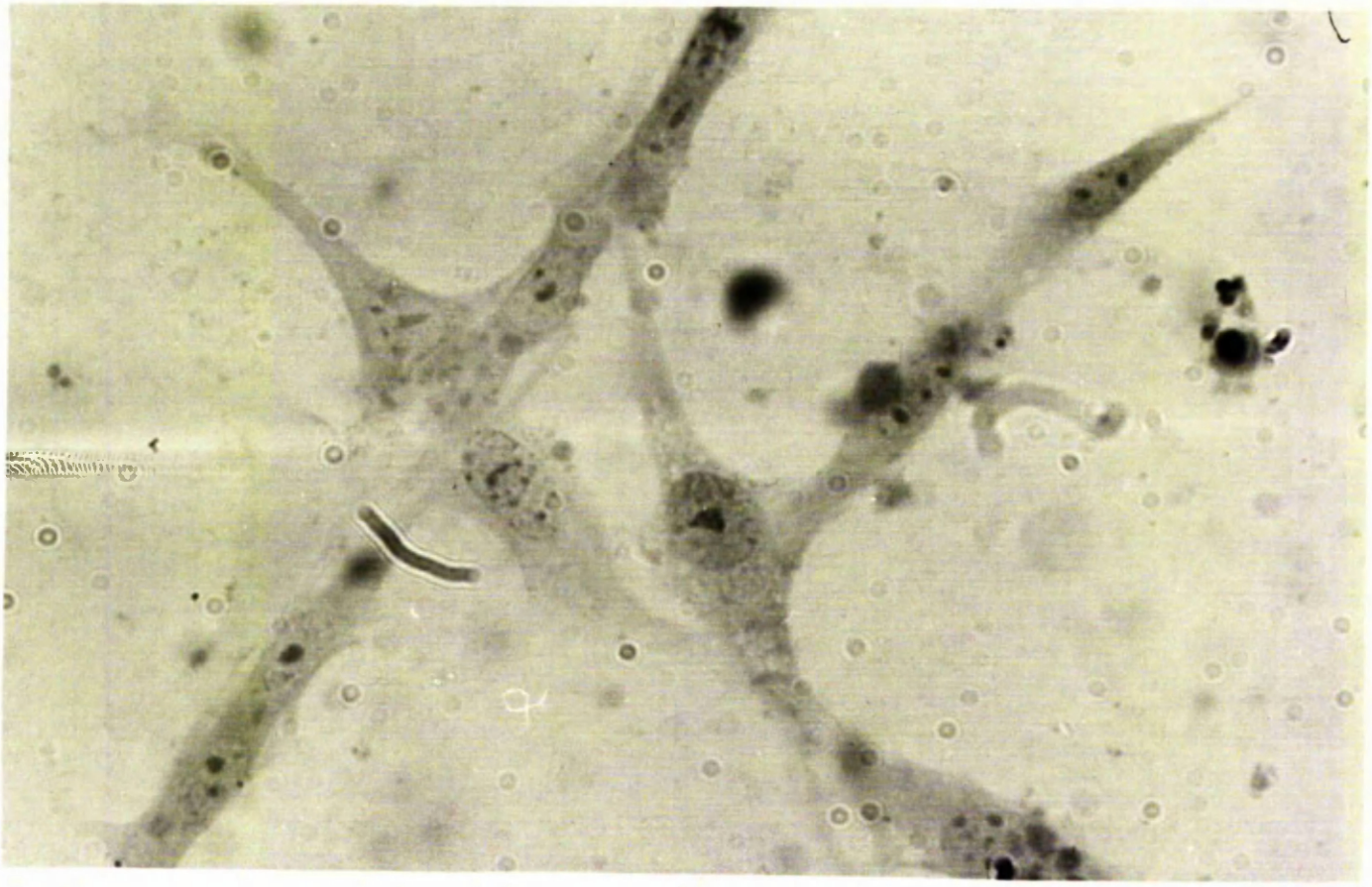


Table 3.3

Protein nitrogen of cultured cell strains

Cell Strain	Total Nitrogen (Mean ± S.D.)	Soluble Nitrogen (Mean ± S.D.)	Ratio of Total to Soluble
HeLa	11.6(± 2.6)	5.1(± 1.5)	2.3
HEM	9.3(± 2.9)	3.7(± 1.1)	2.5
L	6.4(± 1.6)	4.2(± 1.0)	1.4
Landschutz	6.4(± 1.6)	2.8(± 1.1)	2.3
Y-5	5.9(± 3.5)	2.6(± 0.4)	2.3
L5178Y (Ascites)	1.3(± 0.5)	0.8(± 0.4)	1.6
L5178Y (Cultured)	1.7(± 0.4)	0.6(± 0.1)	2.8

Nitrogen determinations were performed on sulphuric acid digests as described in "Materials and Methods."

Total nitrogen was determined in uncentrifuged extracts prepared by freezing and thawing a suspension of cells (5×10^7/ml) in 2% sodium deoxycholate in 0.85% NaCl. Soluble nitrogen was determined similarly in centrifuged, frozen and thawed extracts in 0.85% NaCl alone. Values are mg./10^8 cells.

3.2. Stable features of cultured cells

The appearance of the cultured cells used in the present experiments could be partially correlated with the origins of the cells, and had remained characteristic of the strains since their establishment (Figs. 3.4; 3.5). Those of epithelial origin, strains HMI and Hela, were round, flattened and irregular; lines of fibroblastic origin, e.g. strain L mouse fibroblasts and strain V-5 Chinese hamster fibroblasts, were usually elongated and often assumed a spindle shape. Suspension cultures of Landschutz and L5178Y cells could be distinguished from each other by cell size.

These cultured cell strains could also be distinguished by their protein content (Table 3.3). The epithelial lines had the highest total nitrogen, about 7-16 mg. per 10^8 cells, whereas the fibroblastic cells had only 4-10 mg. per 10^8 cells. The L5178Y ascitic cells had less than 2 mg. per 10^8 cells. Although there was considerable variation from specimen to specimen, it was clear that the different types fell within certain distinct limits.

The fibroblastic and epithelial cell lines had approximately equal amounts of soluble protein, and both were greater than in the ascitic cells. Strain L fibroblasts and L5178Y ascites cells in culture, had a lower ratio of total to soluble protein, about 1.5:1, than the other lines which had ratios of 2.3-2.6:1.

Enzyme activities of cultured cells might be expected to allow a more precise characterisation than morphological description. That this

is so is apparent from a survey of twelve enzymes in five cultured cell strains and two ascitic tumours. The results of this survey are presented in Figs. 3.6-3.17.

The epithelial lines were distinguishable from the rest by their high phosphatase activity, as measured by non-specific acid and alkaline phosphatase, and by glucose-6-phosphatase. HLM had greater activity of each phosphatase than HeLa (Figs. 3.6, 3.7, 3.8); its high glucose-6-phosphatase activity may be correlated with its origin from liver. Glucose-6-phosphate-dehydrogenase (Fig. 3.13) and thiosulphate sulphur-transferase (Fig. 3.14) were higher in HLM and HeLa cells than in the other lines. Landschutz ascites cells could be distinguished from the L5178Y ascites by their higher thiosulphate sulphur transferase (Fig. 3.14).

When the activities of the various enzymes were considered on the basis of cell number, lactic dehydrogenase (Fig. 3.12), cathepsin-C (Fig. 3.9), arylesterase (Fig. 3.11), and glutamyl transferase (Fig.3.17) were all higher in the larger cells, HeLa, HLM, L strain, and L-5. When these activities were calculated on the basis of soluble protein nitrogen, all the cell lines had approximately the same activity. Nevertheless, even when calculated in relation to soluble protein, some characteristic differences remained. These were the activities of glucose-6-phosphatase, acid and alkaline phosphatase, glucose-6-phosphate dehydrogenase, and thiosulphate sulphur transferase in HeLa, HLM, and Landschutz ascites mentioned above. The persistence of these stable differences, which allow these lines to be distinguished, implies

Figs. 3.6 - 3.17

Enzyme activity of cultured cells and two ascites tumours.

Fig. 3.6 Alkaline phosphatase.
3.7 Glucose-6-phosphatase.
3.8 Acid phosphatase.
3.9 Cathepsin-C.
3.10 β-glucuronidase.
3.11 Arylsulfatase.
3.12 Lactate dehydrogenase.
3.13 Glucose-6-phosphate dehydrogenase.
3.14 Thiosulphate sulphur transferase.
3.15 Arginase.
3.16 Aspartate amino transferase.
3.17 D-glutamyl transferase.

Alkaline phosphatase and aspartate amino-transferase were estimated in cell extract prepared by freezing and thawing in 2% sodium deoxycholate. The other enzymes were estimated in frozen and thawed extracts in 0.5% NaCl alone.

⊖—⊖ specific activity by protein nitrogen.
●—● " " cell number.
—┼— Norm.

Each point is the engyme activity of a duplicated sample. Each sample was harvested at different time; several days to several weeks elapsed between samples.

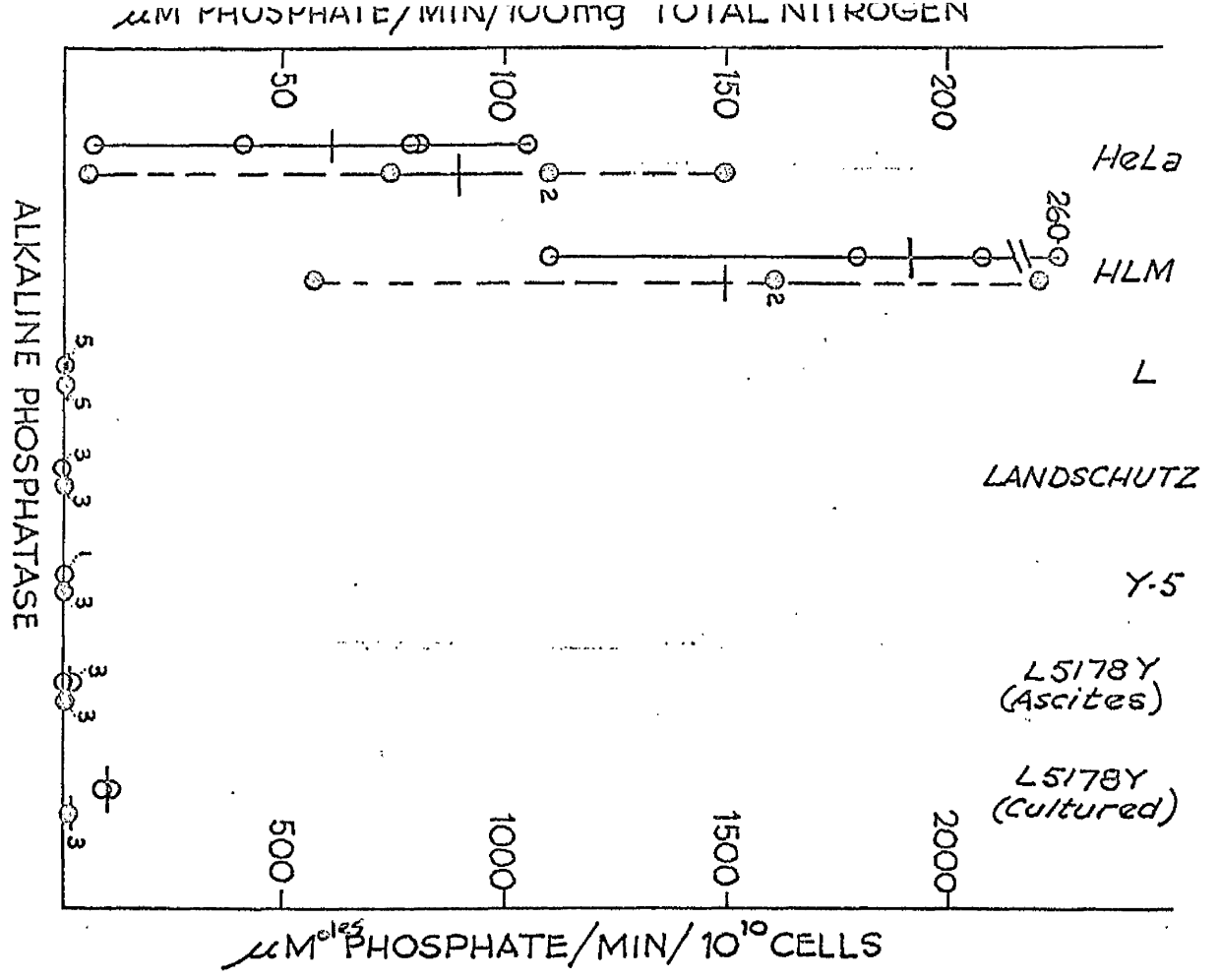

μM PHOSPHATE/MIN/100mg TOTAL NITROGEN
50
100
150
200
HeLa
260
HLM
L
LANDSCHUTZ
Y-5
L5178Y (Ascites)
L5178Y (Cultured)
ALKALINE PHOSPHATASE
500
1000
1500
2000
μMoles PHOSPHATE/MIN/10^10 CELLS

Fig. 3.6.

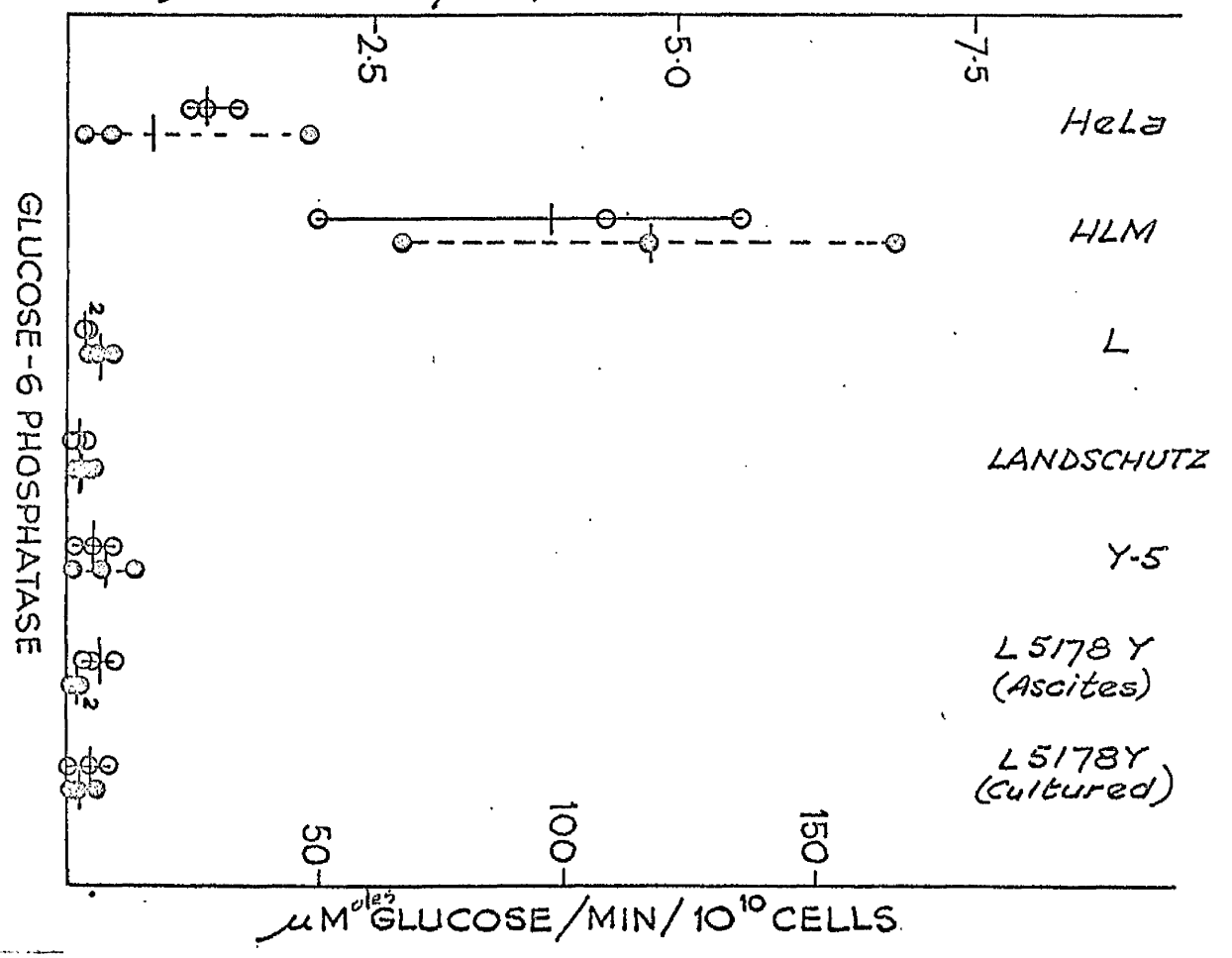

μMoles GLUCOSE/MIN/100mg SOLUBLE NITROGEN
2.5
5.0
7.5
HeLa
HLM
L
LANDSCHUTZ
Y-5
L5178Y (Ascites)
L5178Y (Cultured)
GLUCOSE-6 PHOSPHATASE
50
100
150
μMoles GLUCOSE/MIN/10^10 CELLS

Fig. 3.7.

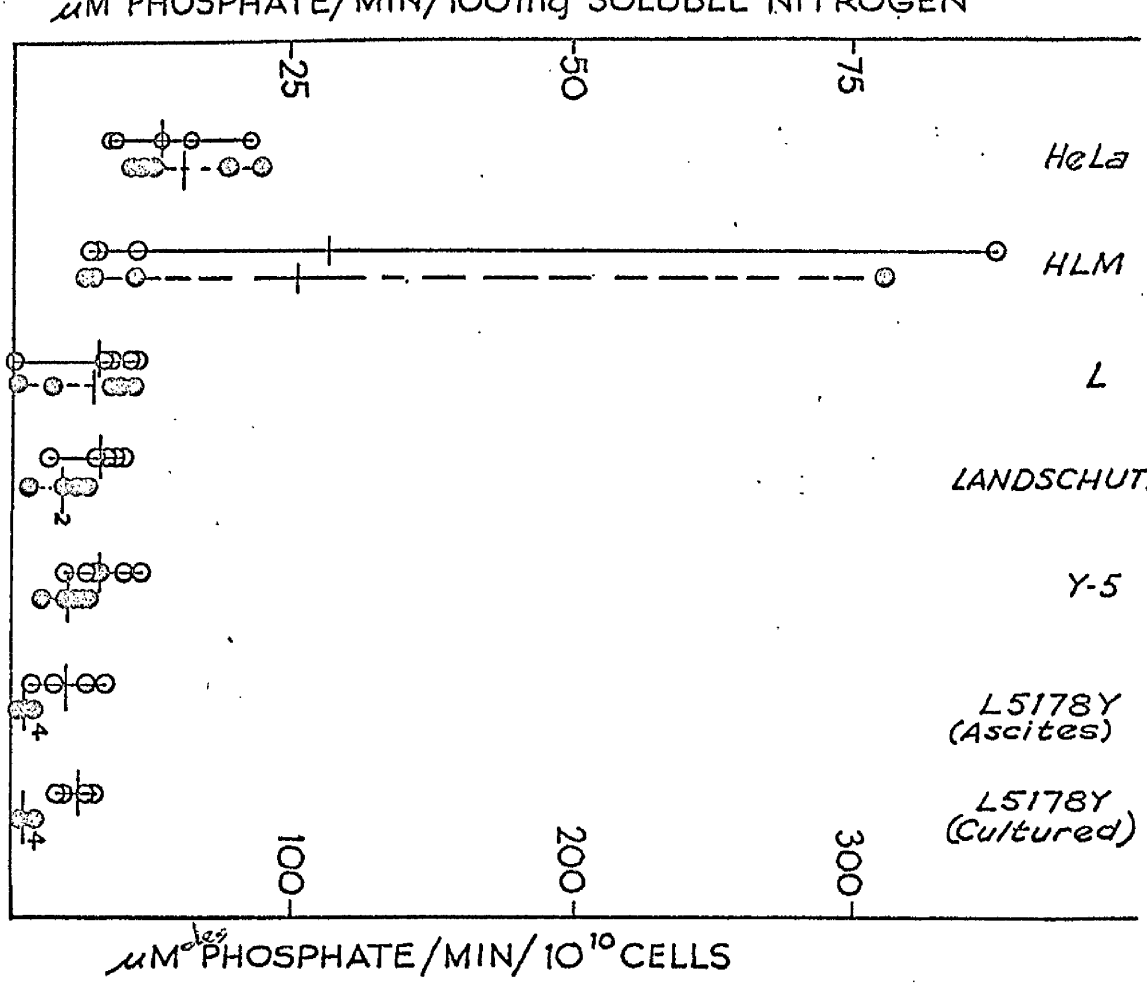

μM PHOSPHATE/MIN/100mg SOLUBLE NITROGEN
ACID PHOSPHATASE
25
50
75
HeLa
HLM
L
LANDSCHUTZ
Y-5
L5178Y (Ascites)
L5178Y (Cultured)
100
200
300
μM moles PHOSPHATE/MIN/10^10 CELLS

Fig. 3.8

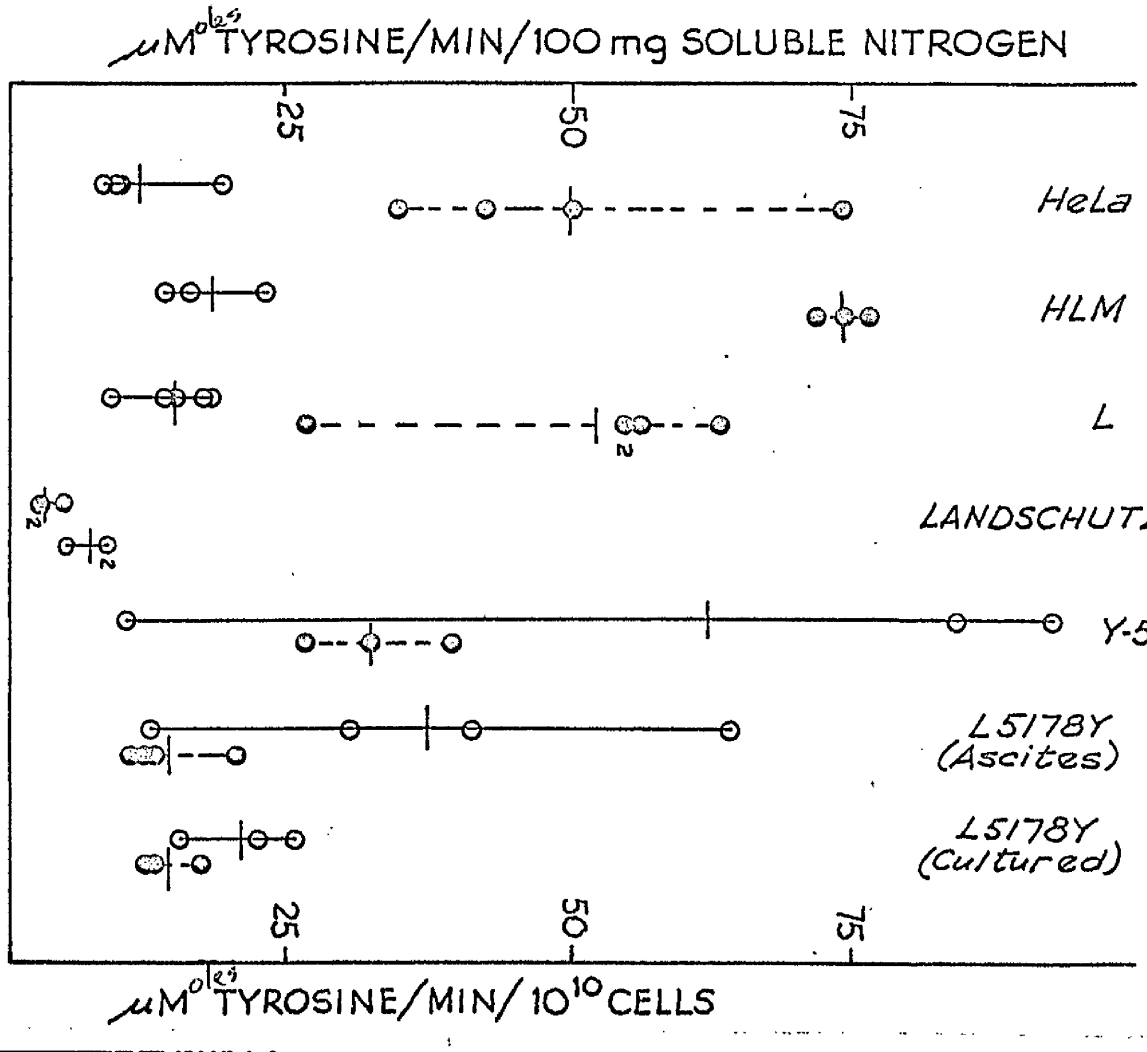

μM moles TYROSINE/MIN/100 mg SOLUBLE NITROGEN
CATHEPSIN-C
25
50
75
HeLa
HLM
L
LANDSCHUTZ
Y-5
L5178Y (Ascites)
L5178Y (Cultured)
25
50
75
μM moles TYROSINE/MIN/10^10 CELLS

Fig. 3.9

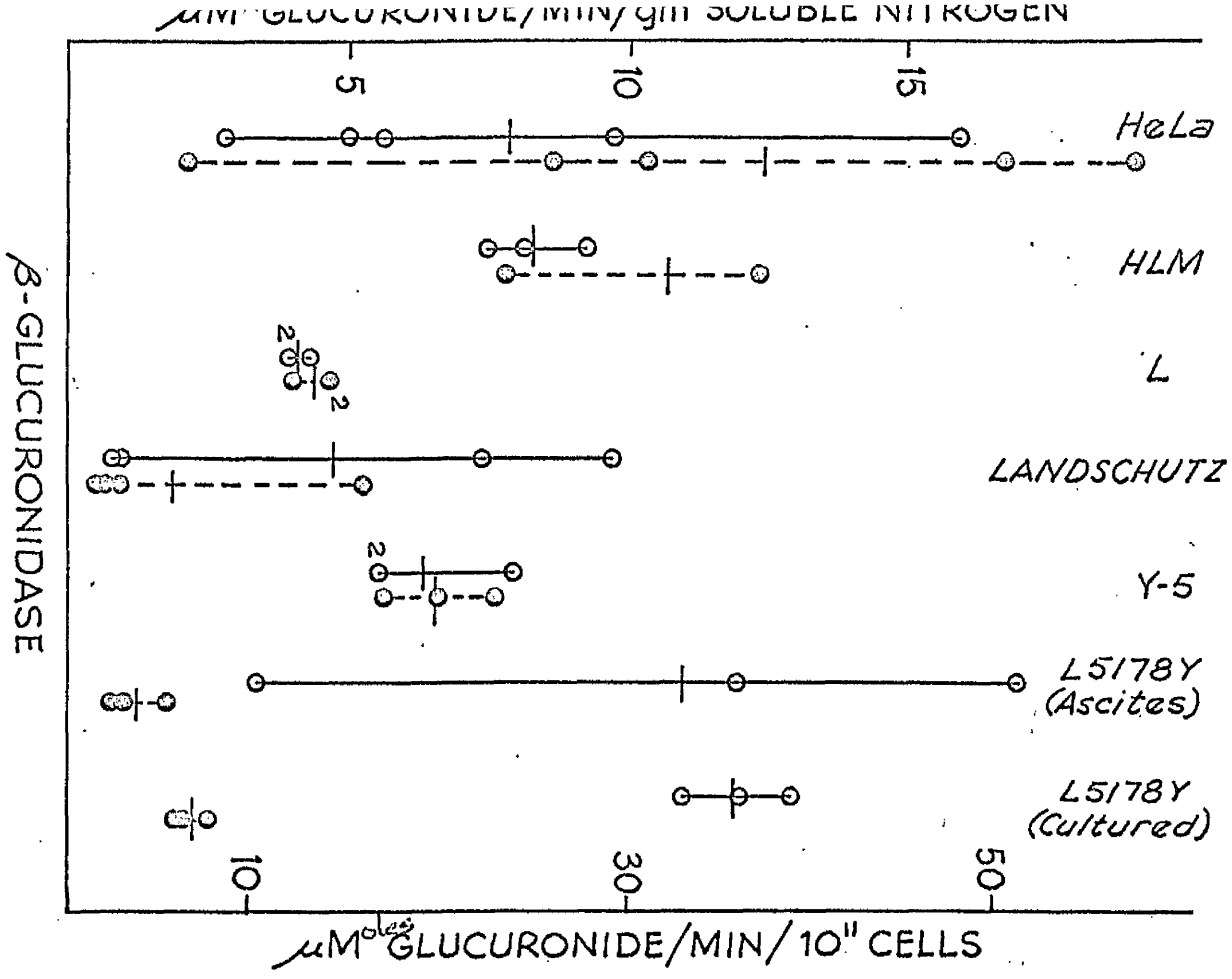


Fig. 3.10

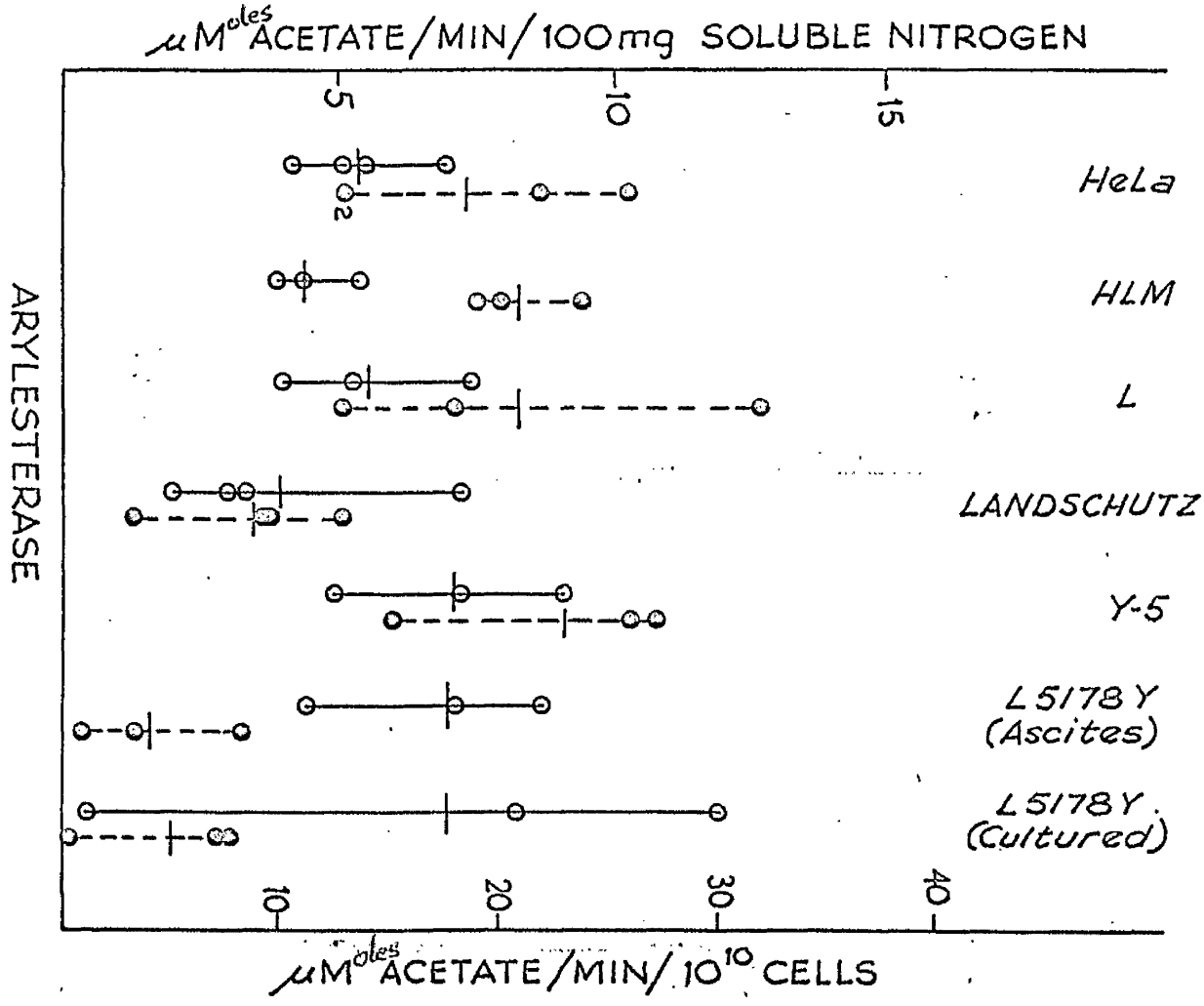


Fig. 3.11

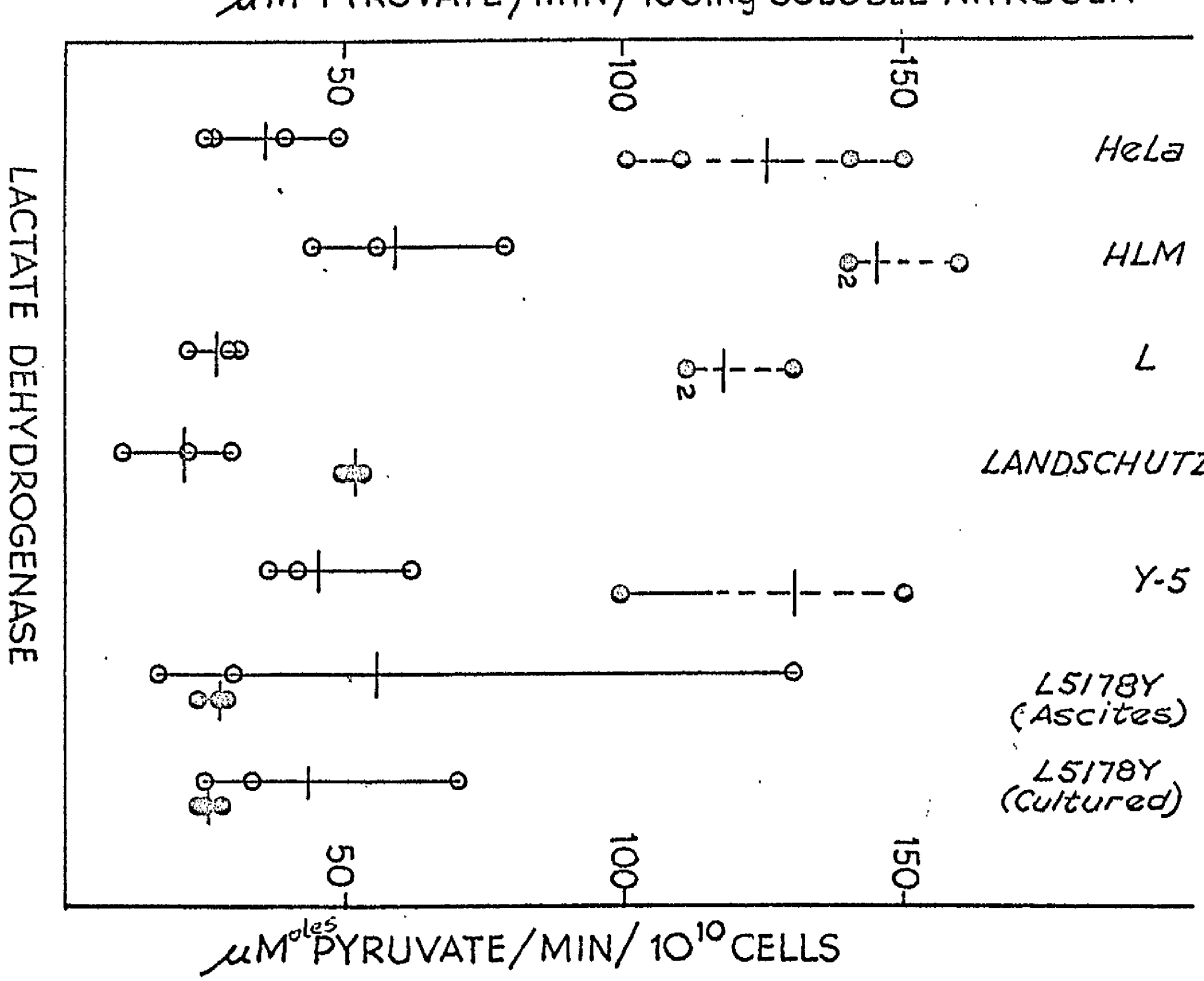


Fig. 3.12

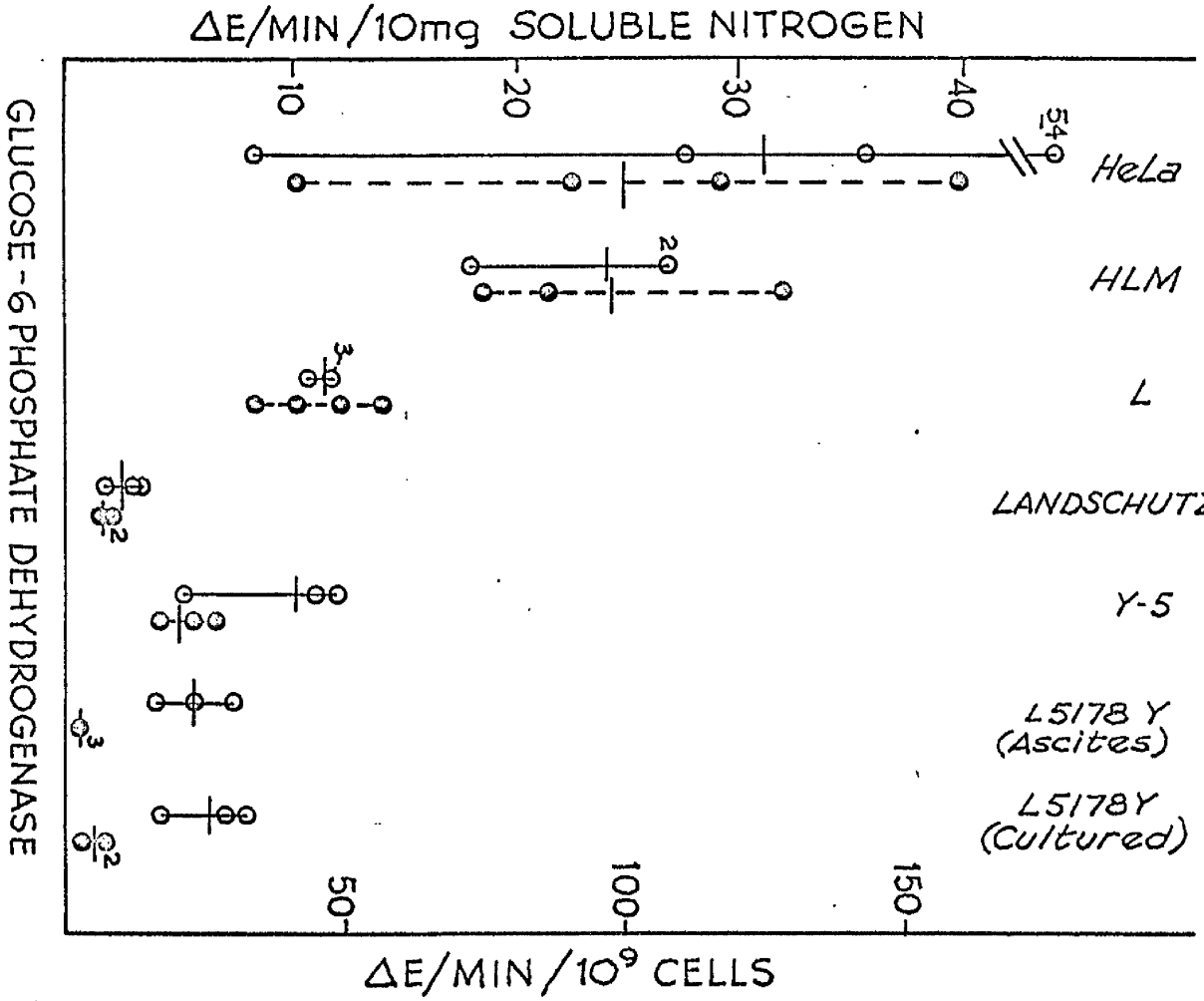


Fig. 3.13

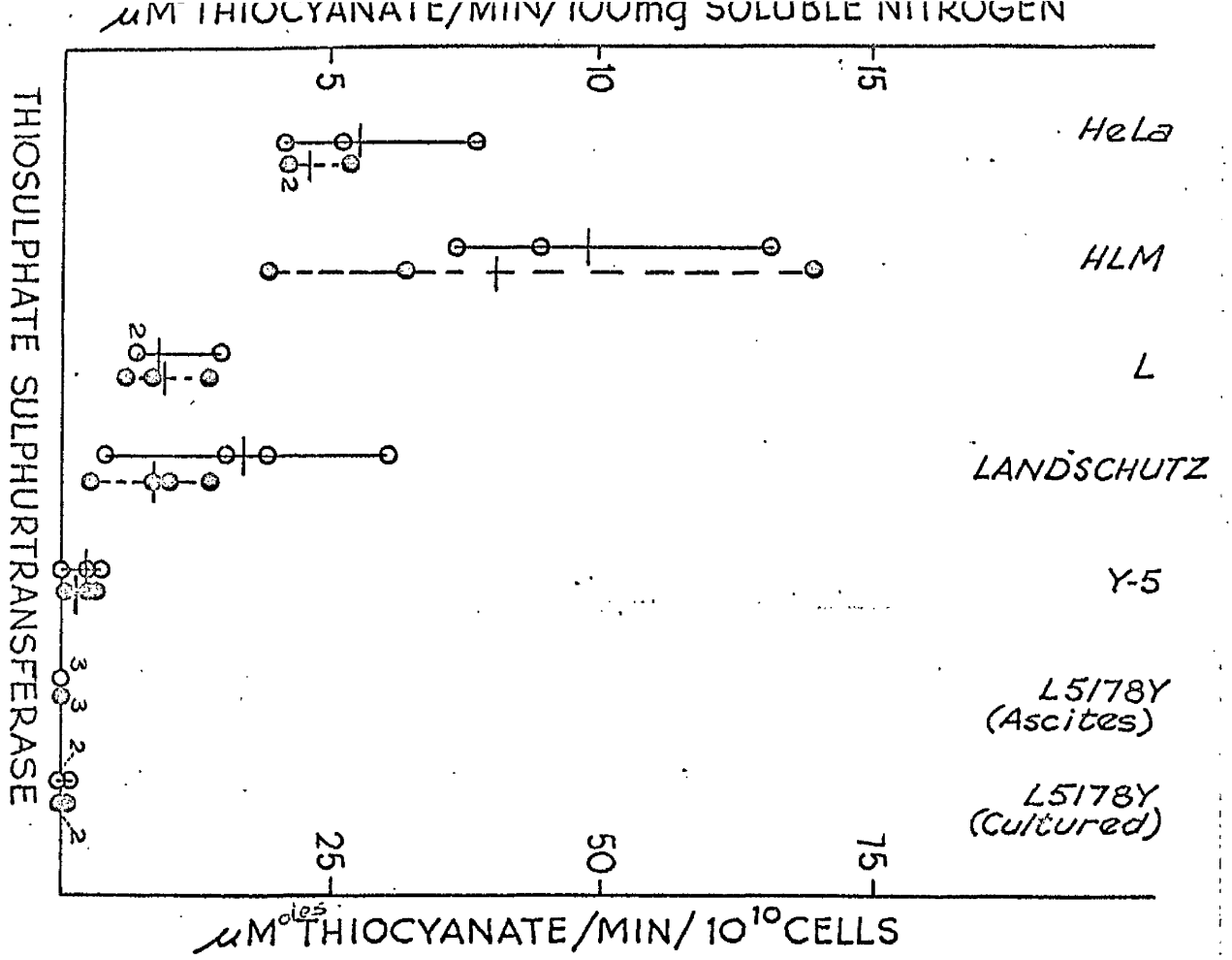


Fig. 3.14

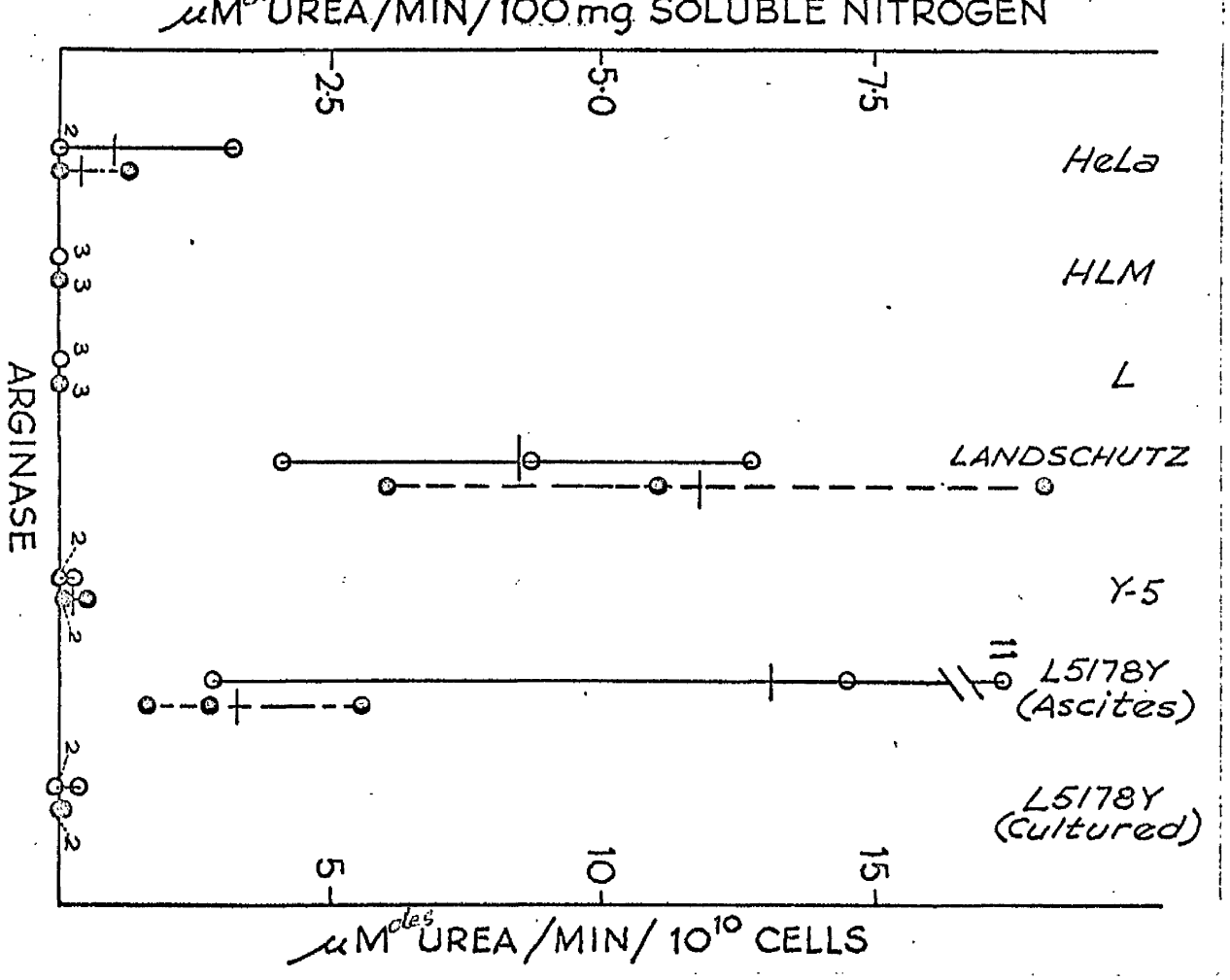


Fig. 3.15

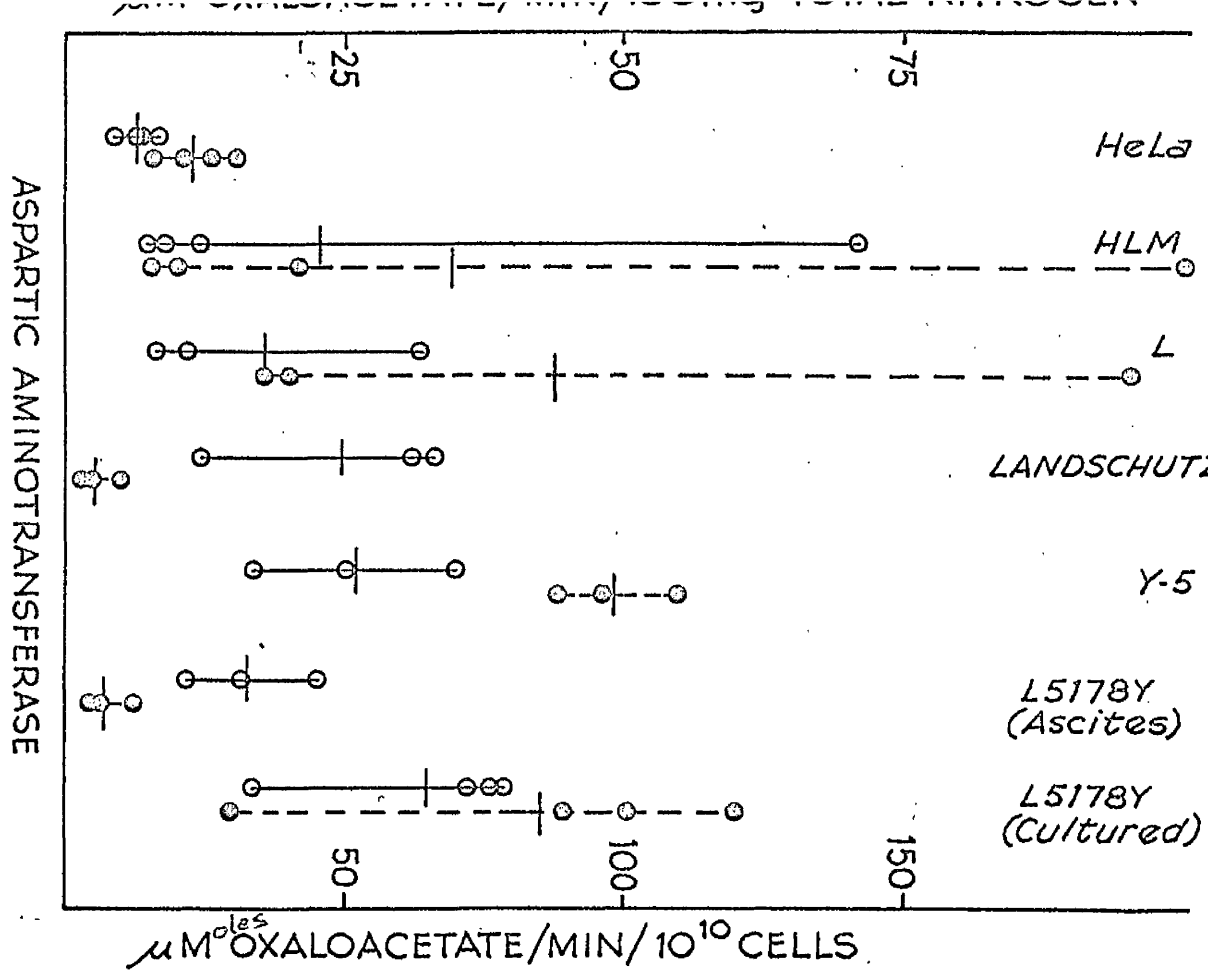


Fig. 5.16

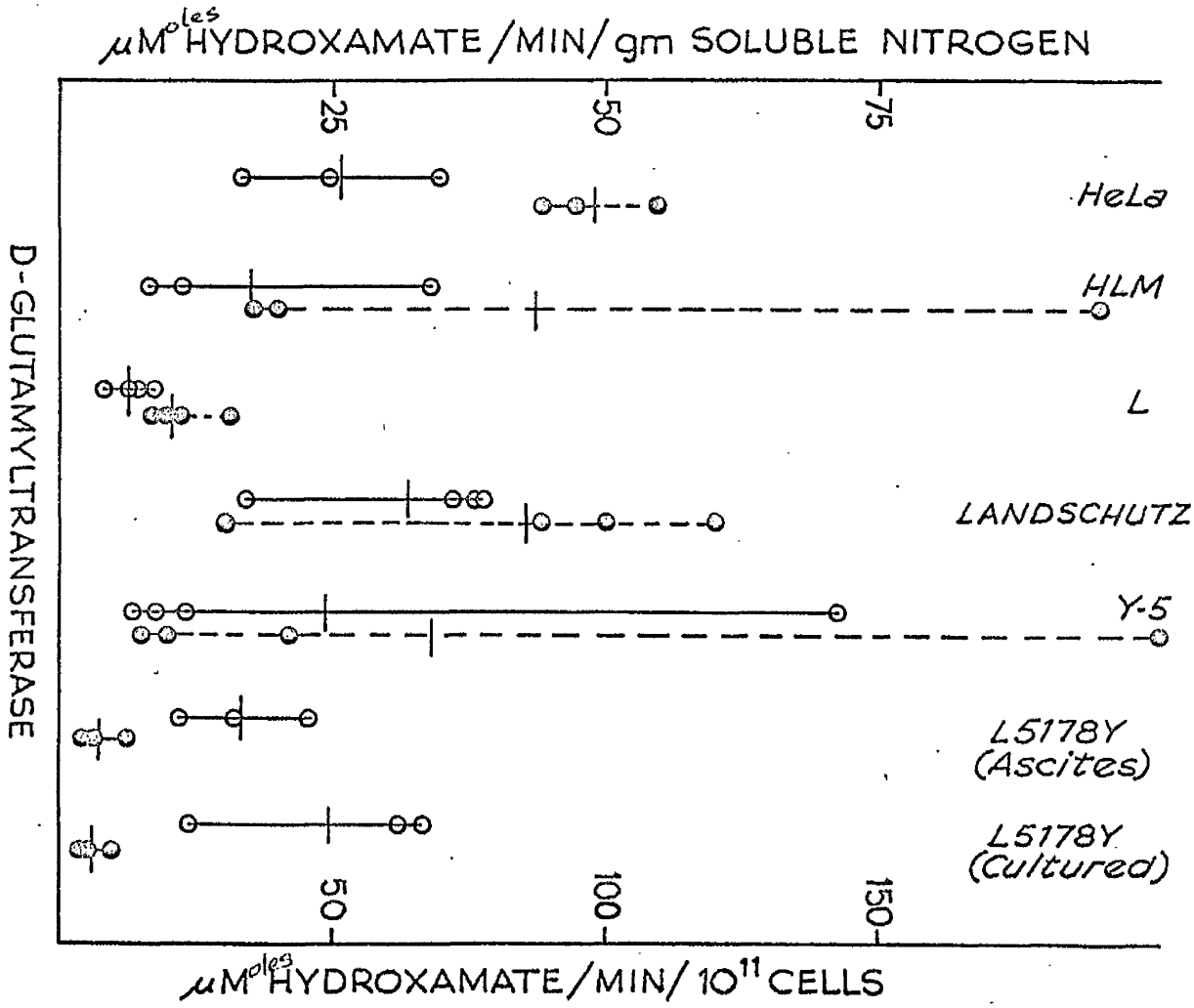


Fig. 5.17

that cultured cells do not assume a common dedifferentiated form.

3.3. Fluctuation of enzymes in cultured and ascites cells

In addition to the reproducible differences in enzyme activity that characterise certain cell strains, fluctuations in some enzymes were noted within the same strain. Thiosulphate sulphur transferase activity varied over threefold in HLM and Landschutz ascites cells (Fig.3.14); glucose-6-phosphate dehydrogenase activity varied fourfold in Hela cells (Fig.13); alkaline phosphatase activity varied tenfold in Hela cells and fourfold in HLM cells (Fig. 3.6); glucose-6-phosphatase activity varied threefold in HLM cells (Fig. 3.7); β-glucuronidase activity varied sixfold in Hela cells (Fig. 3.10); aspartic amino-transferase activity varied tenfold in Hela cells (Fig. 3.16); and D-glutamyl transferase activity varied sixfold in L-5 cells (Fig. 3.17). Arginase activity fluctuated about threefold in the Landschutz and L5178Y ascites cells in the animal, and was absent altogether from both these strains in cell culture (Fig. 3.15; Table 3.13).

These fluctuations in the absence of any deliberate alterations of the respective substrates in the medium imply that the activities of some enzymes are sensitive to certain changes in the culture medium. This suggests that they may be adaptive.

On comparing some cell lines with their tissues of origin, many workers have claimed that the cells become dedifferentiated (see Introduction), and thereby lose their characteristic enzymes. The uncertainty of the true original identity of any strain makes correlation of cultured cells

with their origin very difficult, but the evidence of fluctuations in enzyme activities presented above suggests that cells which lack certain enzymes while in culture, may do so as a result of the absence of the appropriate inducers.

One striking example of apparent adaptation in the transfer of cells from the animal to cell culture is the difference in arginase activity of the L5178Y lymphoma cells. Arginase was not detected in cultured cells but was present in considerable and variable amounts in ascites cells (Fig. 3.15) taken from mice. Two other strains showed the presence of arginase, the Landschutz ascites in vivo, and strain HeLa in cell culture. In each the amount of activity differed between assays of different samples. Although little evidence of arginase induction was obtained with the L5178Y strain in cell culture, Fottrell (1962) did achieve increased arginase activity in HeLa cells after incubation with arginine.

Fluctuation of arginase activity in HeLa cells

The aim of the following experiments was to determine if the fluctuations of enzyme activity in cultured cells were due to enzyme adaptation. Arginase was selected for these experiments because of the results quoted above.

Schimke (1962c,1964) has suggested that arginase is substrate-induced in HeLa cells and Fottrell (1962) obtained increases in arginase activity in HeLa cells incubated with arginine and citrulline. Early experiments in the present series were designed to confirm these reports.

Five million HeLa cells were inoculated into each of two 16 oz. bottles, one with 25 ml. control medium (Eagle's + 5% calf serum + 2% human serum) and one with medium containing 10 ml arginine and 10 ml citrulline. The cells were harvested after three days and their arginase activity assayed. A very small increase in arginase activity was observed (Table 3.4).

Three days may have been insufficient to promote induction. Pottrell (1962) showed that if the medium was changed after three days, the increase he obtained in arginase activity at five days was reduced. He suggested that some conditioning of the medium might be required to promote any increase in arginase activity in the presence of an inducer. It is therefore possible that three days were insufficient to allow such conditioning in the above experiment.

To test this conclusion, two million cells were inoculated into each of eight baby feeding bottles, four with control medium, and four with 10 ml citrulline. Samples were taken at 1, 2, 3 and 5 days to determine if the conditioning of the medium promoted any increase in arginase activity by five days. A sixfold increase in arginase activity was obtained (Fig. 3.18). However, this observation could not be repeated, and a subsequent five-day incubation with arginine and citrulline produced only a small increase in activity (Table 3.5).

These results conflict with the results of Pottrell (1962), who obtained tenfold increases in similar incubation conditions, and suggest that an additional factor is involved in induction besides arginine and

Table 3.4

Arginase activity of strain HeLa cells grown in 0.6mM arginine and in 10mM arginine and 10mM citrulline I. After 3 days incubation

	0.6mM Arginine	10mM Arginine 10mM Citrulline
Enzyme units	21 14	16 27

$5X10^6$ were grown in 16 oz. bottles for three days and harvested by trypsinisation.

Enzyme units are expressed as µM urea produced per minute per 10^{10} cells and are the mean of two samples.

Table 3.5

Arginase activity of strain HeLa cells grown in 0.6mM arginine and in 10mM arginine and 10mM citrulline II. After 1 and 5 days incubation

Day	0.6mM Arginine	10mM Arginine 10mM Citrulline
1	19	24
5	20	25

$4X10^6$ HeLa were grown in 4 oz. bottles and samples harvested by trypsinisation at one and five days.

Enzyme units are as in Table 3.4.

Fig. 3.18

Arginase activity of HeLa cells grown in 10mM arginine and 10mM citrulline for 5 days.

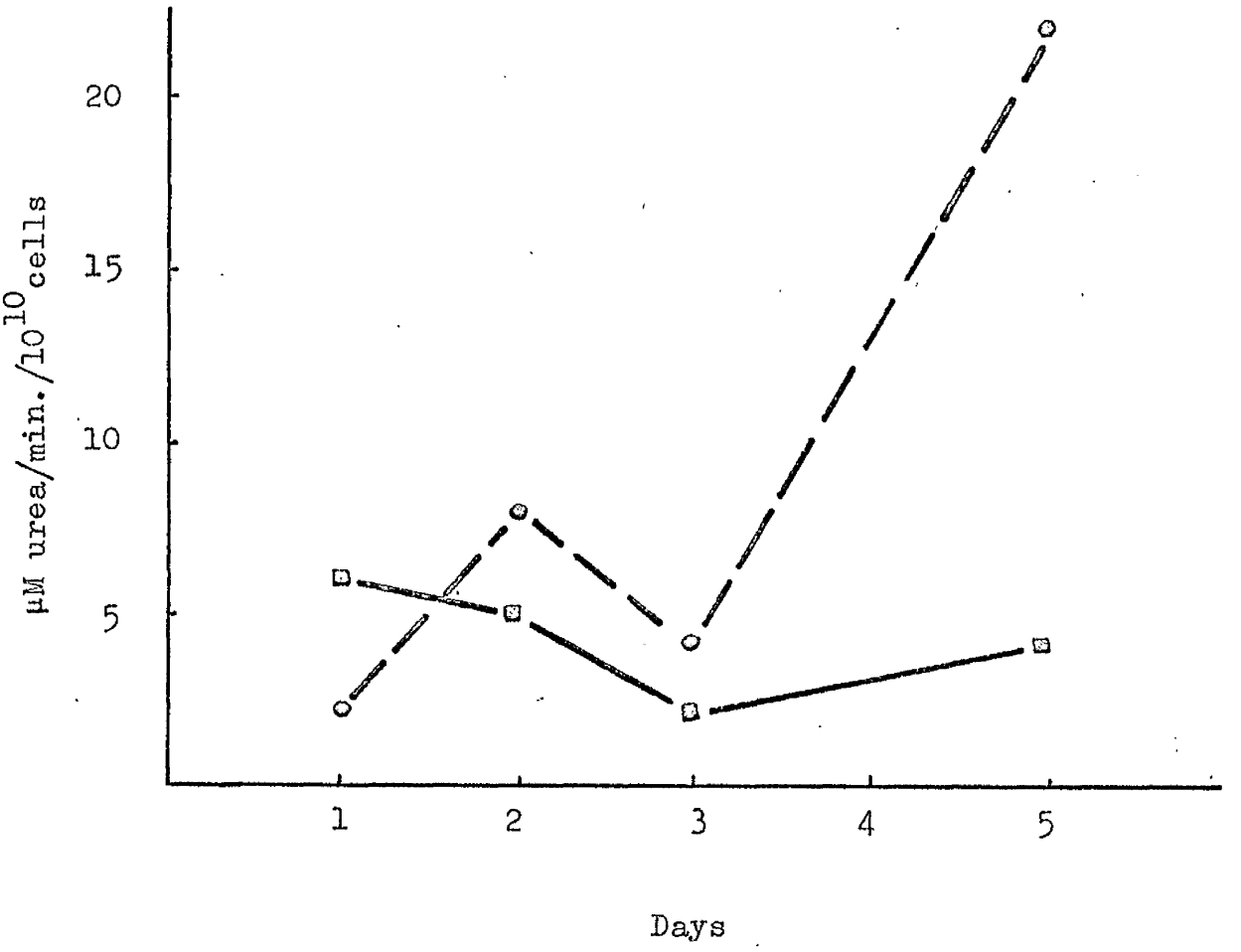
2×10^6 HeLa cells were inoculated into each of 8 baby feeding bottles, and harvested on the days shown.

The arginase activity of this and subsequent experiments was determined by the micro-method. Cell samples were collected by trypsinization.

■—■ grown in Eagle's medium plus 5% calf serum plus 2% human serum.

●--● grown in Eagle's medium plus 5% calf serum plus 2% human serum containing 10mM arginine and 10mM citrulline.

Fig. 3.18



citrulline.

The arginine concentration during these experiments was 10 mM, about seventeen times the level in ordinary culture medium. It is possible, however, that the intracellular concentration of arginine was limited by restriction of its rate of entry through the cell membrane. Differences in the medium (e.g. serum, pH and osmolarity) may have altered the permeability of the membrane in such a way as to alter the uptake of arginine. Such variations may have been responsible for the differences between individual experiments, and between the present results and those of Pottrell (1962).

Two experiments were performed with variations in the culture media and might be expected to result in alterations in the permeability of the cell membrane. The conditions used, and the results, are outlined in Tables 3.6 and 3.7.

Klein (1961) observed an increase of the arginase activity of HeLa cells when they were cultured in the presence of excess arginine and yeast RNA. She suggested that this might be related to the number of coding units of messenger RNA available for initiating arginase synthesis, when induced cells are incubated in the presence of RNA. However, this seems unlikely on considering the great phylogenetic differences between yeast cells and HeLa cells.

In Klein's experiments RNA might have acted as a polyanion. It is possible that the multiple charges of a polyanion might facilitate the access of arginine molecules to entry sites on the membrane.

The small increase in arginase activity in the present results may have been due to a similar effect. However, incubation in the presence of the polyanion gum acacia (polygalacturonic acid) had no effect on arginase activity.

Raising the pH of the incubation medium resulted in an increase in the level of arginase activity to about twice the activity at pH 7.4. In both the BUdR-treated cells and those grown at pH 7.8 the increased activity was observed both in cells grown in 10 mM arginine and in cells grown in 0.6 mM arginine. This implies that, although the extracellular arginine concentration may have influenced arginase activity, it was not limiting. One effect of raising the pH may have been to increase the permeability of the membrane, and the arginine present in both test and control medium may have been sufficient to produce a small increase in arginase activity. Arginase activity also increased after cold shock (Table 3.6) and this may have been due to increased membrane permeability.

Since the level of arginine (0.6 mM) used in control cultures may already have been high enough to result in induction of arginine, HeLa cells were cultured in 0.05 mM arginine, in an attempt to reduce the arginase activity to a completely uninduced level. No decrease in arginase activity was observed (Table 3.6). This suggests that, if arginine does induce arginase, then the intracellular arginine concentration may remain high even when the extracellular arginine concentration is reduced. This would require active concentration of arginine by the cell at low arginine concentrations in the culture medium.

Table 3.6

Effect of cold shock on arginase activity of HeLa cells

10mM Arginine	10mM Citrulline	Cold Shock	Enzyme Units
-	-	-	9 4
+	+	-	4 8
-	-	+	8 0
+	+	+	24 24

200,000 HeLa cells were incubated in 2 ml medium, with and without excess arginine and citrulline. 4 tubes were placed at 4°C for 16 hours and then returned to 37°C. The cultures were harvested at 48 hours by scraping with platinum wire.

Enzyme units are expressed as μM urea produced per minute, per sample.

Table 3.7

The effect of high and low pH and incubation with gum acacia and yeast RNA on the arginase activity of HeLa cells

10mM Arg.	10mM Cit.	Other additions	concs.	pH	Enzyme units
-	-	-	-	7.4	23 24
+	+	-	-	7.4	28 28
-	-	-	-	6.9	27 25
+	+	-	-	6.9	29 29
-	-	-	-	7.8	44 39
+	+	-	-	7.8	56 44
-	-	gum acacia	100ug/ml	7.4	22 27
+	+	gum acacia	100µg/ml	7.4	17 23
-	-	yeast RNA	100µg/ml	7.4	36 26
+	+	yeast RNA	100µg/ml	7.4	32 44

$2X10^6$ HeLa cells were inoculated into 4 oz. bottles and harvested at 96 hours by scraping with a rubber coated glass rod. Enzyme units are expressed as µM urea/minute/10 mg. DNA-phosphorus.

Table 3.8

Arginase activity of HeLa cells cultured in a low arginine concentration

Arginine concentration	Arginase activity: µM Urea/min/10^{10} cells	Cell multiplication in week prior to assay
0.6mM (Eagles Medium)	28	10X
	25	
0.05mM	29	None
	24	

HeLa cells were grown in Roux flasks at an inoculum of 100,000 cells/ml medium. Those in 0.6mM arginine were trypsinised weekly and reinoculated at 100,000/ml; those in 0.05mM arginine were trypsinised less frequently and on each occasion the whole population was returned to the culture flask. Although cultures in 0.6mM arginine increased tenfold in each week, there was no significant increase in cells grown in 0.05mM arginine.

Samples were collected by trypsinisation after 5 weeks.

This conclusion, and the earlier results implicating membrane permeability, suggests that the mode of arginine uptake may be an important consideration in the study of arginase adaptation. Experiments on arginine uptake were therefore undertaken and will be reported later.

Fluctuation of arginase activity of cultured golden hamster cells

Shope papilloma virus is reputed to increase the arginase activity of rabbit papilloma during viral transformation (Rogers and Moore, 1963). Transformation by polyoma virus, another DNA virus giving rise to tumours, has been demonstrated in the BHK21 hamster kidney cell strain (Stoker, 1962; Macpherson, 1963). Several transformed and untransformed clones of this strain were tested for arginase activity to determine whether an alteration in arginase activity occurred similar to that found in the rabbit papilloma. Although two untransformed clones had no arginase activity, detectable by the present method, all the other clones tested had varying amounts of arginase activity regardless of whether they were transformed or not (Fig.19). Apparently viral transformation has no clearcut effect on arginase activity in these cells.

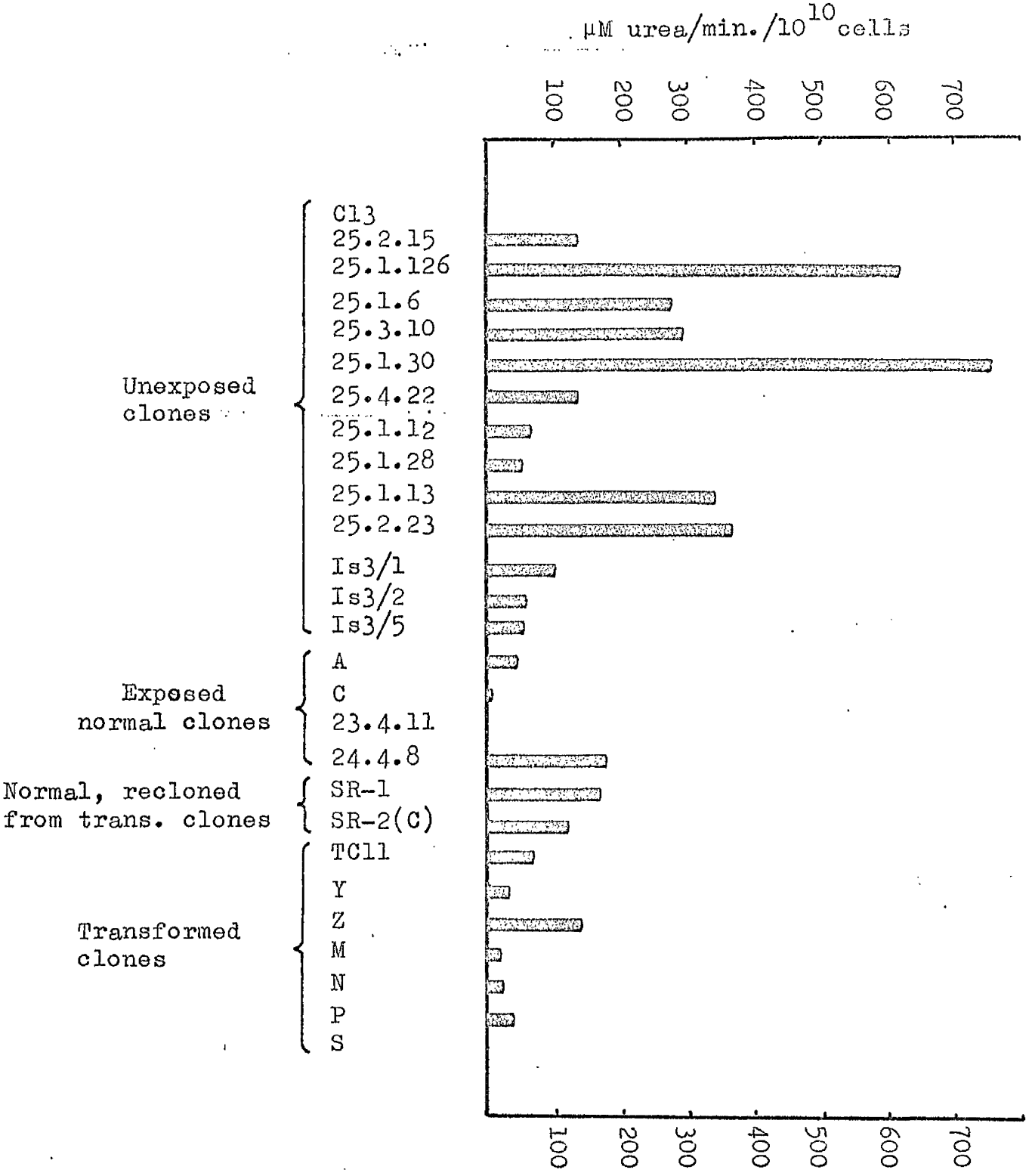
During these investigations, however, considerable variation was observed among clones of the same strain. This could represent genetic differences among the lines, particularly between clone C13 (untransformed) and clone TC11 (transformed) and between clone C13U (untransformed) and C13S (transformed) where arginase is absent from the first in each case. However, a more likely explanation is that the enzyme is adaptive in these cells. This is supported by examination of clones after an

Fig. 3.19

Arginase activity of normal and transformed BHK21 golden hamster fibroblasts.

The arginase activity of several clones of BHK21 cells was determined in cells collected by trypsinisation. "Exposed normal clones" are clones of normal morphology and growth characteristics recovered from a cell population exposed to polyoma virus. SR-1 and SR-2(c) were normal clones obtained when transformed clones were recloned.

Fig. 3.19


μM urea/min./10^{10} cells
100
200
300
400
500
600
700
Unexposed clones
C13
25.2.15
25.1.126
25.1.6
25.3.10
25.1.30
25.4.22
25.1.12
25.1.28
25.1.13
25.2.23
Is3/1
Is3/2
Is3/5
Exposed normal clones
A
C
23.4.11
24.4.8
Normal, recloned from trans. clones
SR-1
SR-2(C)
Transformed clones
TC11
Y
Z
M
N
P
S

interval of seven days in culture in two different laboratories (Table 3.9). The differences between consecutive samples in the same medium are great irrespective of the cell concentration in the growth medium, or of the time of the growth cycle at which the cells were harvested.

When C13C and C13Z cells were incubated in 10 mM arginine for five days in an attempt to discover the basis of arginase fluctuations in these cells, there was still no arginase activity in C13C cells, and only a slight increase was observed in C13Z cells (Table 3.10). When C13Z cells were incubated for one day in similar conditions, a twofold increase was observed in the arginase activity of test cultures (Table 3.11,1), but on repeating this experiment no increase was obtained (Table 3.11,2). The arginase activity of C13Z cells in both these experiments (Table 3.11, 1 & 2) was less than 10% of the activity first recorded (Table 3.10).

Further experiments were carried out on strain Is3/1 cells (hamster fibroblasts, untransformed) which had been maintained in a medium containing a low arginine concentration (0.05 mM) for several weeks in an attempt to reduce the activity to the fully repressed level. The arginase activity per cell at this time was lower than in cultures grown in 0.6 mM arginine (Table 3.12), and lower than at any time measured. However, the total protein nitrogen of the cells was also low, and the reduced arginase activity may have been a reflection of a mtt decrease in protein synthesis, brought about by arginine starvation.

Table 3.9

Arginase activity of clones of golden hamster cells, strain BHK21 before and after culture for one week in different laboratories

Clones	Initial Activity	After 1 wk. Lab. A.	After 1 wk. Lab. B.
Ic3/1	204 181	736	990
Ic3/2	51 63	202	60
Ic3/5	58 53	203	49
25.1.126	610	242	-
25.1.6	274	140	-
25.3.10	302	271	-
25.1.30	750	642	-
25.2.15	127	1180	-

Some clones were maintained in both the Department of Biochemistry, Glasgow University (Lab.A) and Institute of Virology, Glasgow University (Lab.B). Culture media and routine were similar but the serum supplement was different. Enzyme activity was expressed as in table 4.

Table 3.10

Arginase activity of strain C13C and C13% cells after incubation in 0.6mM arginine and 10mM arginine for 5 days

Clone	0.6mM Arginine (Control)	10mM Arginine
C13C	0	0
C13%	354	467

$2x10^6$ C13C and C13% cells were grown for 5 days in 10ml medium containing 0.6mM and 10mM arginine.

Enzyme units are as in table 3.4.

Table 3.11

Arginase activity of strain C13% after incubation in 0.6mM and 10mM arginine for 24 hours

Experiment	0.6mM Arginine (Control)	10mM Arginine
1	13 23	34 37
2	10 10	11 13

$2x10^6$ C13% cells were grown in 10ml medium containing 0.6mM and 10mM arginine for 24 hours. The experiments were done at different times.

Enzyme units are as in table 3.4.

When 10^6 of these cells were incubated with 10 mM arginine and citrulline for six days, no significant difference in arginase activity was observed between cells cultured in 0.05 mM arginine and in 10 mM arginine and citrulline (Table 3.13). However, the arginase activity of both groups was higher than the activity recorded in the previous experiment (Table 3.12).

Hence the effect of incubation of Is3/1 and C132 cells with excess arginine and citrulline is insignificant when compared with the fluctuation in activity observed between different assays. As this spontaneous fluctuation has been recorded on several different occasions (cf. Table 3.9) it is likely that this enzyme may be adaptive in cultured hamster kidney fibroblasts. However, arginine alone or with citrulline does not seem capable of inducing these fluctuations; all these results therefore point to the operation of an unidentified factor.

Since, as will be discussed later, differences in arginase activity were observed between ascites cells grown in the animal and in cell culture (see below and Fig. 3.15), the arginase activity of a hamster tumour, produced by cultured hamster fibroblast cells, was determined. The strain Is3/Col. 11 cells readily produce tumours in hamster brain (Jarret pers. comm.). The arginase activity of one such tumour was compared with the arginase activity of cells from the same tumour after one week in cell culture (Table 3.14). Cultured cells had higher activity. This contrasts with the results with L5178Y and Landschutz ascites tumour cells, where a much higher arginase activity was found in the animal

Table 3.12

Arginase activity of Ie3/1 cells grown in different concentrations of [illegible]

Arginase activity of Ie3/1 cells grown in different concentrations of arginine for 5 weeks

Concentration of Arginine in Medium	Arginase activity µM urea/minute	
	per 10^{10} cells	per 1 gm Protein N
0.6mM	301	194
0.05mM	144	235
	144	288

Ie3/1 cells were cultured in Roux flasks in different concentrations of arginine and the arginase activity determined after 5 weeks. Cells cultured in 0.05mM arginine grew more slowly than control cultures in 0.6mM arginine.

Table 3.13

Culture of Le3/1 cells in 0.05mM arginine and 10mM arginine and citrulline after 5 weeks' culture in medium containing 0.05mM arginine

	µM urea/min. per 1gm Protein N
0.05mM Arginine	809 516
10mM Arginine and Citrulline	905 870

10^6 Le3/1 cells were inoculated into each of four 4 oz. bottles containing 10ml Eagles medium plus 10% calf serum and 0.1% tryptose. The arginine concentration in two bottles was 10mM and in the other two it was 0.05mM. The cells were trypsinised after 6 days and their arginase activity determined.

Table 3.14

Arginase activity of strain Is3/col.11, grown in vivo (brain tumour) and in cell culture

	Arginase activity µM urea/minute	
	per 10^{10} cells	per Enzyme units/1gm Protein N
Tumour	17 20	16 17
Culture	42 46	47 52

The tumour was produced by implantation of one cell of strain Is3/col.11 in the brain of a golden hamster. The tumour was excised and trypsinised (Jarret, pers. comm.). Arginase estimations were performed on the trypsinised suspensions and on a one week old culture of an aliquot of the same cells.

Table 3.15

Effect of culture in ascitic fluid on the arginase activity of Landschutz ascites cells

Time (hrs.)	0	3	6	24
Ascitic Fluid		0 6	0 5	0 6
Eagle's Medium + 10% CS + 0.1% Tr.	0 0	0 0	0 0	0 0
Ascites cells *in vivo*	0	75	75	280

Landschutz ascitic tumour was withdrawn from Porton white mice and centrifuged (2,000g) to remove cells. 20 mg glucose and 1 mg heparin were added per ml.

4×10^6 Landschutz cells were added to each of 6, 25 ml conical flasks with 3 ml of ascitic fluid plus glucose and heparin or 3 ml Eagle's medium plus 10% calf serum and 0.1% tryptose.

The arginase activity was determined in the cells at the times shown.

Enzyme units are expressed as µM urea/min. of 10^{10} cells.

Values for "ascites cells *in vivo*" are taken from fig.2.23.

(Fig. 3.15; see below).

Arginase activity of Landschutz cells culture in vitro and maintained as ascites tumours

While Landschutz ascites cells have considerable arginase activity after growth in the animal (Fig. 3.15) it was not possible to detect any activity in a strain growing in cell culture. Two experiments were performed to determine whether arginase activity could be induced in cultured cells when they were injected intraperitoneally into mice. For short-term samples (3-48 hours) 10^8 cells, in 1 ml. of Landschutz ascitic fluid (supernatant of centrifuged ascites tumour), were inoculated intraperitoneally into each mouse. For later samples 2×10^7 cells were inoculated in 0.2 ml. ascitic fluid. Short-term samples were harvested aseptically by washing out the peritoneum with sterile tris-citrate buffered Eagle's medium (as used for culture of these cells). Long term tumours (7-day) were harvested aseptically in their own fluid, centrifuged at 900 g and resuspended in medium. 16×10^6 cells, from each sample, were inoculated into 80 ml. medium in Roux flasks. 10 ml. were removed immediately as the first sample, and further 10 ml. aliquots were removed at intervals thereafter.

About 90% of the cells were recovered in 24 and 48 hour samples. Seven-day samples showed a 25-fold increase and 5×10^8 cells were harvested per mouse. The viability of these cells, estimated by vital staining with 0.1% naphthalene black, was found to be 100% on inoculation of Roux flasks, and 60-80% after seven days in culture. The results

of these experiments are presented in Figs. 3.20 - 3.28.

A sharp rise in arginase activity was obtained after inoculation into the animal, reaching a maximum at 12 - 48 hours (Figs. 3.20; 3.23). There was no further increase in total arginase activity by seven days during a twentyfold increase in cell number (Figs. 3.20 & 3.22), which suggests that there may be a limiting concentration of an inducing substance in the peritoneal exudate.

When cells that had been grown *in vivo* were transferred to cell culture arginase activity continued to increase. The increase was greatest when cells were transferred after 3 - 24 hours. If transferred at 24 - 48 hours no further increase was obtained in culture. During rapid cell growth, arginase activity of 48-hour samples was diluted out in culture. Tumour cells extracted from the animal at seven days did not have maximum arginase activity and showed an increase in culture (Figs. 3.20). There was no evidence of a change in the rate of arginase synthesis in culture (Fig.3.22).

The continued increase of arginase activity in cell culture, after transfer from the animal, implies that the cells have been conditioned in some way whilst in the peritoneum, to enable them to respond to a common factor found in ascitic fluid and culture medium. This common factor could be arginine, and could be the limiting factor that prevents a further rise in arginase activity after two days in the animal (the rate of cell growth in the peritoneal cavity is likely to lead to an excessive demand for amino acids). The peritoneal environment of

Fig. 3.20

Increase in arginase activity of cultured Landschutz ascites cells after inoculation into mice. (Experiment 1.)

10^8 Landschutz ascites cells were inoculated into the peritoneal cavity of each mouse. 2 mice were sacrificed at 24 and 48 hours and the ascites cells were washed from their peritoneal cavities with a culture medium (Eagle's plus 5% calf serum plus 0.1% tryptose) and counted. 2×10^7 cells cultured ascites cells were inoculated into mice and tumour was collected after seven days. The cells were washed with culture medium, resuspended in the same medium and counted.

16×10^6 cells from each sample were inoculated into 80 ml culture medium and aliquots of 10 ml (2×10^6 cells) removed immediately and at intervals thereafter. The arginase activity of these samples was determined by the micro-method.

■ 24 hours *in vivo*
▲ 48 hours *in vivo*
● 7 days *in vivo*

Whole lines *in vivo*
Broken " *in vitro*

Points are means of a total of four samples, 2 from each mouse.

Fig. 3.20

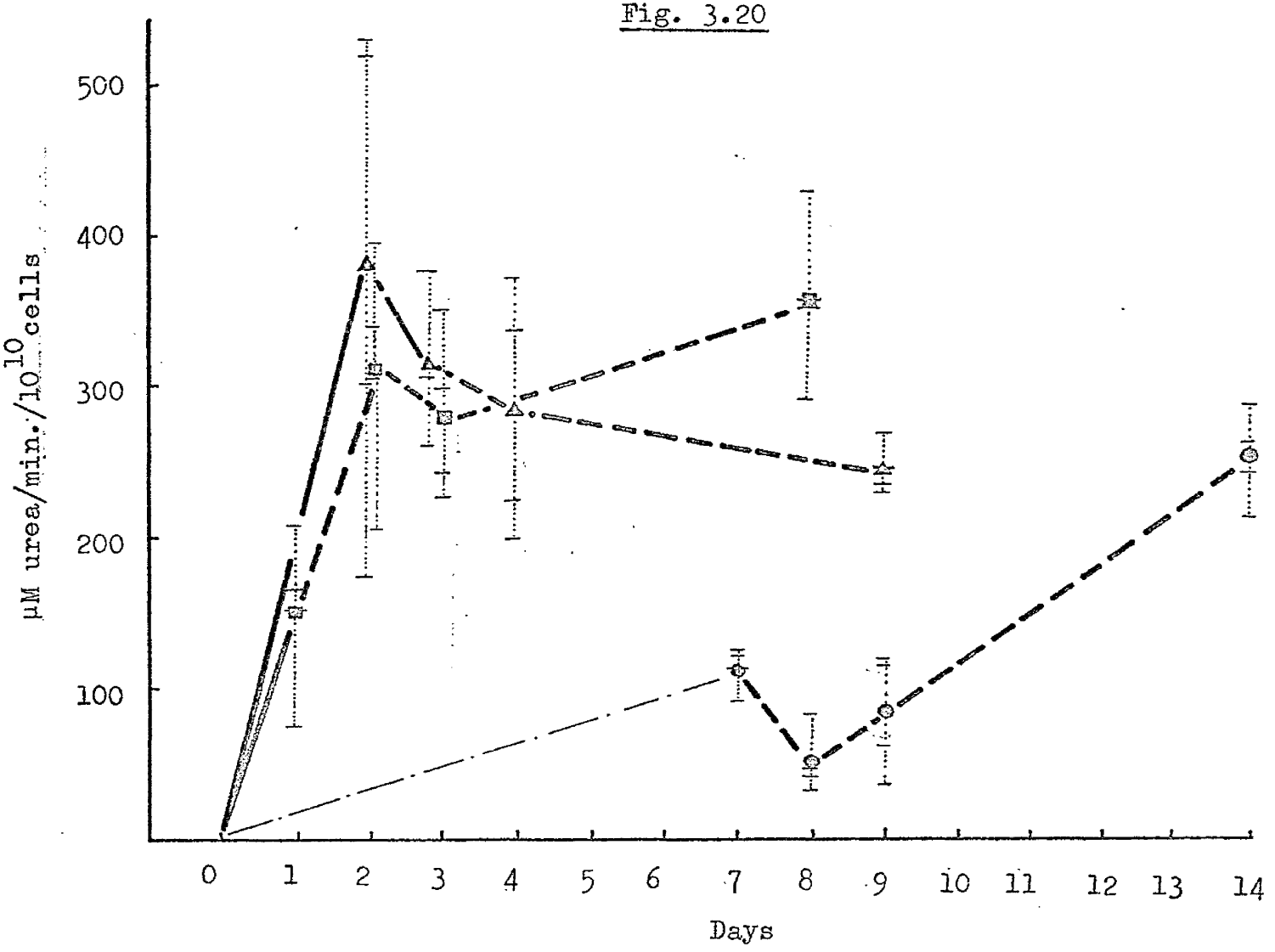

500
400
300
200
100
µM urea/min./10^{10} cells
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days

Fig. 3.21

Growth of ascites cells in culture and in the animal during experiment 1. (Fig. 3.20.)

The means of the cell counts of each sample were taken.

Whole line — growth in the animal.

Broken line — " " culture.

■ after 24 hours in the mouse.

▲ " 48 " " " "

o " 7 days " " "

Fig. 3.21

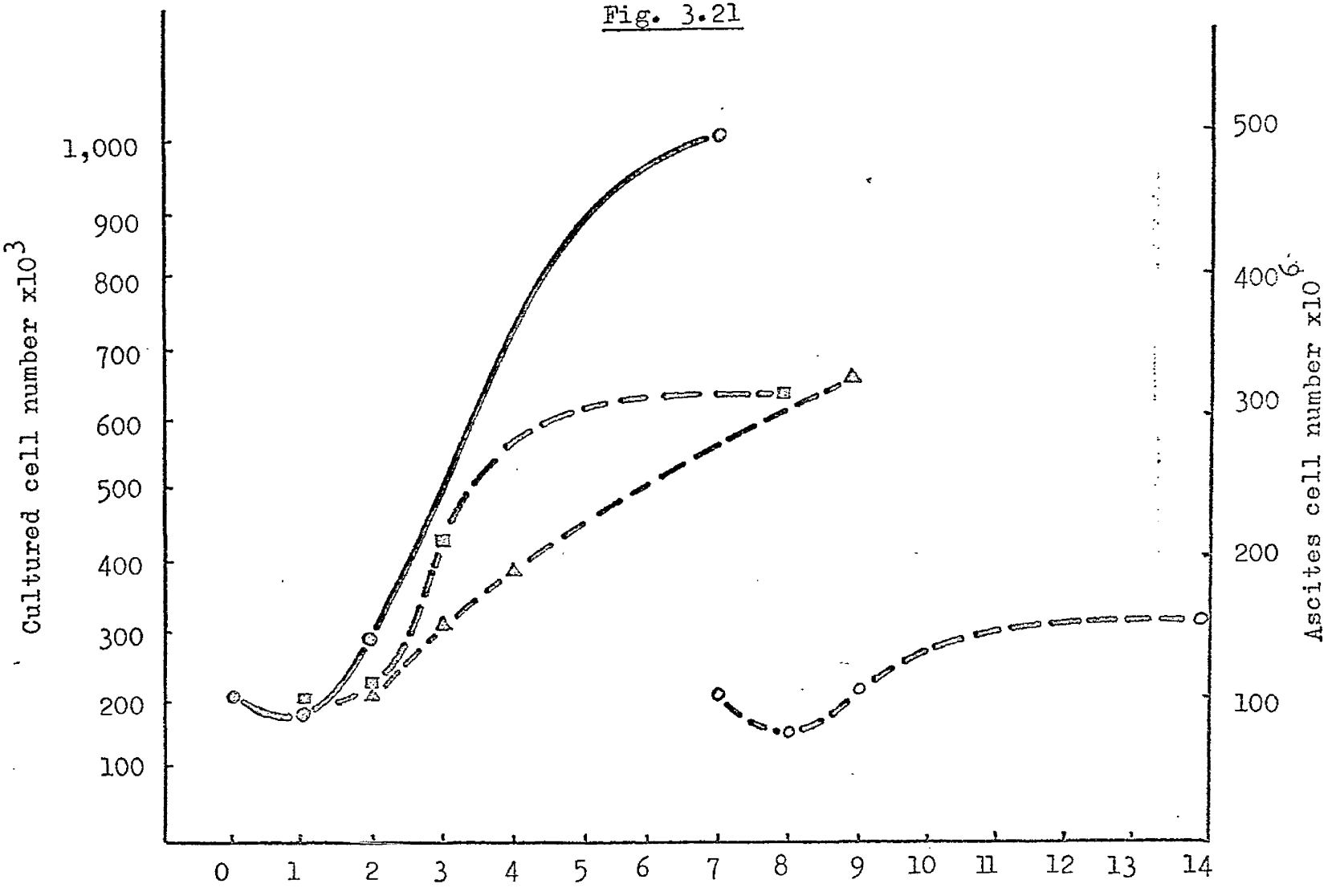

Cultured cell number x10^3
1,000
900
800
700
600
500
400
300
200
100
Ascites cell number x10^6
500
400
300
200
100
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14

Fig. 3.22

Increase in arginase activity of cultured Landschütz ascites cells after inoculation into mice. (experiment 1). Total enzyme activity plotted against total population.

Experimental procedure as in fig. 3.20

- ● in the animal.
- ■ in cell culture after 24 hours in the animal.
- ▲ in cell culture after 48 hours in the animal.

Fig. 3.22.

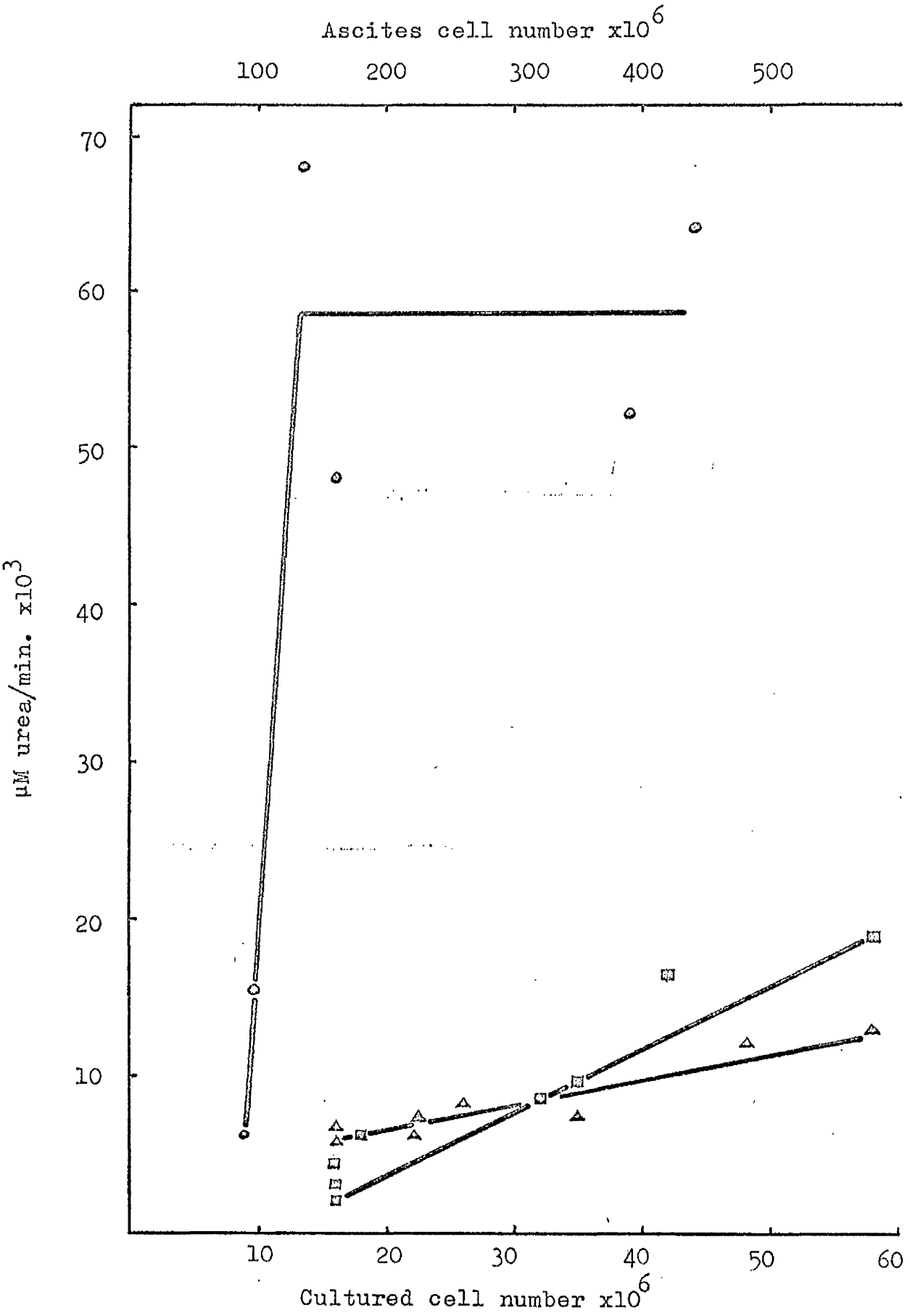

Ascites cell number x10^6
100
200
300
400
500
70
60
50
40
30
20
10
μM urea/min. x10^3
10
20
30
40
50
60
Cultured cell number x10^6

Fig. 3.23

Increase in arginase activity of cultured Landschutz ascites cells after inoculation into mice (Experiment 2). First 48 hours.

Experimental procedure as in fig. 3.20.

○ mean of 4 samples, 2 from each of 2 mice.

● mean of 2 samples from same mouse.

Fig. 3.23

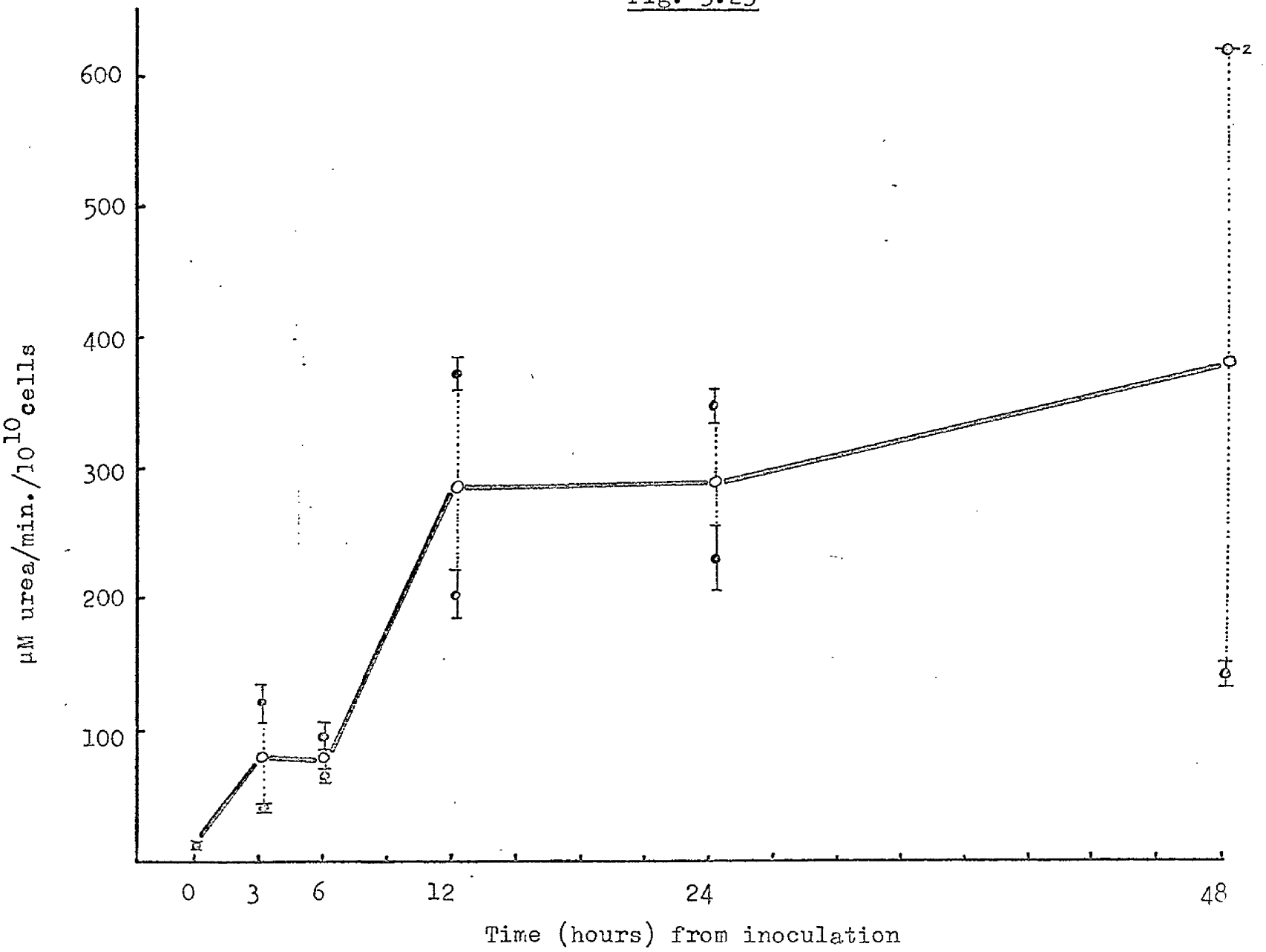

600
500
400
300
200
100
µM urea/min./10^{10}cells
0
3
6
12
24
48
Time (hours) from inoculation

Fig. 3.24

Growth of Landschutz cells in culture during experiment 2. (fig. 3.23).

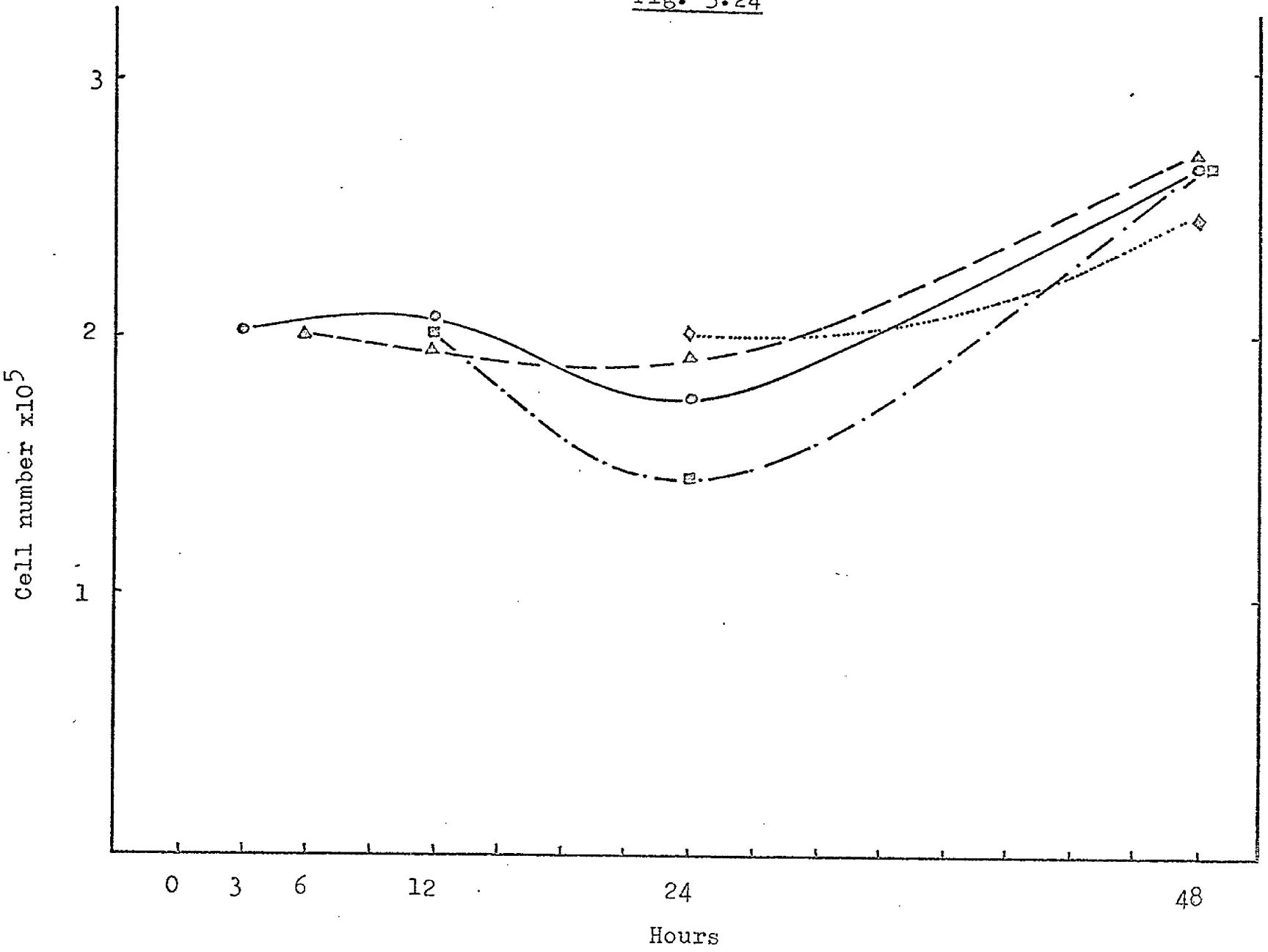
●——● After 3 hours in the animal.

▲- - -▲ " 6 " " " "

■—·—■ " 12 " " " "

◆·····◆ " 24 " " " "

Fig. 3.24



Figs. 3.25 - 3.28

Arginase activity of Landschütz cells transferred from the animal to culture. (Experiment 2).

Fig. 3.25	After	3	hours	in	the	animal.
3.26	"	6	"	"	"	"
3.27	"	12	"	"	"	"
3.28	"	24	"	"	"	"

Experimental procedure as in Fig. 3.20.

Whole line, course of increase in the animal prior to transfer (means from Fig. 3.23).

Broken line, course in culture.

Separate traces represent samples from different animals; two samples were taken from each animal.

Fig. 3.25

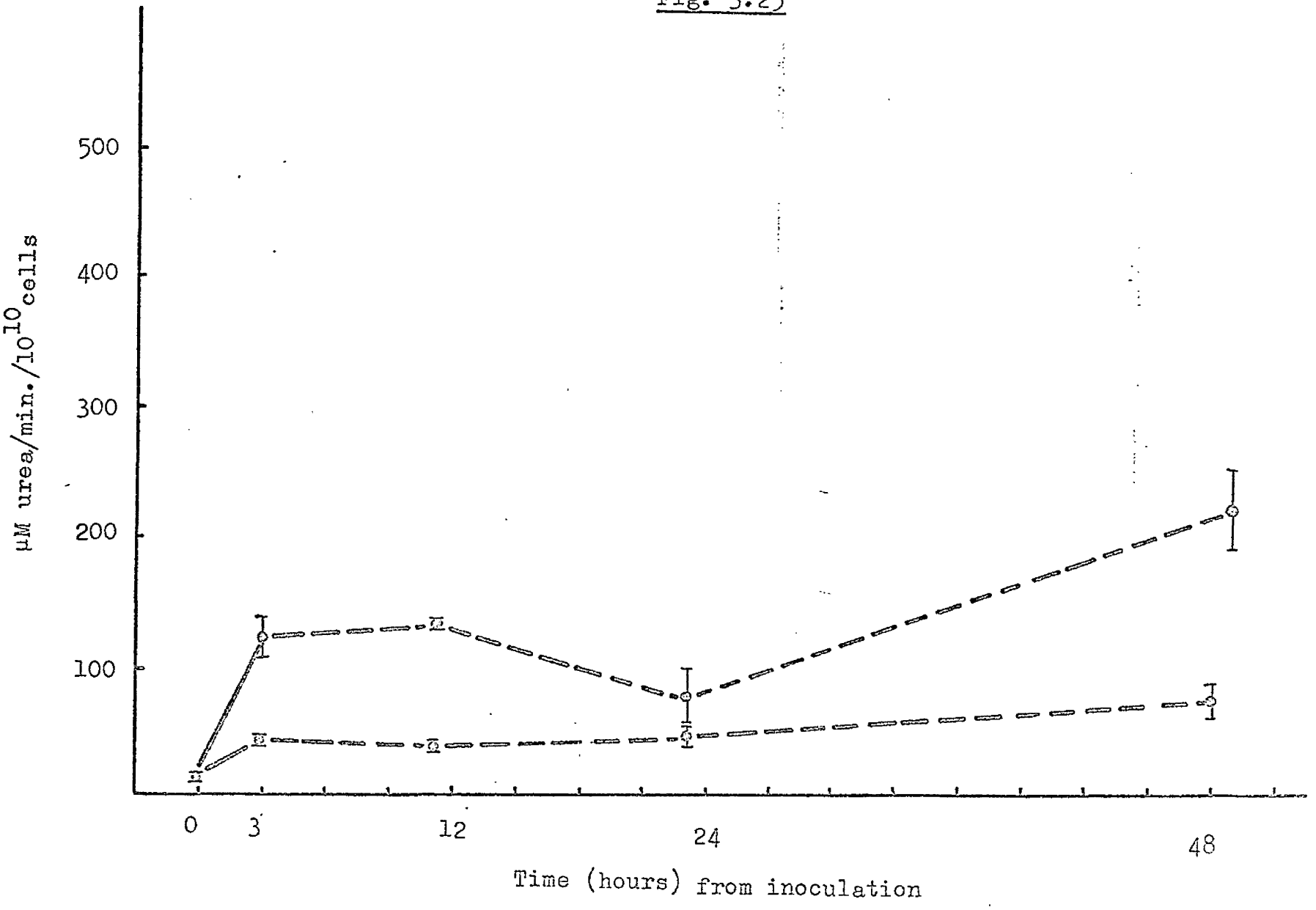

500
400
300
200
100
µM urea/min./10^{10} cells
0
3
12
24
48
Time (hours) from inoculation

Fig. 3.26

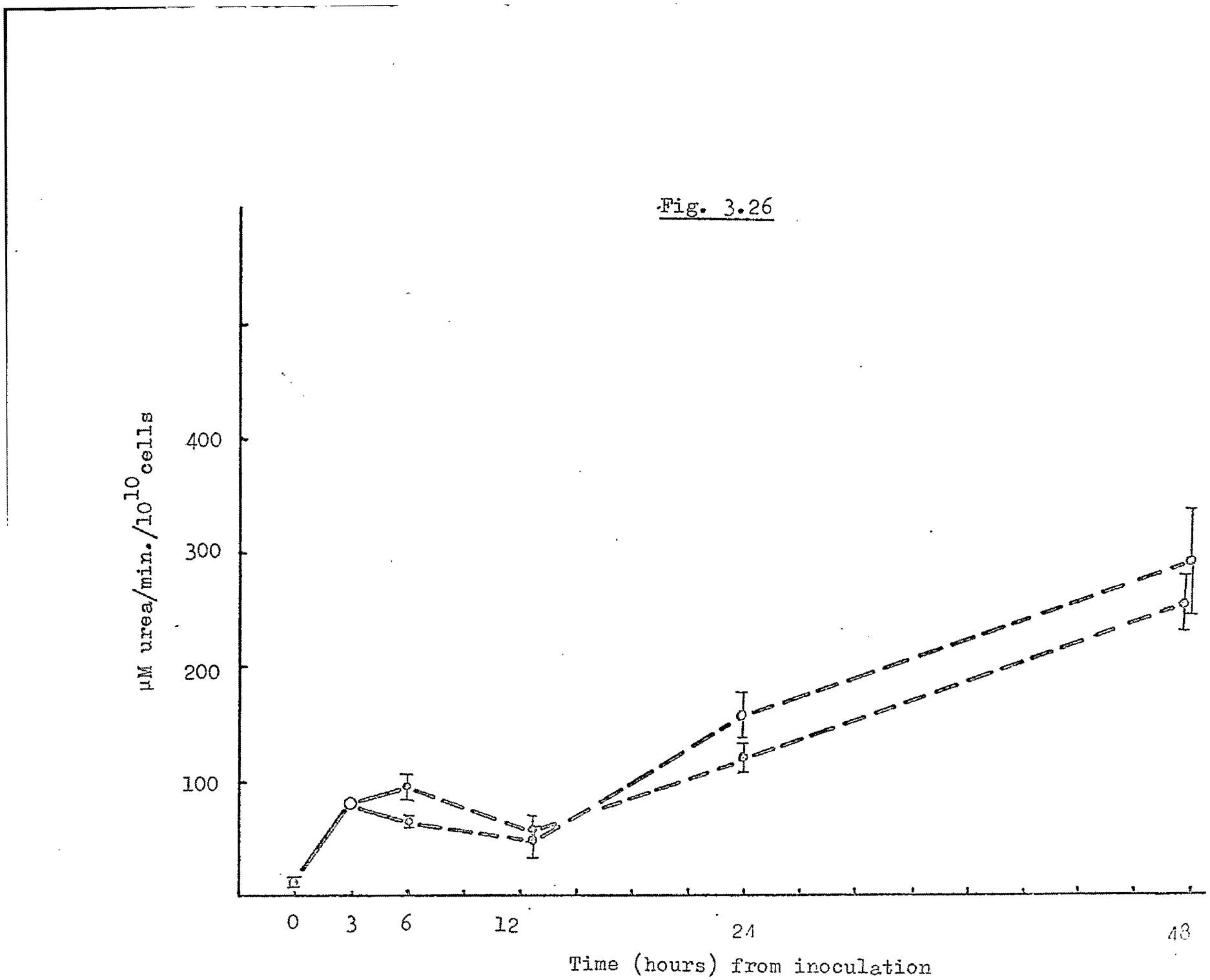

µM urea/min./10^10 cells
400
300
200
100
0
3
6
12
24
48
Time (hours) from inoculation

Fig. 3.27

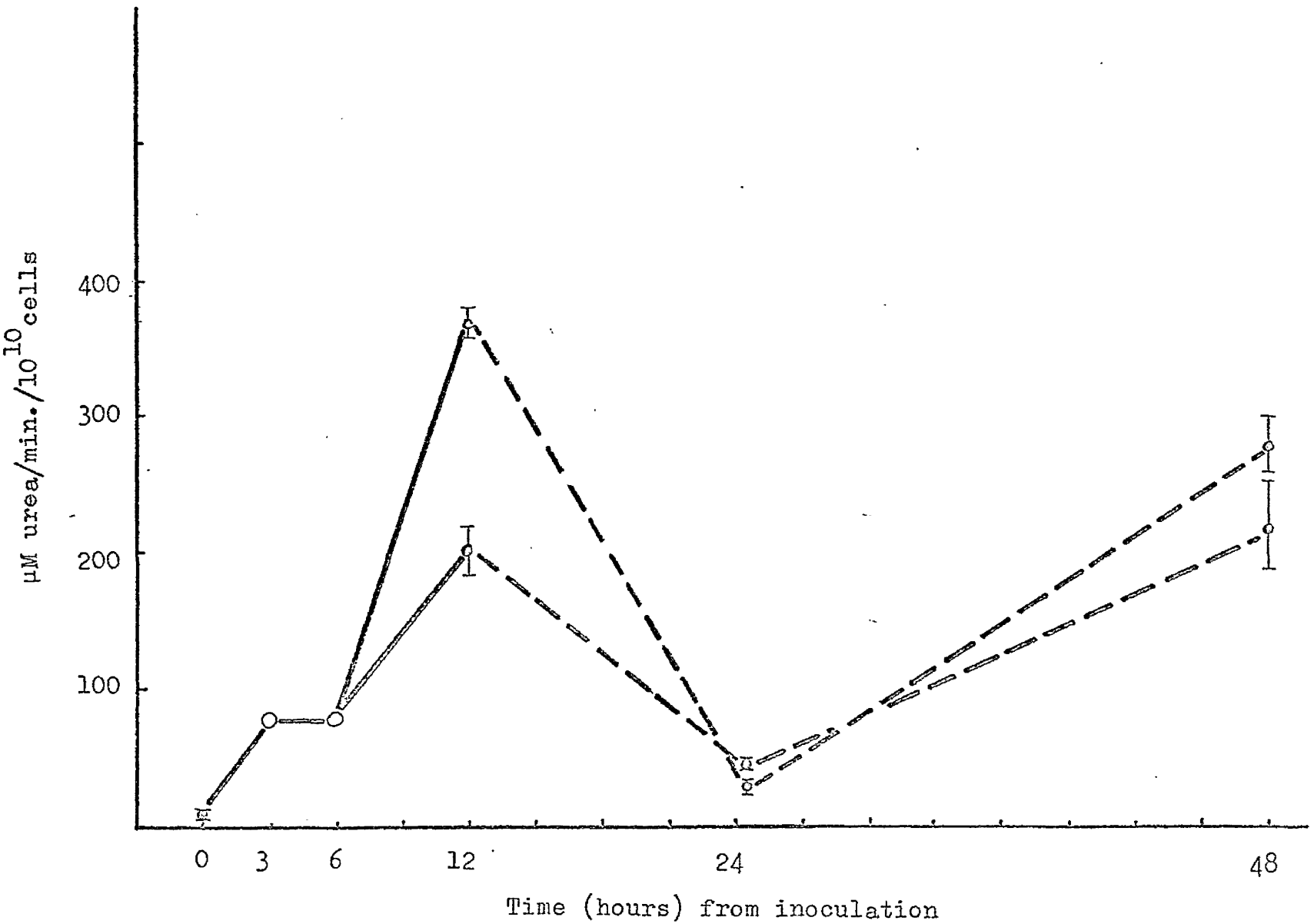
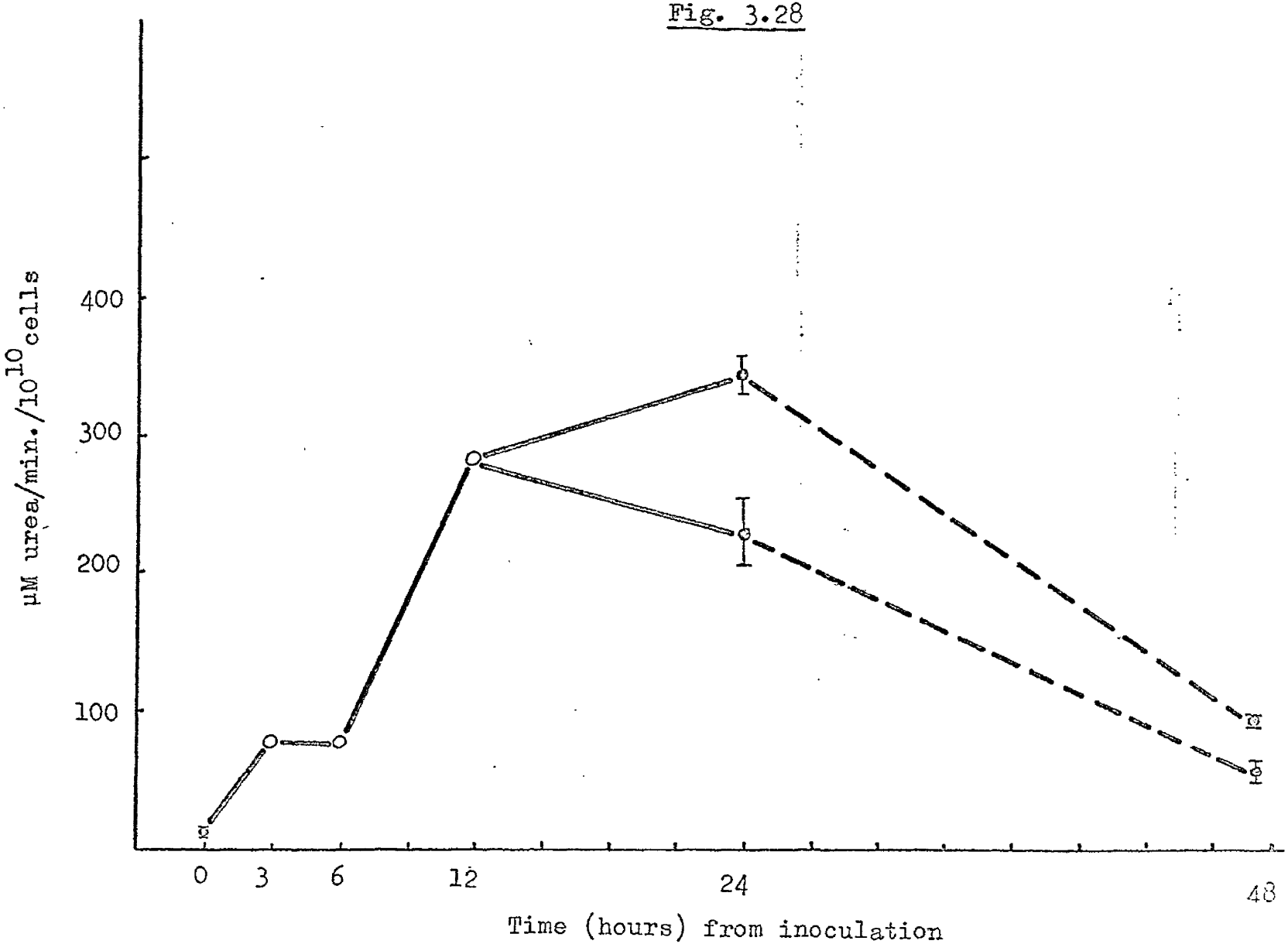

µM urea/min./10^{10} cells
400
300
200
100
0
3
6
12
24
48
Time (hours) from inoculation

Fig. 3.28


µM urea/min./10^{10} cells
400
300
200
100
0
3
6
12
24
48
Time (hours) from inoculation

ascites cells may differ from culture conditions in many ways - e.g. by the presence of hormones, by pH, or by concentration of certain ions; any one of these may cause an increase in arginase activity.

Cells taken from different animals show different rates of increase in arginase activity, and, as the culture conditions are standard, this indicates that the conditioning process may vary from animal to animal. Similarly the degree of increase obtained *in vivo* may also reflect differences in the ascitic environment between animals.

It has not been possible to simulate the effects of growth in the peritoneum by incubating cells in fresh ascitic fluid (Table 3.15). The reason for this is not apparent.

These results emphasise the participation of an influence other than arginine concentration upon the possible induction of arginase.

3.4 The uptake of arginine in HeLa cells.

Results from the previous section have demonstrated that there is considerable variation in the degree of increase in arginase activity obtained at different times. This has prompted an investigation of membrane permeability to determine whether it plays a part in the control of arginase activity by regulation of the entry of arginine.

Experiments were performed to determine whether the kinetics of arginine uptake would support the existence of a specific transport mechanism. The first of these experiments has been described in the "Materials and Methods" section. It involves the incubation of 25 ml. conical flask cultures of 1-2 x 10^6 HeLa cells with 2.5 μc/ml. ^{3}H-arginine for times ranging from 2 minutes to 2 hours. Each sample was duplicated. The level of ^{3}H-arginine in the acid soluble extract of these cells rose rapidly to reach a maximum at 30 minutes, and then fell to a slightly lower level, which probably represented equilibrium of influx and efflux (Fig. 3.27) (Fig. 3.29).

Tracer was removed from eight flasks after incorporation for one hour. The cells were washed briefly with 5 ml. balanced salt solution, and then incubated for 5, 15, 30 and 60 minutes with 5 ml. of tris-citrate buffered balanced salt solution containing 100 μg/ml. glucose and 0.1 mM arginine. The radioactivity of the acid soluble extract of these samples decreased at a rate comparable to the rate of incorporation, which suggests that influx and efflux proceed at a similar rate (Fig. 3.29, 30).

The uptake of ^{3}H arginine was then recorded at different times,

Fig. 3.29

Rate of ^{3}H-arginine uptake in HeLa cells at different times after addition of tracer.

Rate ($V_{max.} - V$) (mean values from fig. 2.7) plotted against log.10 time.

Fig. 3.30

Efflux of ^{3}H-arginine from HeLa cells incubated with tracer for 1 hour.

Cultures were labelled as for 1 hour samples in fig. 2.7. They were then rinsed briefly in 5 ml preincubation medium, and incubated for a further 5, 15, 30 and 60 minutes in preincubation medium. The decrease in the radioactivity of acid soluble extracts was determined as in fig. 2.4, and efflux was expressed as the rate of decrease.

Fig. 3.29

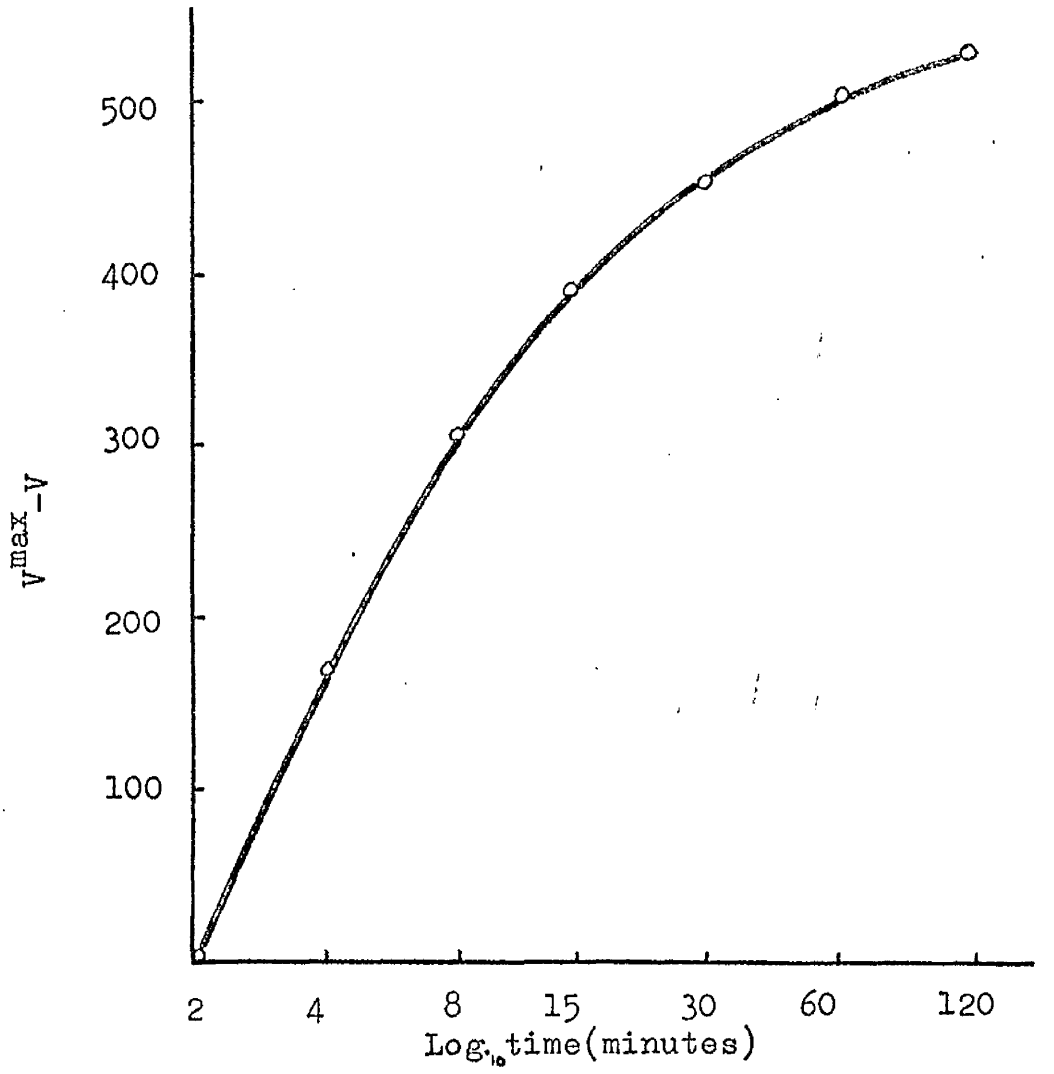
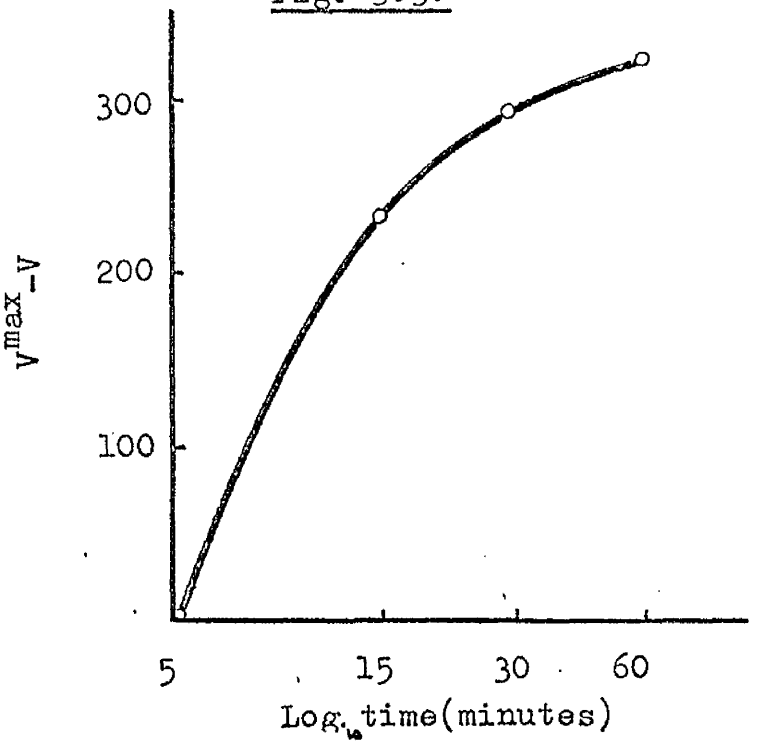


Fig. 3.30



over the same range, in different concentrations of arginine, to determine the effect on the initial rate of uptake and on the level of equilibrium between influx and efflux.

The protocol was as in the previous experiment (although efflux was not measured), and the results are displayed in Fig. 3.31. Influx and efflux reached equilibrium at 30 minutes during incubation with 0.01, 0.02 and 0.1 mM arginine, but did not reach equilibrium until 2 hours during incubation with 1 mM arginine. The equilibrium levels were not proportional to the concentration of arginine. This suggests that arginine uptake does not occur by simple diffusion.

When the equilibrium levels are compared there is more than a twofold difference in the ratio of intracellular to extracellular arginine between cells cultured in 10 µM and in 1 mM extracellular concentrations of arginine (Table 3.15). This suggests that while arginine is not concentrated within cells when the concentration in the medium is 1 mM it is concentrated to some extent at lower concentrations. If active concentration of arginine does occur, the degree would be so small that it would not be expected to have a significant effect on arginase activity, assuming that this enzyme is substrate induced.

The reciprocal of the rate of uptake, determined after two minutes incorporation, plotted against the reciprocal of the concentration was linear (Fig. 3.32). Hence the kinetics of the process suggest that specific receptor sites catalyse arginine entry.

Specific sites have been proposed for the uptake of other amino

Fig. 3.31

Uptake of ^{3}H-arginine in HeLa cells in different extracellular concentrations of arginine.

Cultures were incubated in 1mM, 0.1mM and 0.01mM arginine. Experimental procedure was as in Fig. 2.7. In this and the following uptake experiments, radioactivity was determined on a Nuclear Chicago scintillation spectrometer.

■ 1mM arginine

▲ 0.1mM "

● 0.01mM "

Fig. 3.31

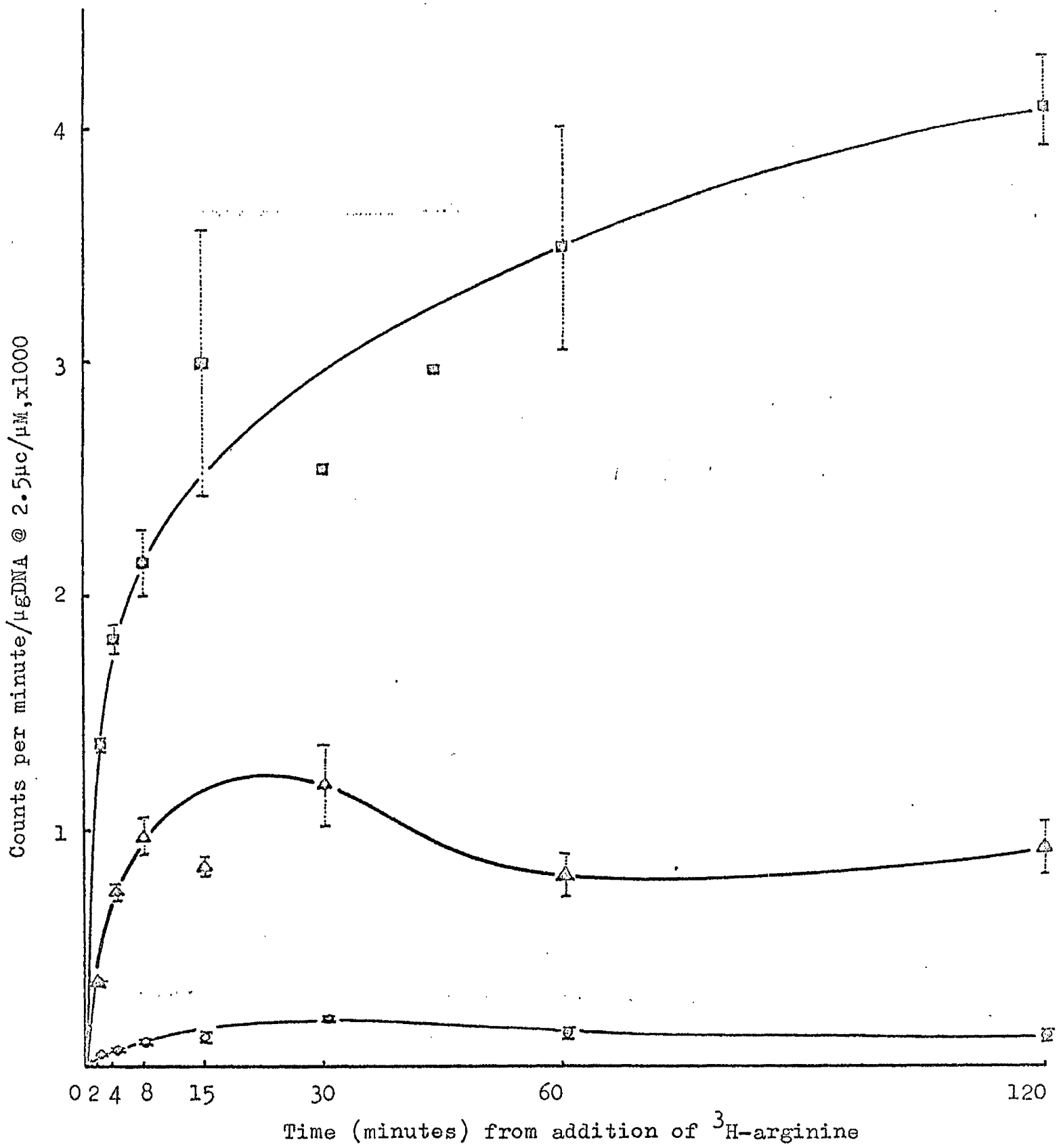

Counts per minute/µgDNA @ 2.5µc/µM, x1000
4
3
2
1
0 2 4 8 15 30 60 120
Time (minutes) from addition of ^{3}H-arginine

Table 3.16A

Ratio of intracellular to extracellular activity after equilibration of HeLa cells with ^{3}H-arginine (2 hours incubation at 37°C)

Extracellular Arginine concn.	1mM	0.1mM	0.01mM
Concn. of ^{3}H-arginine in medium µc/ml	2.5µc	2.5µc	2.5µc
Concn. of ^{3}H-arginine in cells µc/ml	3.52µc	7.25µc	9.5µc
Ratio $\frac{\text{inside}}{\text{outside}}$	1.4	3.0	3.8

The intracellular activity in µc/ml was calculated from the counts per minute in the acid soluble extract of samples equilibrated with ^{3}H-arginine (2.5µc/ml). The number of cells in each sample was calculated from the DNA phosphorus value (table 3.16B). 2.5×10^6 cells were taken as equivalent to 1ml packed cell volume (PCV). 320c.p.m. are equivalent to 1muc at 14% counting efficiency.

Table 3.16B

DNA-phosphorus of cultured HeLa cells

Sample	DNA-phosphorus/ 10^6 cells	Mean
1	0.92	
2	0.83	0.95
3	1.11	

Flasks of HeLa cells were trypsinised; the cells counted with an electronic cell counter and the DNA-phosphorus determined.

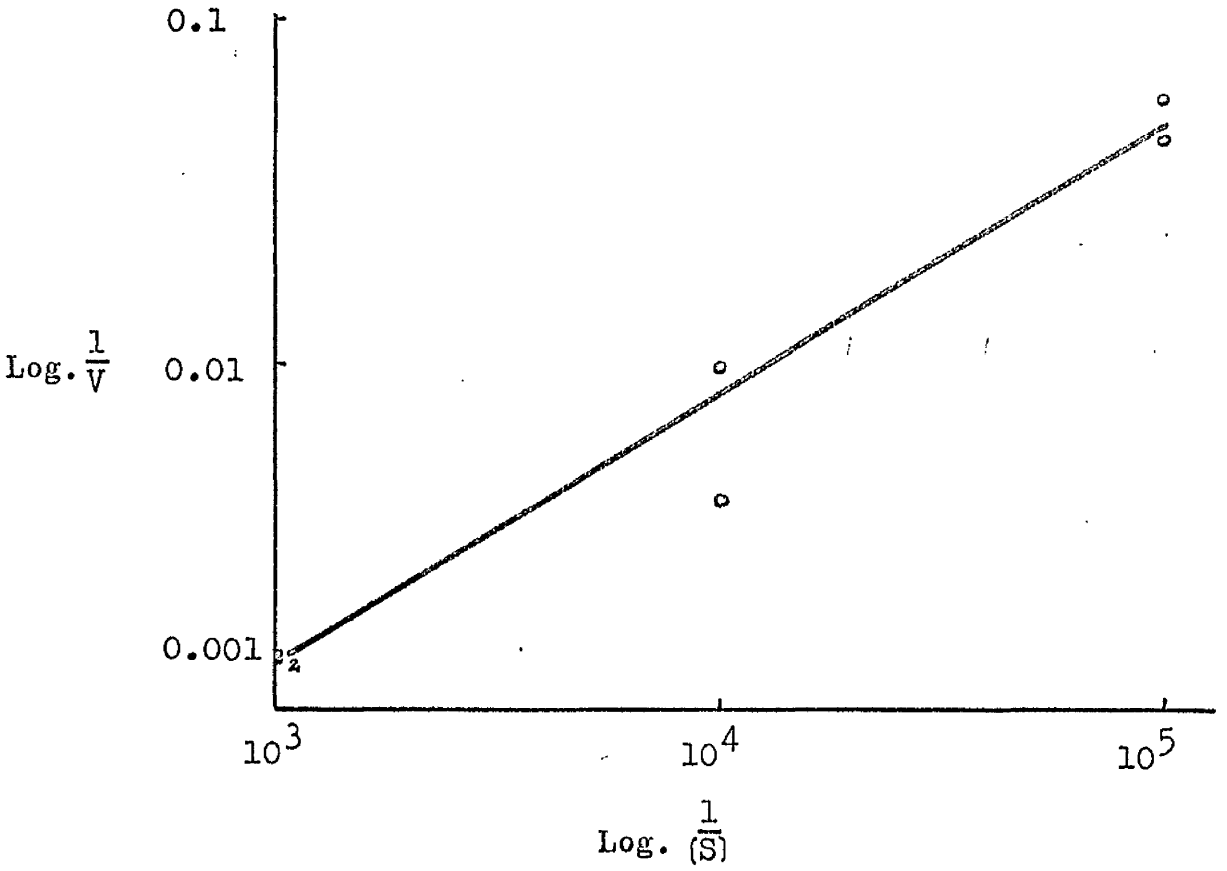
The DNA-phosphorus is expressed per 10^6 cells.

Fig. 3.32

Reciprocal of the rate of uptake of ^{3}H-arginine in HeLa cells plotted against the reciprocal of the extracellular concentration of arginine.

Results from 2 minutes' incubation in ^{3}H-arginine (Fig. 3.31) were used to calculate these values.

Fig. 3.32



acids (Christensen, 1961a) and acidic, basic, and neutral amino acids may each utilise a separate mechanism (Hokin and Hokin, 1963). Competition for the uptake sites has been demonstrated between amino acids within one group (Christensen, 1961a; Scholefield, 1962) and Rosenberg et al. (1963) showed that competition exists between ornithine, lysine and arginine. To determine whether similar competition existed in the present system, HeLa cells were incubated with 0.1 mM ^{3}H-arginine in the presence of equimolar concentration of ornithine, citrulline, lysine or glutamine. Each of these basic amino acids inhibited arginase uptake, glutamine more strongly than the others (Table 3.17). This suggests that a common entry mechanism exists for these five basic amino acids. It is a little surprising, however, to find that glutamine was the strongest inhibitor, although its molecular configuration does not resemble arginine, as much as lysine, ornithine and citrulline.

If arginine uptake is mediated by specific sites incorporating an enzyme-like system then the Q_{10} of the process would be expected to be higher than 2, the average value obtained for most chemical reactions. To determine Q_{10} HeLa cell cultures were incubated for 25 minutes at 37^{o}C in preincubation medium. They were then transferred to differing temperatures, from 4^{o}C - 39^{o}C, for 5 minutes. Incubation with 0.5 µc/ml. ^{3}H-arginine was carried out at the appropriate temperature for 2 minutes, and the activity of the acid soluble fraction of the cells determined as before.

The uptake of ^{3}H-arginine at different temperatures is shown in

Table 3.17

Inhibition of arginine uptake in HeLa cells by basic amino acids

	Uptake counts/min/μg DNA-P	Mean
Control	3150 2930 2880	2990
Lysine	2210 2080	2150
Citrulline	1860 2030 2170	2020
Ornithine	2192 1950 1810	1980
Glutamine	1160 1400 1520	1360

HeLa cultures were incubated with 0.1mM ^{3}H-arginine (10μc/μM) and equimolar concentrations of other basic amino acids.

Uptake was measured as counts per minute (corrected to 100% counting efficiency) in 0.5ml of the acid soluble extract, per μg of cell DNA-phosphorus after incubation for two minutes.

Figure 3.33. When the Q_{10} is calculated, a value of 3.5 is obtained between 4°C and 25°C; above 25°C the Q_{10} drops to 1.7. A similar observation was made by Christensen and Riggs (1952) where they found a low temperature coefficient for the uptake of glycine in Ehrlich's ascites cells between 20 - 33°C. This may mean that an enzymatic component is present and is working maximally by 34°C.

When cells were kept on ice for 1½ hours and then ^{3}H-arginine incorporation was measured at 37°C, an increase of arginine uptake of more than 100% over controls was observed (Table 3.18). This may be of significance in relation to the experiment suggesting increased arginase activity in cells incubated in a high arginine concentration after cold shock (Table 3.6).

The results of the experiments in Table 3.7 suggested that a high pH might alter the uptake of arginine. To test this, and to determine the pH optimum of the process, the following experiment was carried out. Incubation media, covering a pH range of 6.8 - 7.8, were prepared by adding 0.1 N HCl or 6% $NaHCO_3$. Labelled medium was prepared by adding ^{3}H-arginine to aliquots of media at each pH, to give 0.5 μc/ml. HeLa cultures were incubated for 30 minutes in preincubation medium at the appropriate pH, and then transferred to labelled medium at the same pH for 2 minutes. The radioactivity of the acid soluble material was then determined as before. There was no indication of a distinct optimum pH (Table 3.19), in the extent of the range examined. Neither was there any indication that arginine uptake could be significantly increased by raising the pH above pH 7.4.

Table 3.18

Effect of cold shock on the uptake of arginine in HeLa cells

Preincubation Time (mins.)	Temp	Uptake counts/min/µg DNA-P	Mean
30	36°C	2880 3030 2710	2870
90	4°C	4100 6040 5610	5825

HeLa cultures were incubated with ^{3}H-arginine (5µc/µM) for two minutes after preincubation at 36°C and 4°C in tris-citrate buffered BSS containing 0.1mM arginine and 1mg/ml glucose. Uptake was measured as the counts per minute/µg cell DNA-phosphorus in 1ml acid soluble extract at a counting efficiency of 100%.

Table 3.19

The effect of pH on arginine uptake in HeLa cells

pH	6.8	7.0	7.2	7.4	7.6	7.8
Uptake (counts/min/ µg DNA-P)	2680 2680	2310 2340 2380	2590 2380 2010	3360 2570 2900	2420 2610 2350	3140 2240 3500
Mean	2680	2340	2320	2940	2470	2960

Cultures were incubated with 0.1mM ^{3}H-arginine (5µc/µM).

The appropriate pH was achieved by addition of HCl and $NaHCO_3$ to incubation media. pH was determined by comparison with phenol red standards after equilibration with air at 37°C. Uptake was measured as in table 3.18.

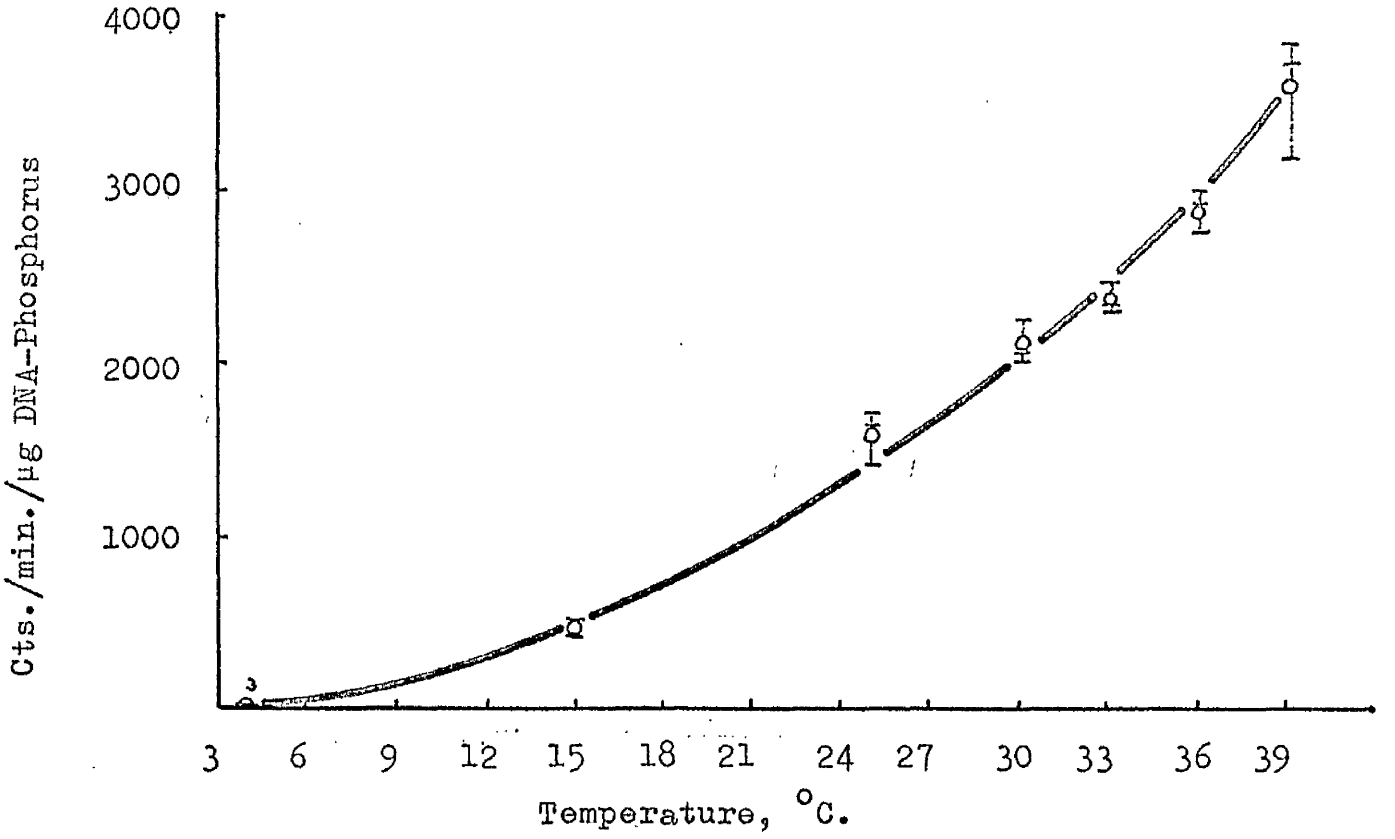
Fig. 3.33

Effect of temperature of the uptake of ^{3}H-arginine in HeLa cells.

3 day old cultures were incubated for 30 minutes in preincubation medium at 37°C and then transferred to preincubation medium at 4°, 15°, 25°, 30°, 33°, 36° and 39°C. for 5 mins. The preincubation medium was replaced with labelled medium containing 0.5 μc/ml ^{3}H-arginine and the incubations were continued for 2 minutes at the same temperatures.

Uptake of ^{3}H-arginine was determined by measuring the radioactivity of 1 ml of an 0.2N perchloric acid extract.

Fig. 3.33


4000
3000
2000
1000
Cts./min./µg DNA-Phosphorus
3
3
6
9
12
15
18
21
24
27
30
33
36
39
Temperature, °C.

Metabolic inhibitors were used to determine (1) whether the uptake process was energy dependent, and (2) whether the specific sites mediating uptake were sensitive to enzyme inhibitors. HeLa cell cultures were incubated for 30 minutes in preincubation medium containing the inhibitor, and then for a further 2 minutes in the same media with 0.5 μc/ml. ^{3}H-arginine. The radioactivity of acid soluble extracts was determined (Table 3.20).

Since each inhibitor used has several known effects and may also have had other unpredictable effects, it is not possible to correlate inhibition with specific metabolic pathways. However, some suggestions may be made on the basis of some of the predictable effects of those inhibitors. The absence of inhibition by DNP, dinitrophenol, sodium azide, iodoacetamide, phlorizin and phlorotin, suggests that arginine uptake does not require energy from aerobic processes. Ouabain, which inhibits ATPase (Schwartz and Lasater, 1963), and sodium fluoride, which inhibits ATPase and enolase, both inhibit uptake. This suggests that energy for arginine transport may be derived from anaerobic glycolysis.

Ouabain also inhibits the "sodium pump", and may consequently reduce the influx of potassium. As ouabain inhibits arginine uptake, this suggests that this process is not accompanied by an efflux of potassium.

N-ethyl maleimide, parachloromercuribenzoic acid, mercuric chloride, and ouabain may all inhibit the function of sulphydryl groups (Webb, 1963; Schwartz and Lasater, 1963), and each, except the first, caused considerable inhibition of arginine uptake. Mercuric chloride,

Table 3.20

The effect of metabolic inhibitors on arginine uptake in HeLa cells

Inhibitor	Concn.	Uptake counts/min./µg. DNA-P	Mean
(Control)	-	6,600 6,400 6,900	6,600
Potassium Cyanide	1 mM	7,000 6,600 9,700	7,800
Dinitro-phenol	0.25 mM	8,100 10,000 6,700	8,300
Sodium Azide	10 mM	4,600 8,000 6,100	6,000
Iodoacetamide	0.05 mM	6,300 8,700 6,700	7,200
Iodoacetate	1 mM	4,100 6,100 5,000	5,300
Phlorizin	3 mM	7,800 6,700 5,400	6,600
Phloretin	0.1 mM	11,600 10,400 11,000	11,000
Sodium Fluoride	10 mM	3,700 4,500 4,200	4,000
N-Ethyl Maleimide	0.01 mM	7,000 6,200 7,500	6,900
Parachloro-mercuri-benzoic Acid	5 µM	3,300 2,600 3,100	3,000
Mercuric Chloride	0.01 mM	1,900 1,000 1,100	1,300
Ouabain	1 mM	2,900 3,800 5,000	3,900

Measurement of uptake and labelling and counting procedure as in Table 3.18.

which has a profound effect on many sulphydryl enzymes, inhibited arginine uptake by 80%. This suggests that sulphydryl groups may be involved in the specific sites mediating arginine entry.

Following the report of Nitowsky et al. (1963) that alkaline phosphatase could be induced by alteration of the osmolarity of the incubating medium, the effect of osmolarity on arginine uptake was tested to determine whether osmotic control could participate in arginase induction by an effect of arginine uptake. HeLa cell cultures were incubated for 30 minutes in preincubation media with different concentrations of balanced salt solution and then for 2 minutes with ^{3}H-arginine in the same media. Small increases were obtained in hypotonic and hypertonic saline, but it is questionable if they can be considered significant (Table 3.21).

The entry of some amino acids is accompanied by K^{+} efflux (Christensen, 1961a). To determine whether changes in the Na^{+}: K^{+} ratio affected arginine uptake, HeLa cell cultures were incubated in 4 different Na^{+}: K^{+} ratios (Table 3.22). Although an increase in the proportion of extracellular potassium, to the exclusion of sodium, slightly inhibited arginine uptake, a 2:1 Na^{+}: K^{+} ratio produced higher arginine uptake than a 24:1 ratio. Also an increased Na^{+}: K^{+} ratio did not increase uptake. Furthermore, ouabain, which inhibits the sodium pump, and may consequently reduce the influx of potassium, inhibits arginine uptake (Table 3.20). Hence potassium may not be involved in arginine transport, and the inhibitory effects recorded, may only be evidence of a sub-optimal

Table 3.21

The effect of different osmotic pressures on arginine uptake in Hela cells

% Normal Saline	50	75	100	185	150
Uptake counts/min/µg DNA-P	2970	2200	1980	2320	2210
	2260	2150	1780	3310	2500
	2190	2340	1930	2600	4150
Mean	2470	2230	1900	2740	2950

The osmotic pressure of the medium was altered by addition of distilled water or 10X stock BSS to normal balanced salt solution.

Measurement of uptake and labelling and counting procedure as in table 3.18.

Table 3.22

The effect of altering the Na^+: K^+ ratio on arginine uptake in HeLa cells

Na:K ratio	Uptake counts/min/µg DNA-P	Mean
1:0	4920 3230 3580	3940
0:1	2460 3360 3200	3000
2:1	5200 4550 4750	4830
24:1 (Control)	3800 3310 3500	3540

The Na^+: K^+ ratio was changed by substituting different proportions of 11.5% KCl and 9.5% NaCl for 10X BSS stock (cf. table 2.4). Phosphate was added as sodium or potassium salt, as appropriate.

Measurement of uptake and labelling and counting procedure as in table 3.18.

K:Na ratio, the effect of which is not known.

A similar experiment was performed, varying the Mg^{++} and Ca^{++} concentration during incubation. Increase and decrease of the magnesium and calcium ion concentrations led to inhibition of arginine uptake (Table 3.33). This suggests that, in common with many enzyme reactions, the uptake mechanism for arginine requires an optimum concentration of these ions.

As a further test of the hypothesis that polyionic material might influence arginine uptake by alteration of the surface charge of the cells (c.f. Table 3.7), protamine (a poly-cation) and heparin (a polyanion) were included in incubation media to test their effect on arginine uptake. Both of these substances produced a 30% inhibition of arginine uptake (Table 3.34). This suggests that the alteration of the surface charge of the membrane does not increase arginine entry into HeLa cells, and the use of polyanions would not be expected to increase the internal arginine concentration (c.f. Table 3.7).

Since oestradiol, hydrocortisone, and insulin have been shown to influence amino acid uptake in Ehrlich's ascites cells and in rat liver and uterus (Noall et al. 1957; Christensen, 1961) these hormones (or derivatives) were tested in the present system. The hormones were dissolved directly in the incubation media, and samples were incubated for 30 minutes in preincubation medium, and for 2 minutes in the same medium containing 1 μc ^{3}H-arginine. Estimation of the radioactivity of acid soluble extracts gave the following results (Table 3.35).

Table 3.23

The effect of altering the Mg^{++} and Ca^{++} concentration of arginine uptake in Hela cells

Ionic conc.		Uptake counts/min/µg DNA-P	Mean
Mg^{++}	Ca^{++}		
-	1.3mM	4400 3790 3840	3900
0.9mM	-	2870 2900 2890	2890
1.8mM	1.3mM	1680 2520 2260	2150
0.9mM	13mM	2610 2490 2330	2370
(Control) 0.9mM	1.3mM	3800 3310 3500	3540

Alterations to incubation media were made independently with 14% $CaCl_2$ and 25% $MgCl_2$. Phosphate was added as equal proportions of the sodium and potassium salt and isotonicity was maintained by adding NaCl and KCl in the correct proportions (24:1).

Measurement of uptake, and labelling and counting procedure as in table 3.18.

Table 3.24

The effect of polyions on the uptake of arginine in HeLa cells

Polyion	Conc.	Uptake counts/min/µg DNA-P	Mean
(Control)	-	3150 2930 2860	2990
Protamine	30µg/ml	2780 1770 2150	2230
Heparin	1µg/ml	1890 1770 1830	1830

Measurement of uptake, and labelling and counting procedure as in table 3.18.

Table 3.25

The effect of stilboestrol, insulin and prednisolone on the uptake of arginine in HeLa cells

Hormone	Conc.	Uptake counts/min/µg DNA-P	Mean
(Control)	-	3150 2930 2890	2990
Stilboestrol	5µg/ml	3860 3370 3670	3630
Insulin	1 unit/ml	2970 2380 2110	2490
Prednisolone	5µg/ml	2430 2380 2190	2330

Measurement of uptake, and labelling and counting procedure as in table 3.10.

Insulin and prednisolone caused a 20% inhibition and stilboestrol a 30% increase in uptake. Although these effects are rather small, it is possible that they represent specific control mechanisms of arginine uptake.

From the results of these experiments it can be concluded that arginine is probably not concentrated in HeLa cells during incubation in 0.1 - 1 mM arginine. Nevertheless, arginine uptake appears to be mediated by specific sites on the membrane, and it is possible that the activity of these sites governs substrate induction of arginase. If the observed uptake represented a relatively passive phase of a potentially active system, induction of arginase by substrate might also involve induction of the appropriate transport mechanism, as has been shown to occur during induction of β-galactosidase in E. coli (Monod et al. 1963). Although hormones had little effect on the present system, it is possible that other hormones may be responsible for induction of specific transport mechanisms participating in enzyme adaptation.

3.5 RNA synthesis in cultured cells

If enzyme adaptation utilises a similar mechanism in animal cells to that described in bacteria (Jacob and Monod, 1961), then a rapidly synthesised messenger RNA may be involved. For such a fraction to function in animal cells it must move from the nucleus to the cytoplasm where the bulk of protein synthesis occurs. The following experiments were designed to follow the movement of rapidly labelled RNA in cultured cells.

As discussed in the "Introduction" kinetic studies of RNA synthesis and movement have not provided unequivocal evidence about the precursor relationships of various fractions of RNA. Before intracellular movement of RNA can be demonstrated, further incorporation, after a defined pulse with a radioactive precursor, must be prevented. In the following experiments, this was attempted by introducing an inhibitor of RNA synthesis after a brief incubation with ^{3}H-uridine.

Initially, 8-azaguanine was used for this purpose. Suspension cultures of strain L"S" cells were incubated for 15 minutes with 20 µc/ml. ^{3}H-uridine, and then labelled medium was removed. The cells were washed in balanced salt solution, and resuspended in culture medium (Eagle's medium plus 5% calf serum) containing 10 mM 8-azaguanine. Samples were removed at intervals and autoradiographs were prepared by method 2.

Nuclear activity built up rapidly for 4 hours and fell off slowly (Fig. 3.34). Label appeared slowly in the cytoplasm and continued to increase up to 24 hours. Since there is no nett gain of RNA during

Fig. 3.34

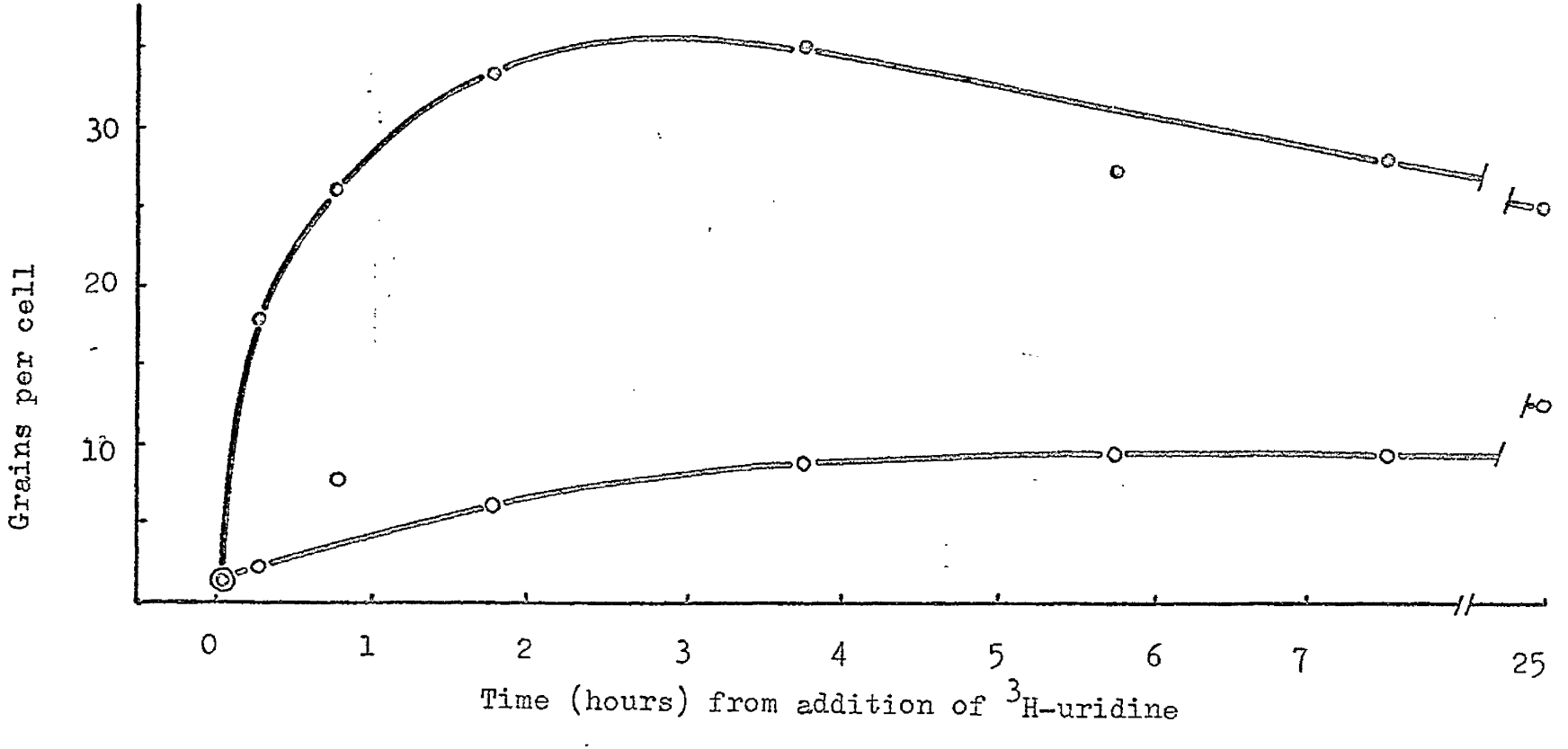
Effect of 8-azaguanine on RNA synthesis in L"S" mouse fibroblasts.

^{3}H-uridine was added to a rotating culture of L"S" cells at 37°C to give a final concentration of 20 μc/ml. After 15 minutes, the cells were washed with balanced salt solution and resuspended in unlabelled medium containing 1mM 8-azaguanine. Samples were removed before addition of ^{3}H-uridine, before addition of 8-azaguanine, and at intervals after addition of 8-azaguanine. Autoradiographs were prepared by method 2.

- ● nuclear activity.
- o cytoplasmic activity.

The specific activity of ^{3}H-uridine in this and subsequent experiments was 2mc/μMole.

Fig. 3.34


Grains per cell
30
20
10
0
1
2
3
4
5
6
7
25
Time (hours) from addition of ^{3}H-uridine

normal metabolism, the bulk of rapidly synthesised RNA would be expected to decay rapidly. Since the disappearance of label in the presence of azaguanine is much slower than the initial incorporation rate, incorporation must have continued in the presence of azaguanine, at a gradually decreasing rate as the labelled precursors were lost from the cell.

Cell fractionation experiments (Paul and Struthers, unpublished) have shown that in the presence of azaguanine, incorporation continues in the nucleus, although at a slower rate than in controls. If complete inhibition does occur the time taken would be too long to guarantee prevention of tracer incorporation after the pulse.

Since actinomycin D has been shown to inhibit DNA-primed RNA synthesis (Hurwitz et al. 1962; Reich, 1963) it was used in the present experiments to inhibit incorporation after the ^{3}H-uridine pulse. Before performing labelling experiments an experiment was done to observe the effects of exposure of L"S" cells to actinomycin D on the cellular RNA detectable by cytochemical methods. Actinomycin D was added to give a final concentration of 0.1 µg./ml., to rotating suspension cultures of L"S" cells and samples were withdrawn immediately and at 2, 8, 24, 48, 74 and 120 hours. These were washed in balanced salt solution, and fixed in ice-cold acetic alcohol for 10 minutes. Drops of suspensions of cells in fixative were then put on to slides, allowed to spread, and dried. Several slides were prepared from each sample. One slide was stained with haemalum and eosin for general structure, one was stained

with methyl green-pyronine (Curr, 1958) for RNA, and one was stained with acridine orange which binds to RNA and emits yellow fluorescence in ultraviolet light with high concentrations of RNA and a red fluorescence with low concentrations (Shatkin et al. 1962). The results are listed in Table 3.36.

In the first sample, the nucleolus stained intensely with pyronin, and the cytoplasm was also stained, though with a lower intensity. However, staining was completely absent from both by 24 hours. Similarly, yellow-orange and red fluorescence, indicative of RNA, was lost from nucleoli and cytoplasm by 24 hours. Nuclear pyenosis increased from 8 hours onwards and about 80% of all nuclei were pyenotic by 180 hours. Mitosis ceased after 24 hours. Green fluorescence in the cytoplasm of 24, 74 and 180 hour samples may have been due to non-specific fluorescence or to the presence of denatured DNA (Shatkin et al. 1962); green fluorescence in the cytoplasm was generally found in cells with pyenotic nuclei.

This indicates that, in the presence of actinomycin, the RNA of the nucleolus and cytoplasm gradually diminished and was not replaced. Most of the cellular RNA was lost by 24 hours.

Autoradiographs were prepared (by method 1) from cells which had been incubated with 20 $\mu c/ml.$ ^{3}H-uridine for 15 minutes and then transferred to 0.1 $\mu g./ml.$ actinomycin D for the rest of the experiment (24 hours). Activity appeared in the nucleus in the first sample and disappeared rapidly in following samples to reach a level of 10% of the level of the first sample by 6 hours (Fig. 3.35). The half-life of this fraction

Table 3.26

Cytochemistry of RNA in Actinomycin D-treated L"S" cells

Time from add'n of Actinomycin D 0.1µg/ml	Nucleolar Chromophilia		Cytoplasmic Chromophilia		Nuclear Pycnosis	Mitosis
	Pyronin	Acridine orange	Pyronin	Acridine orange		
0	++	Y ++	+	R +	-	++
2 hours	+	Y +	+-	-	+-	+
8 hours	+-	G ++	+-	R +-	+	+
24 hours	-	G ++	-	G +	+	+-
48 hours	+-	G ++	-	R +-	++	-
74 hours	-	G +	-	G +	++	-
120 hours	-	G +-	-	G +-	+++	-

Slides were fixed in acetic:alcohol (1:3) and stained as described in the text. The disappearance of chromophilia was recorded most easily from nucleoli and cytoplasm.

Y = yellow
R = red
G = green

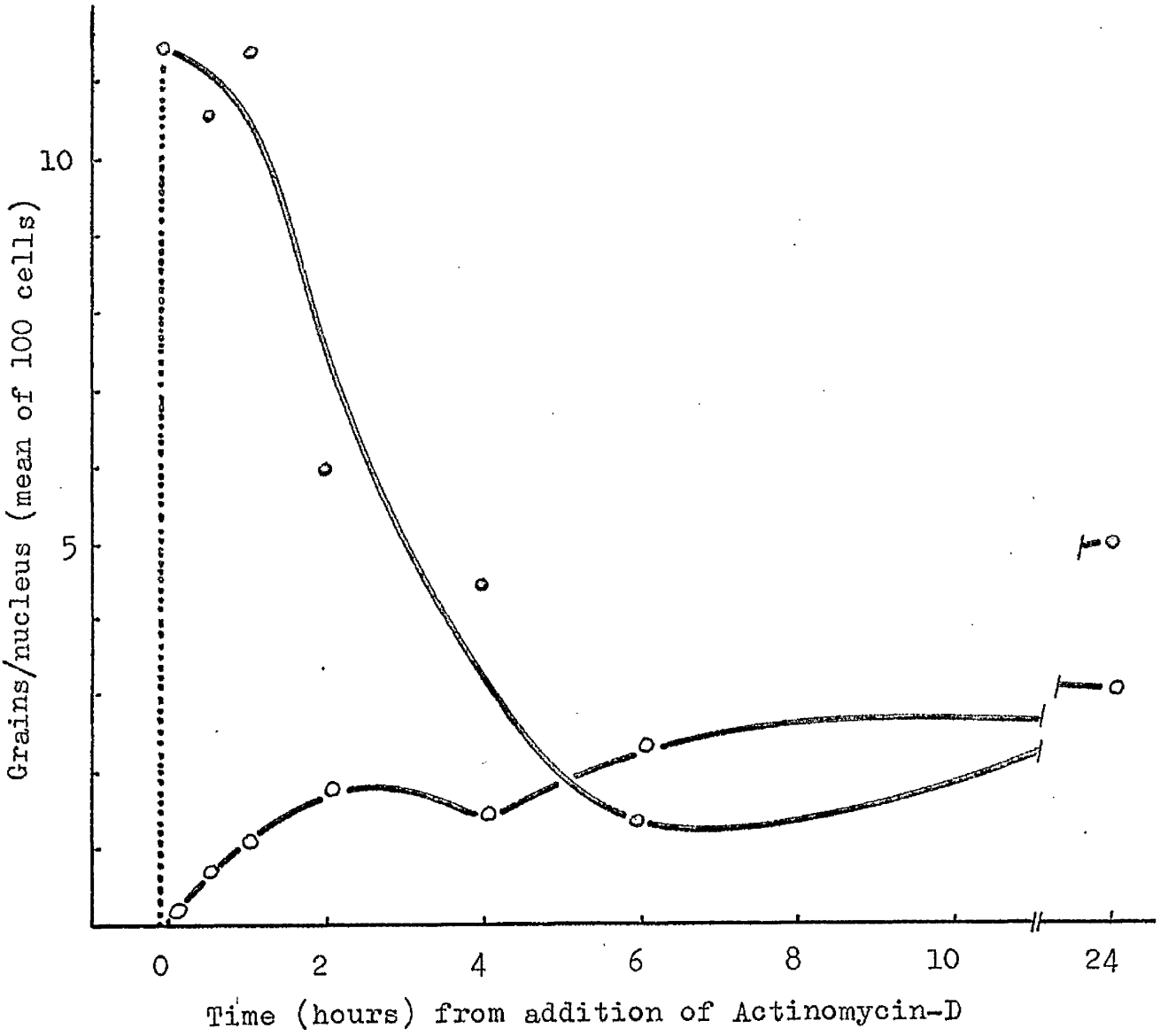
Fig. 3.35

Effect of 0.1 µg/ml actinomycin D on RNA synthesis in L"S" cells.
1. After a 15 minute pulse of ^{3}H-uridine.

L"S" cells were labelled as in fig. 3.34. 0.01 µg/ml actinomycin D was added after removing the tracer and washing the cells. Samples were removed at intervals after addition of actinomycin, and autoradiographs were prepared by method 1.

- ● nuclear activity.
- ○ cytoplasmic activity.

Fig. 3.35


Grains/nucleus (mean of 100 cells)
10
5
0
2
4
6
8
10
24
Time (hours) from addition of Actinomycin-D

was 3 hours.

This fraction is the "rapidly-labelled fraction" discussed in the introduction, and may be a precursor of other more slowly synthesised fractions of RNA (Goldstein and Micou, 1959 a, b; Amano and Leblond, 1960). As the rapidly-labelled peak of activity disappeared from the nucleus, activity built up in the cytoplasm, and reached 30% of the original nuclear activity by 6 hours. The results of this experiment did not show activity in cytoplasm comparable with the activity lost from the nucleus. It is not possible to claim that the activity which did appear in the cytoplasm was derived from the nucleus because of the following experiment.

Similar cultures of 'L' cells to those used above, were incubated with 0.1 µg./ml. actinomycin D for 35 minutes. The cells were then washed with balanced salt solution and incubated for a further 15 minutes with medium containing 10 µc. ^{3}H- uridine per ml. Autoradiographs prepared from these cells (by method 1) at intervals after addition of actinomycin (Fig. 3.36) showed that the activity appearing in both nucleus and cytoplasm was not reduced by incubation with actinomycin at this concentration.

Fig. 3.36 represents the activity which was removed when some slides were incubated with 0.1 mg./ml. RNAse in 0.1 M acetate buffer for 5 minutes. These results showed, in the cytoplasm an RNAse-sensitive, metabolically labile fraction, whose activity reached a maximum at 2 hours, and decayed with a half-life of 30 minutes. This fraction may have been derived from the

Fig. 3.36

Effect of 0.1 μg/ml actinomycin D on RNA synthesis in L"S" cells.

2. Actinomycin added before ^{3}H-uridine.

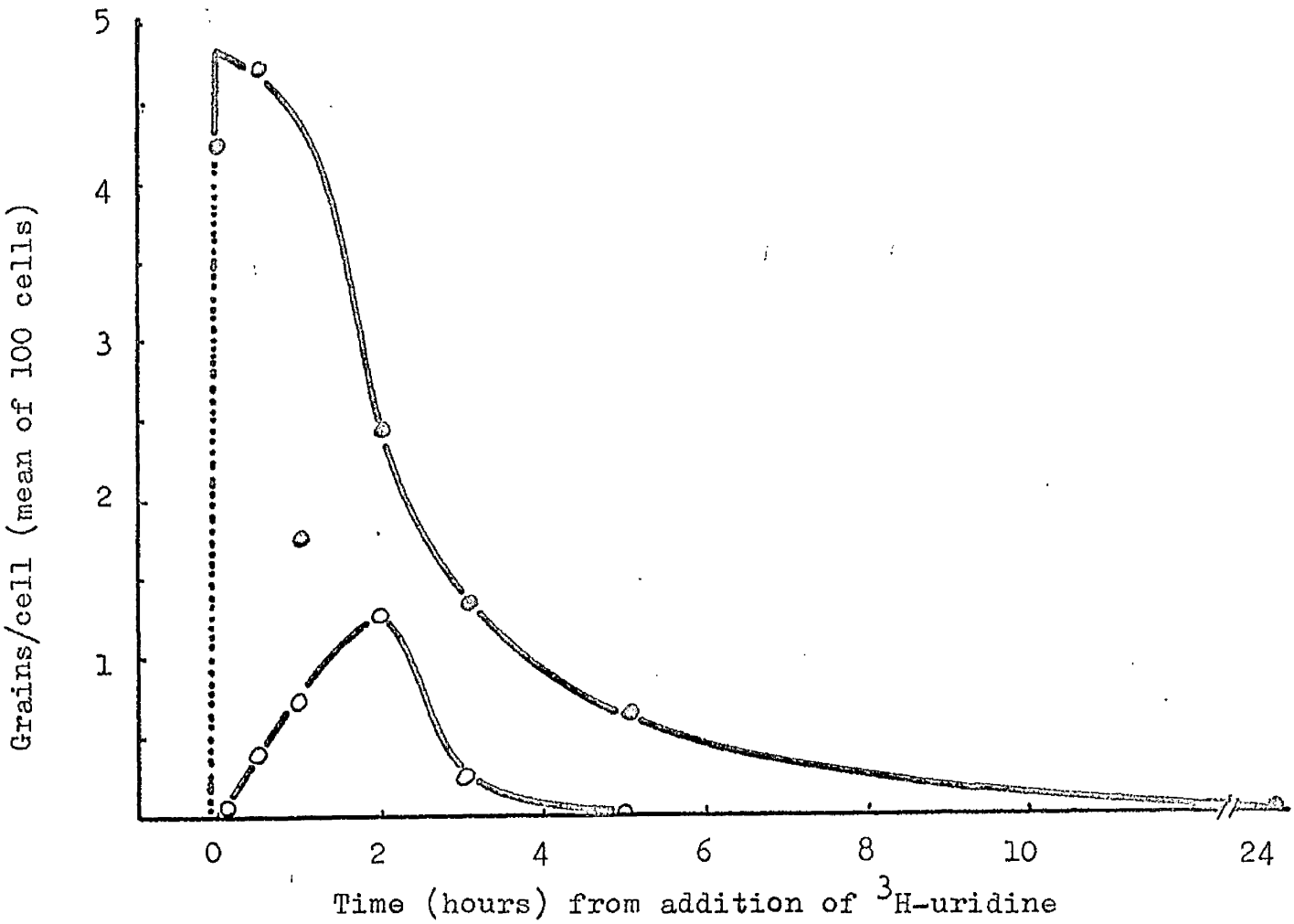
L"S" cells were incubated at 37°C in the presence of 0.1 μg actinomycin D for 35 minutes. The actinomycin was removed and the cells were washed and resuspended in medium containing 10 μc ^{3}H-uridine. They were incubated for 15 minutes and the tracer removed. The cells were washed in balanced salt solution and resuspended in growth medium (Eagle's medium plus 5% calf serum). Samples were removed 15 minutes after addition of tracer and at intervals thereafter. Autoradiographs were prepared by method 1.

● nuclear activity.

o cytoplasmic activity.

The activity is expressed as the number of grains per nucleus removed by incubation with 0.1 mg/ml RNAse.

Fig. 3.36


5
4
3
2
1
Grains/cell (mean of 100 cells)
0
2
4
6
8
10
24
Time (hours) from addition of ^{3}H-uridine

nucleus but this cannot be proved as there is no way of excluding independent cytoplasmic incorporation at this concentration of actinomycin.

The concentration of actinomycin was raised in later experiments to 100 µg./ml. which Paul and Struthers (1963) showed to be sufficient to inhibit 95% of all RNA synthesis in 15 minutes in cultured L'S' cells.

The conduct of these experiments was basically as already described. When the cells were incubated with 5 µc. ^{3}H-uridine per ml. for 10 minutes, and then washed and transferred to 100 µg. actinomycin per ml., auto-radiographs prepared (by method 1) from the cells at intervals after 15 minutes exposure to actinomycin, showed a peak of rapidly-labelled material in the nucleus comparable to those described above. This reached a maximum at 1 hour, but did not decay at the same rate as previously (Fig. 3.37). In this experiment no rapidly-labelled, labile, RNA could be detected in the cytoplasm.

This experiment was repeated using 5 µc/ml. ^{3}H-uridine for 15 minutes and 100 µg./ml. actinomycin for 15 minutes after the pulse. On this occasion, L'S' cells were also incubated for 15 minutes in 100 µg./ml. actinomycin prior to 15 minutes incubation with 5 µc./ml. ^{3}H-uridine. The cells were washed between incubation and after the second in each case. When ^{3}H-uridine was supplied before actinomycin rapidly-labelled material appeared in the nucleus, reaching a maximum at 1 hour and decaying with a half-life of 45 minutes (Fig. 3.38). No rapidly-labelled material appeared in the cytoplasm.

When actinomycin was added before ^{3}H-uridine, the activity of the nuclear rapidly-labelled fraction was reduced by 80% (Fig. 3.39). The

Fig. 3.37

Effect of 100 μg/ml actinomycin D on RNA synthesis in L"S" cells. Experiment 1.

L"S" cells were incubated at 37°C in the presence of 5 μc/ml. ^{3}H-uridine for 10 minutes, washed and incubated for a further 10 minutes in 100μg actinomycin D. The actinomycin was removed and the cells were resuspended in growth medium. Samples were removed 10 minutes after addition of actinomycin, and at intervals thereafter. Autoradiographs were prepared by method 1.

● nuclear activity (total)

• " " (RNAse resistant)

○ cytoplasmic " (total)

∘ " " (RNAse resistant)

Fig. 3.37

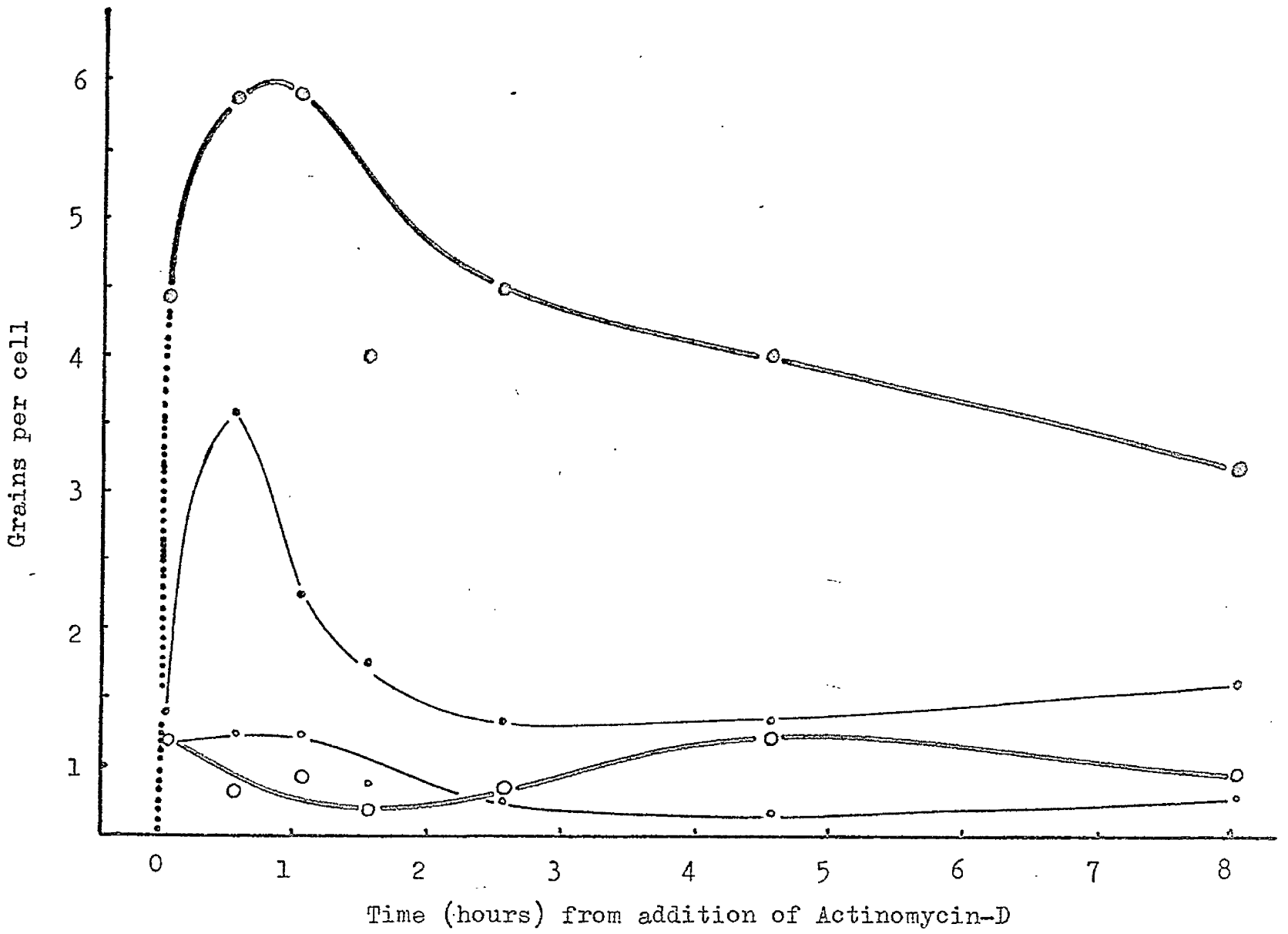

Grains per cell
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
Time (hours) from addition of Actinomycin-D

Fig. 3.38

Effect of 100 µg/ml actinomycin D on RNA synthesis in L"S" cells. Experiment 2.

(1) Actinomycin added after ^{3}H-uridine pulse.

L"S" cells were incubated for 15 minutes at 37°C in the presence of 5 µc/ml ^{3}H-uridine, washed, and incubated for a further 15 minutes in the presence of 100 µg/ml actinomycin. The cells were washed and resuspended in growth medium. Samples were removed 15 minutes after addition of actinomycin and at intervals thereafter. Autoradiographs were prepared by method 1.

Key as in Fig. 3.37.

Fig. 3.38

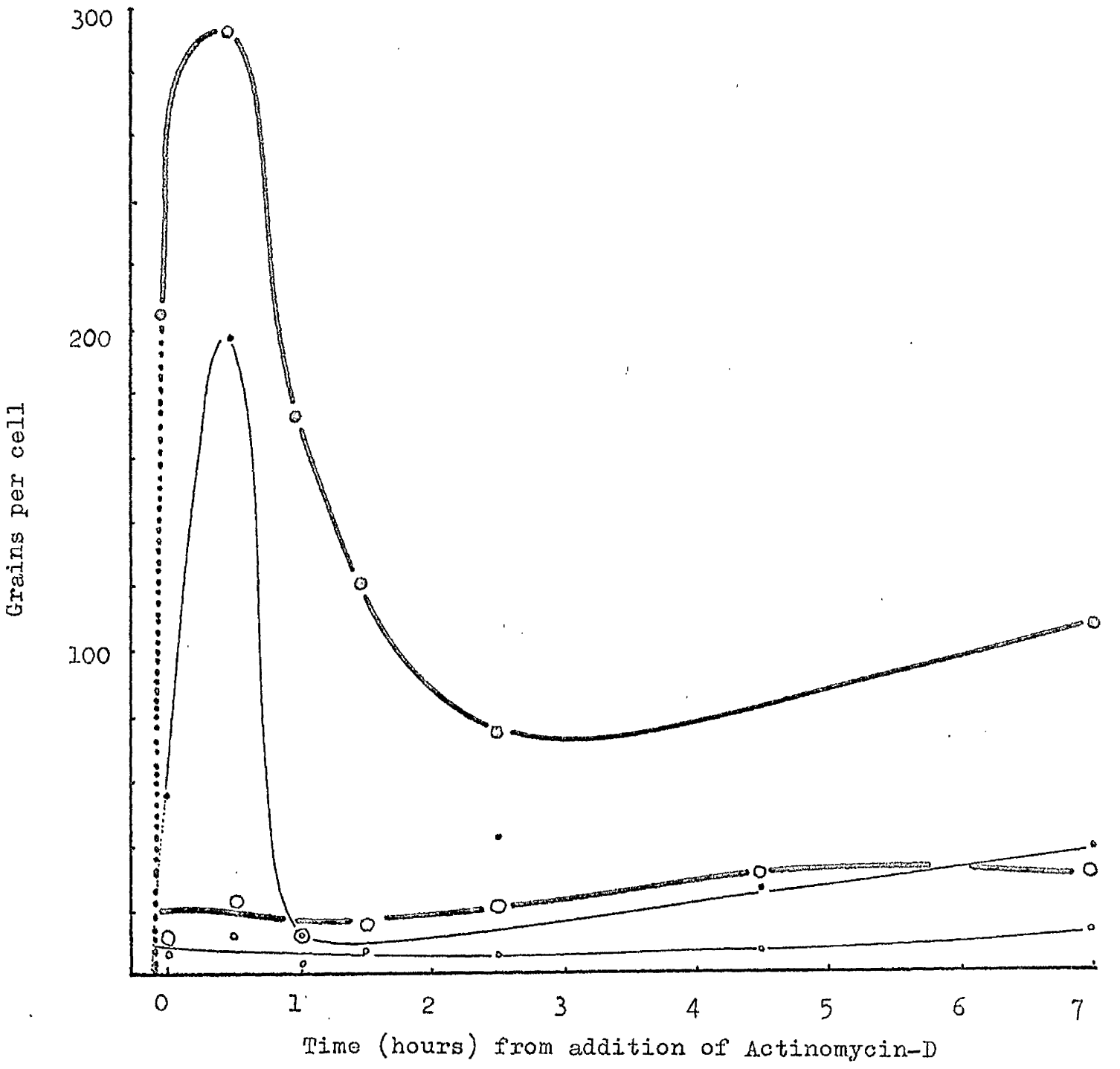

300
200
100
Grains per cell
0
1
2
3
4
5
6
7
Time (hours) from addition of Actinomycin-D

Fig. 3.39

Effect of 100 μg/ml actinomycin D on RNA synthesis in L"S" cells. Experiment 2.

(ii) Actinomycin added before ^{3}H-uridine pulse.

The order of incubation was the reverse of that in fig. 3.38. Conditions of incubation were the same as in fig. 3.38. Samples were removed 15 minutes after addition of ^{3}H-uridine and at intervals thereafter.

Key as in fig. 3.37.

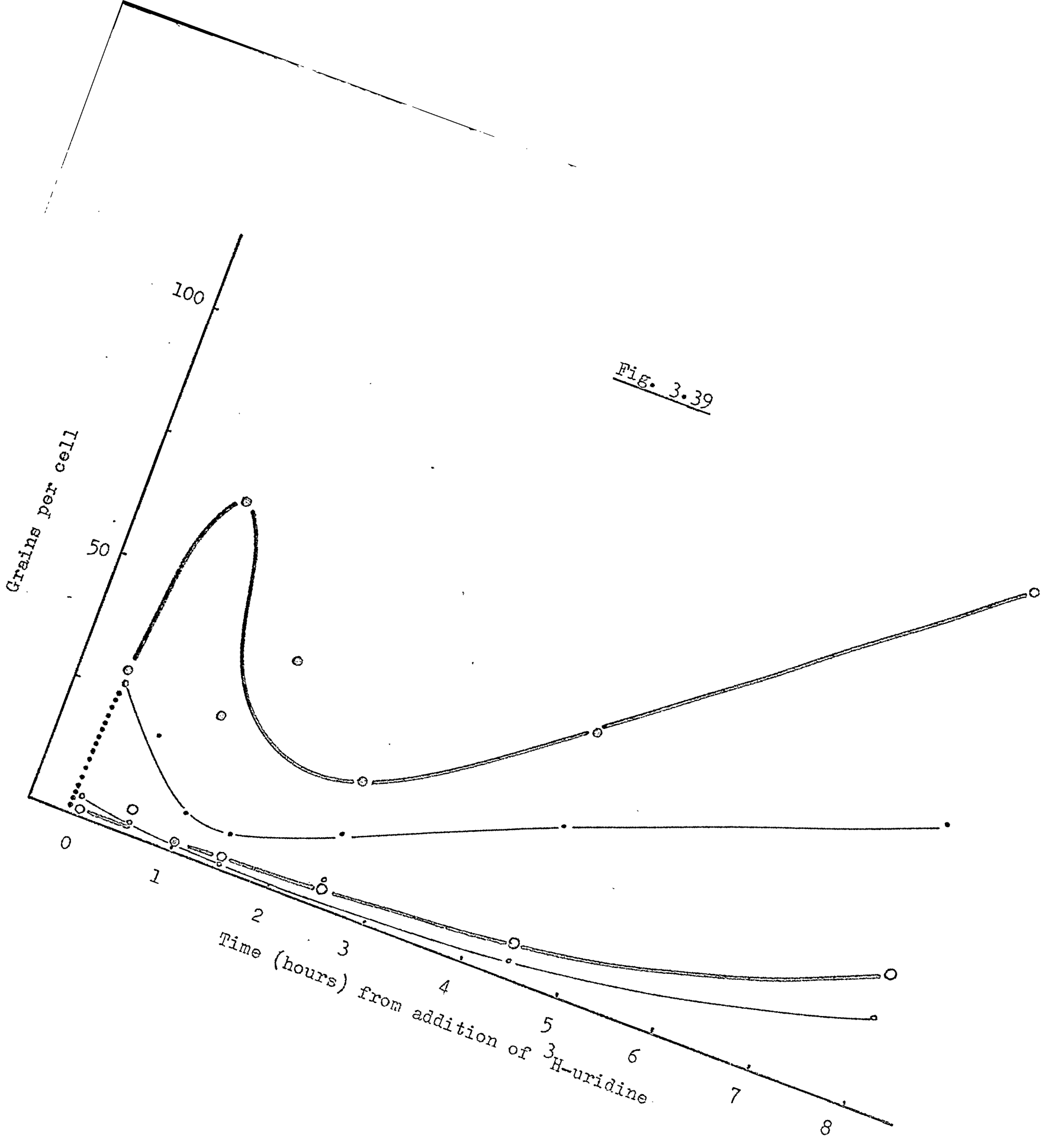

Grains per cell
100
50
0
1
2
3
4
5
6
7
8
Time (hours) from addition of 3H-uridine

Fig. 3.39

half-life of this material was also 45 minutes.

These results suggest that inhibition of the synthesis of the rapidly-labelled material is not complete, even after incubation with 100 µg./ml. actinomycin for 15 minutes. In addition there was evidence for the slow accumulation of labelled material in the nucleus and cytoplasm; this continued to rise during the 7 hours of the experiment. Some of the nuclear incorporation may have been into DNA, but as some of this is RNAse sensitive (Figs. 3.38, 39) and some also appears in the cytoplasm, this suggests that there is a slow incorporation of ^{3}H-uridine into RNA even in the presence of 100 µg./ml. actinomycin D.

Morita (1963) and Paul and Struthers (unpublished) have shown that incorporation of ^{3}H-uridine and cytidine into RNA in the presence of actinomycin may occur by terminal addition of cytidine on to soluble RNA. This labelled soluble RNA could become associated with ribosomes, and the slow appearance of RNA in the presence of actinomycin in these experiments may have been due to such a process. As some of the slowly-labelled material in the cytoplasm is resistant to RNAse (Fig. 3.39) it may be soluble RNA (Cantoni et al. 1963); the RNAse sensitive material may be ribosomal RNA.

There is also a part of the rapidly labelled fraction in the nucleus that is resistant to RNAse. The activity of this fraction diminishes before the whole nuclear peak in the presence of actinomycin. DNA-bound RNA may be resistant to RNAse, and this RNAse-resistant fraction of the rapidly-labelled material may therefore be newly synthesised DNA-bound RNA.

RNA from cultured mammalian cells has been characterised on sucrose density gradients. It has been suggested that the rapidly-labelled material, sedimenting at the 35.45S position, may be a precursor of RNA sedimenting at the 28S and 18S positions, which has been identified as ribosomal RNA (Scherrer and Darnell, 1963; Tamaki and Mueller, 1963). These findings are based on the assumption that no synthesis of RNA occurs in 5 µg./ml. actinomycin D. When RNA from H"S" cells, treated with 5 µg./ml. actinomycin D and then washed and incubated with 4 µc./ml. ^{3}H-uridine for 30 minutes, was centrifuged through a sucrose density gradient, there was evidence of considerable incorporation in all parts of the gradient including the 35S - 45S region (Fig. 3.40i). When the cells were treated with 100 µg./ml. actinomycin before the ^{3}H-uridine pulse, sucrose density gradient analysis of the RNA showed that incorporation into 28S - 45S sedimenting RNA was abolished and that into ribosomal and soluble was greatly reduced (Fig. 3.40ii).

Although incorporation into ribosomal and soluble RNA was slight it increased slowly during the experiment (1 hour). This increase may represent residual synthesis of the fractions already described in the "Introduction" or it may represent synthesis of a specific fraction of RNA. If the latter is true, the resistance to actinomycin may be due to RNA-primed synthesis, as distinct from actinomycin-sensitive DNA primed synthesis.

To test this hypothesis, 2 samples of H"3" cells were incubated

Fig. 3.40

Sucrose density gradients of DNA from L"S" cells incubated in the presence of actinomycin.

(i) 5 μg/ml. actinomycin.

(ii) 100 μg/ml. actinomycin.

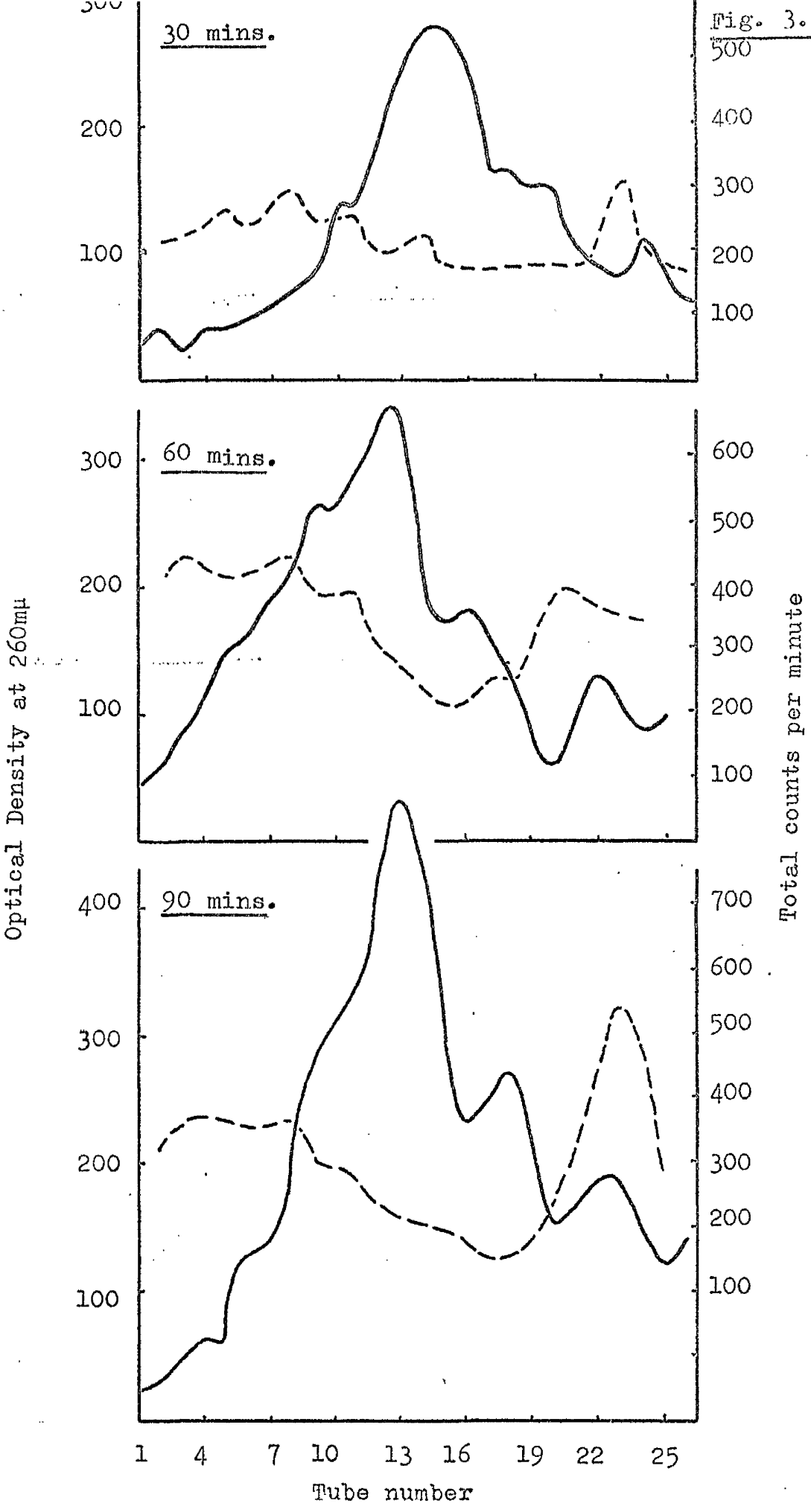
L"S" cells were incubated at 37°C in the presence of 5 μg/ml. and 100 μg/ml. actinomycin D for 15 minutes, washed and incubated for a further 30 minutes in the presence of 4 μc/ml ^{3}H-uridine. Samples were removed 30, 60 and 90 mins. after addition of ^{3}H-uridine. Sucrose gradients were prepared as described in the text.

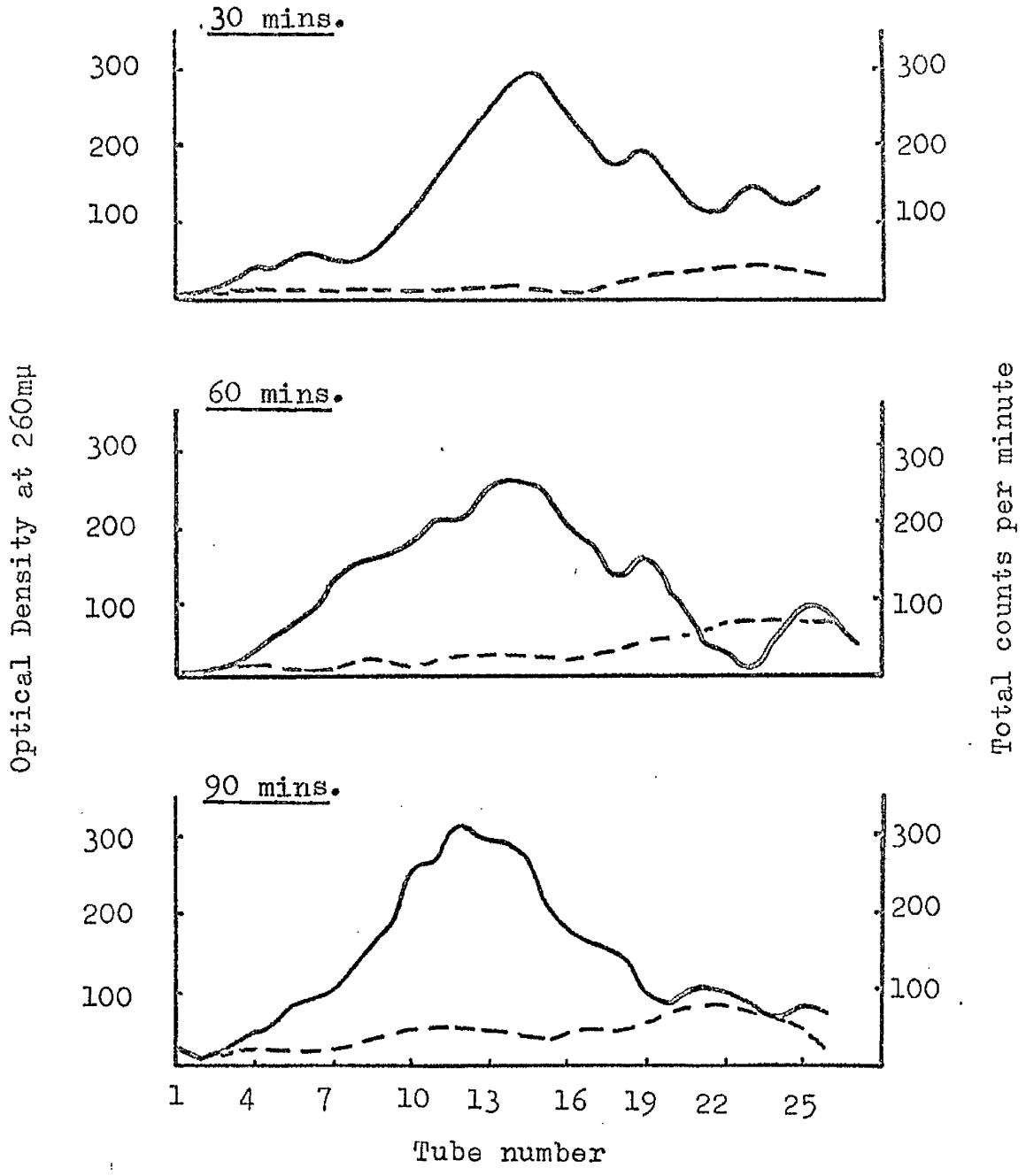
Tube number 1 is the bottom of the gradient.

——— optical density at 260 mμ

— — counts/min.

Fig. 3.40(i)


30 mins.
60 mins.
90 mins.
Optical Density at 260mμ
Total counts per minute
Tube number
1
4
7
10
13
16
19
22
25
100
200
300
400
500
600
700


30 mins.
60 mins.
90 mins.
300
200
100
Optical Density at 260mμ
Total counts per minute
1
4
7
10
13
16
19
22
25
Tube number

in 100 µg./ml. actinomycin D, washed, and then incubated in 50 µc./ml. ^{3}H-uridine for 4 hours. One sample had 2 µg./ml. bacterial RNA (prepared in the same manner as RNA from E'S') added to the culture.

The sucrose density gradient analysis of RNA from the sample incubated in the absence of bacterial RNA, showed greater incorporation into the ribosomal region of the gradient (Fig. 3.41A). However, the position of the peak produced is not typical of ribosomal RNA. Whether this is a specific RNA fraction, or an artefact produced by actinomycin treatment is not apparent from these results.

When the RNA from cells treated with bacterial RNA was centrifuged on sucrose density gradients, the presence of bacterial RNA appeared to have inhibited incorporation into the ribosomal region of the gradient (Fig. 3.41B). The excess RNA may have diluted the precursor pool with unlabelled polymer fragments, which may have been incorporated in preference to labelled nucleotide. Hence, one cannot draw any definite conclusion on the priming of this actinomycin-resistant fraction from these results.

Both gradients showed incorporation into regions corresponding to soluble RNA. As mentioned above, incorporation of cytidine (which can be produced from uridine in the cell) into soluble RNA, may continue in the presence of actinomycin.

In conclusion, there is still some actinomycin resistant material that is slowly formed, RNAse sensitive, and sediments with ribosomal RNA.

Fig. 3.41.

Sucrose density gradients of RNA from L"S" cells, synthesised in the presence of actinomycin D. Effect of incubation in the presence of bacterial RNA.

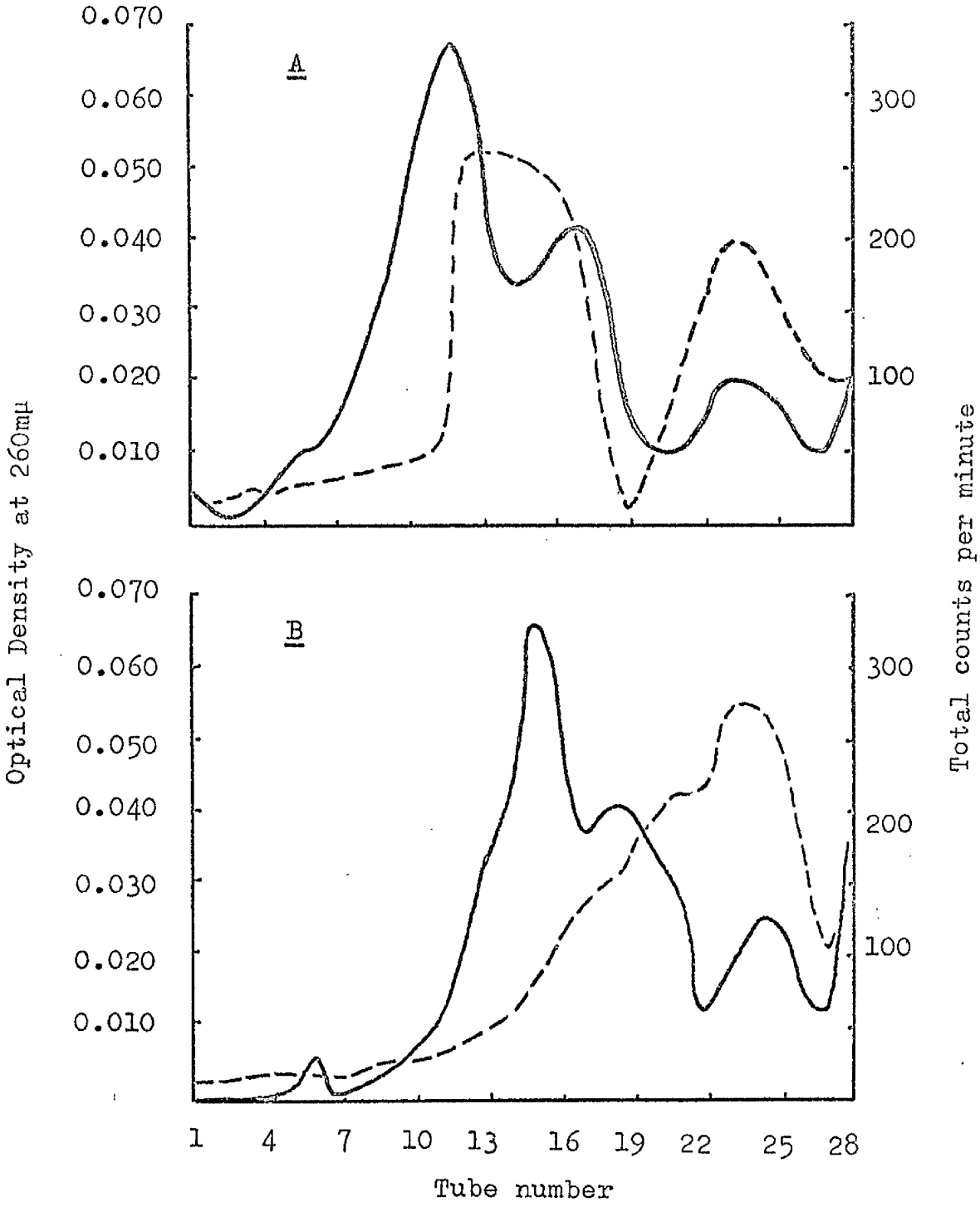
L"S" cells were incubated for 15 mins. at 37°C in the presence of 100 µg/ml actinomycin D, washed and one sample was incubated for a further 4 hours in the presence of 2 µg/ml bacterial RNA and 20 µc/ml ^{3}H-uridine. Another sample was incubated in 20 µc/ml ^{3}H-uridine in the absence of bacterial RNA.

Tube number 1 is the bottom of the gradient.

——— optical density at 260 mµ

— — counts/minute

Fig. 3.41


A
B
0.070
0.060
0.050
0.040
0.030
0.020
0.010
300
200
100
Optical Density at 260mμ
Total counts per minute
1
4
7
10
13
16
19
22
25
28
Tube number

It is still unknown whether this represents a specific fraction or a non-specific residual fraction of normal ribosomal RNA synthesis.

One implication of these results is that previous work using actinomycin D at concentrations lower than 100 µg./ml. must be regarded with caution. There is a considerable amount of incorporation into all fractions at low concentrations, and even at higher concentrations there may be a significant degree of incorporation, particularly in soluble and ribosomal RNA.

At higher concentrations of actinomycin, no rapidly labelled material reaches the cytoplasm, implying either that cytoplasmic RNA is synthesised independently and at a slower rate, or, if of nuclear origin, its transport from the nucleus is inhibited by actinomycin. When autoradiographs were prepared (by method 1) from HeLa cells incubated in 200 µc./ml. for 10 minutes followed by incubation in 180 µg./ml. actinomycin D for 2 hours, nucleolar labelling was absent (Fig. 3.42). This suggests that, if actinomycin does inhibit the movement of rapidly-labelled RNA to the cytoplasm, the inhibition may occur at the nucleolus.

Fig. 3.42

Nucleolar labelling in L"S" cells incubated with ^{3}H-uridine.

(i) Typical pattern of labelling after 15 mins. incubation in 20 µc/ml ^{3}H-uridine.

A. 2 hours after pulse. Label concentrated over nuclei; nucleoli tend to be particularly active.

B. 24 hours after pulse. Label distributed over nuclei and cytoplasm. Not concentrated over nucleoli.

(ii) Inhibition of nucleolar incorporation in the presence of 180 µg/ml actinomycin D.

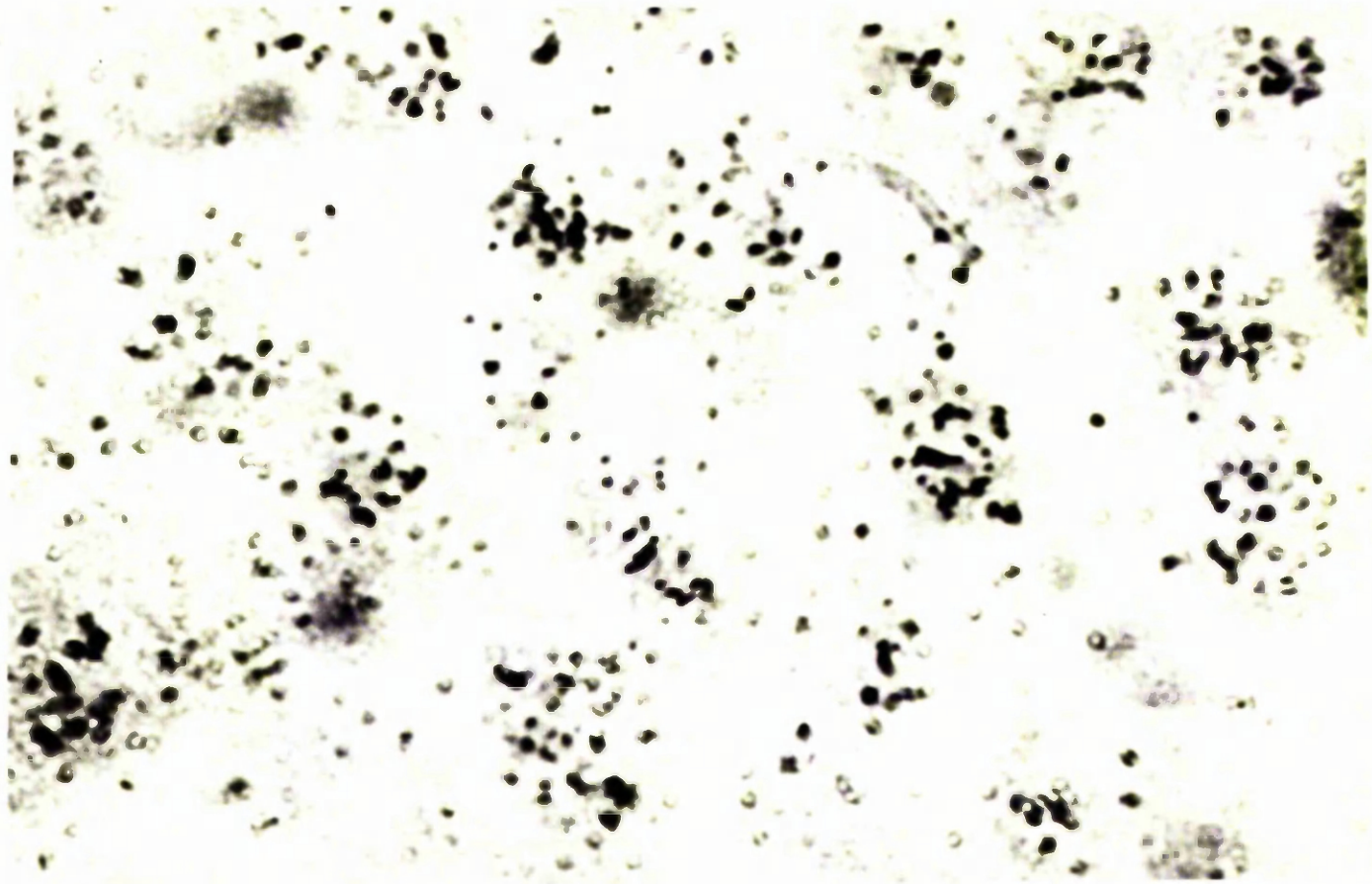
A. 4 hours incorporation after a 10 minute pulse in ^{3}H-uridine.

B. 4 hours incorporation in the presence of 180 µg/ml actinomycin D, after a 10 minutes pulse with ^{3}H-uridine.

(i) Autoradiographs were prepared by method 2.
(ii) " " " " " 1.

Fig. 3.42(i)

A.



B.

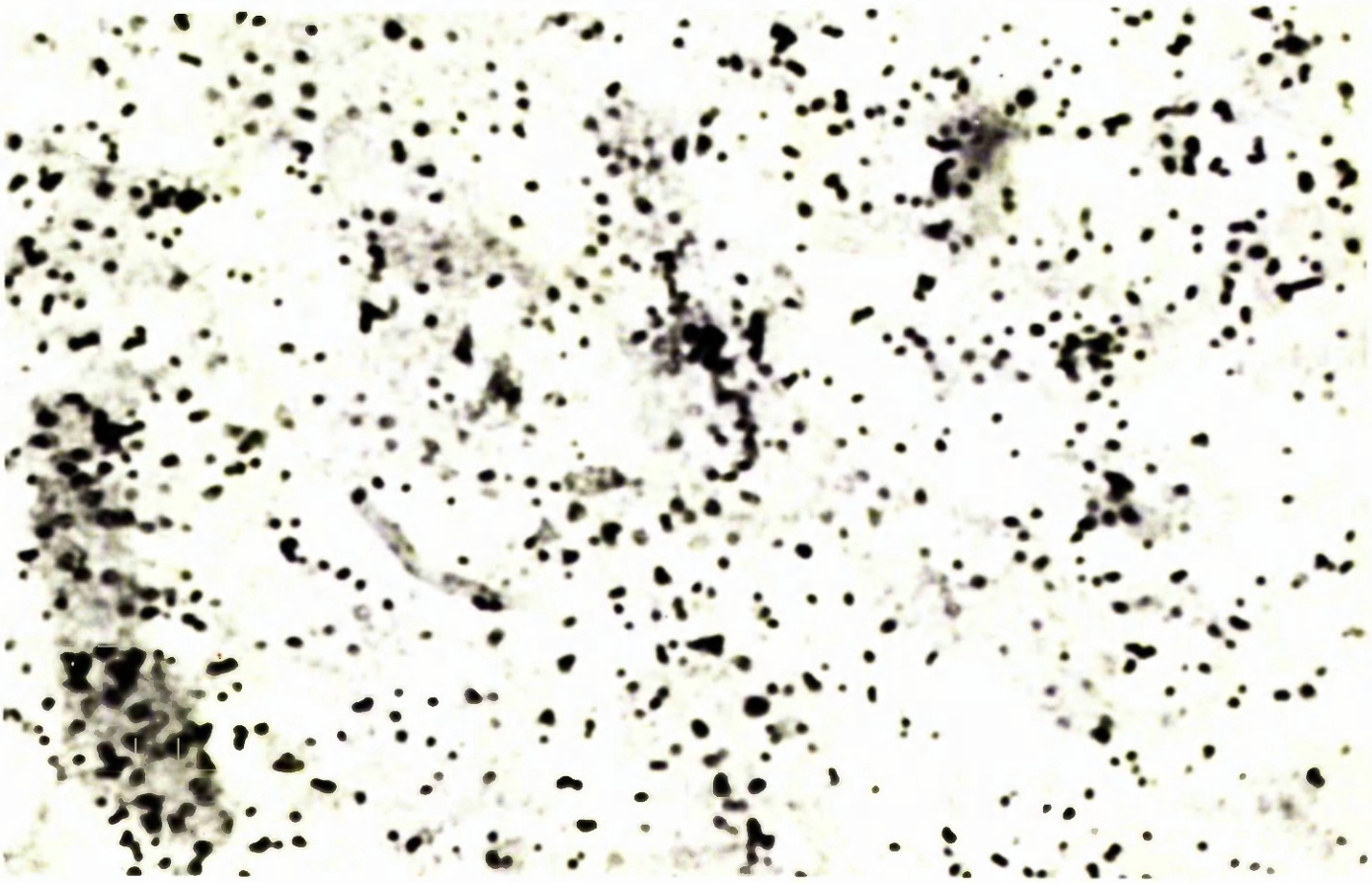
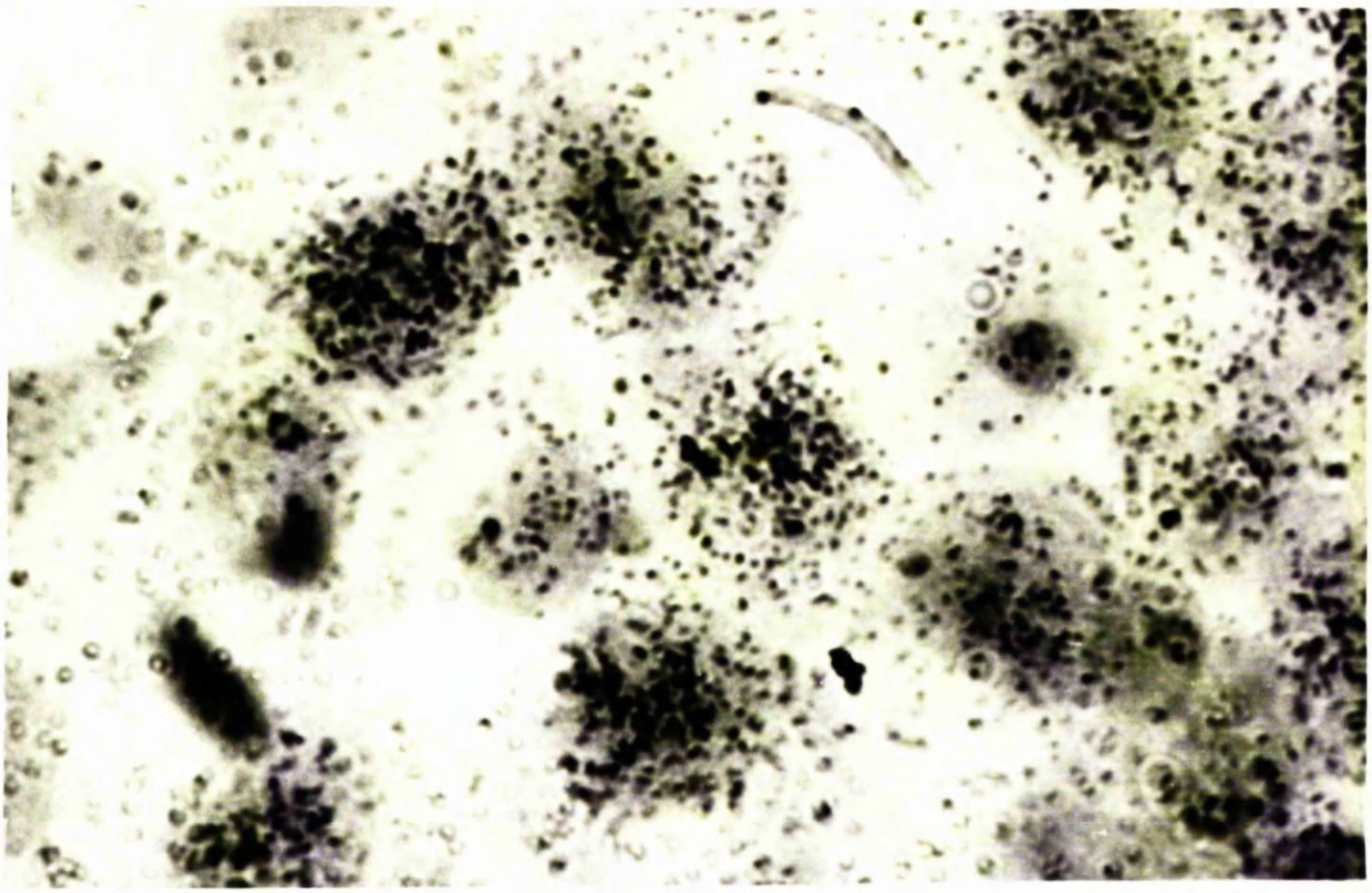
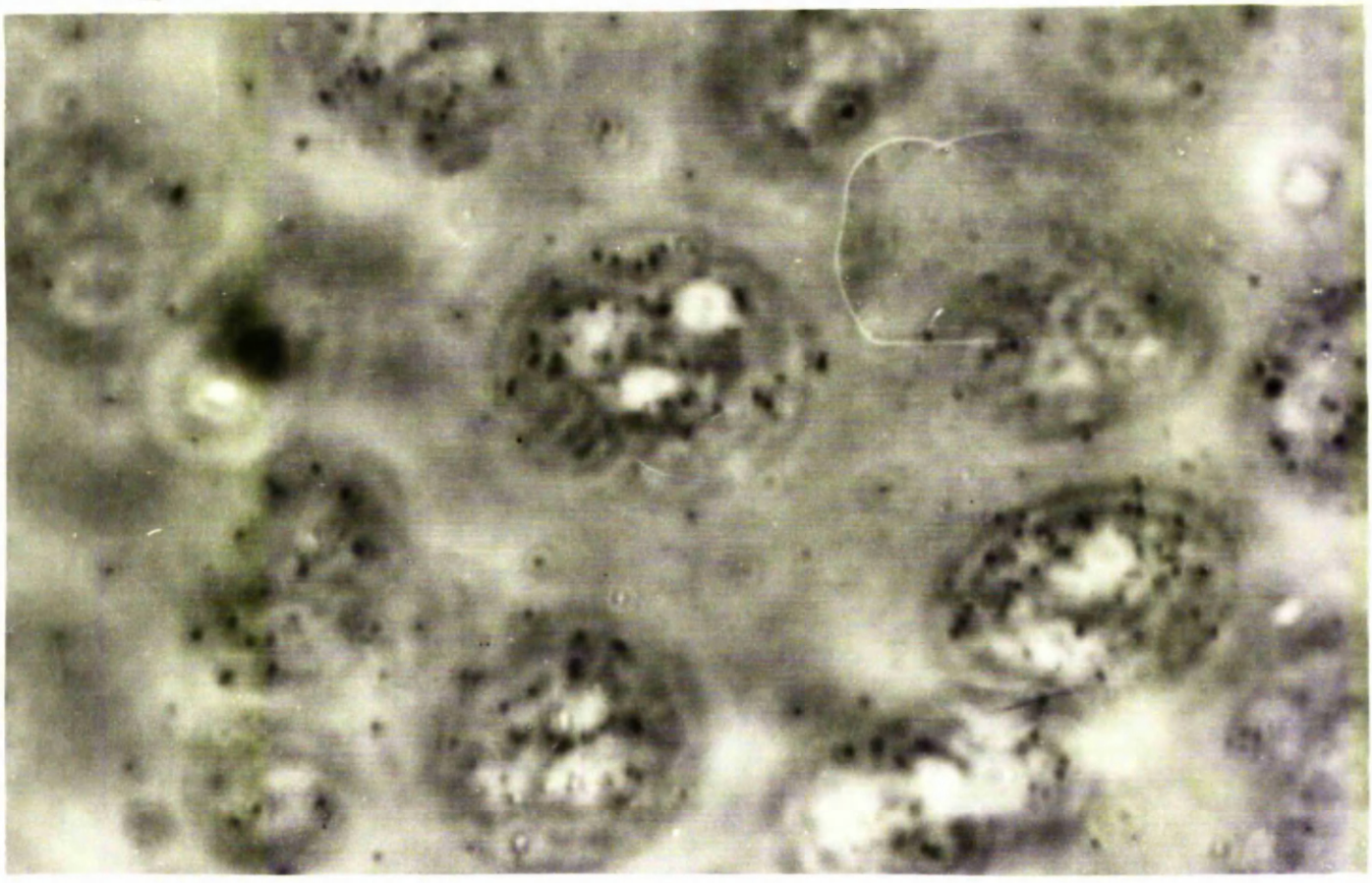


Fig. 3.42(ii)

A.



B.



D I S C U S S I O N

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4.1. Adaptation of animal cells to culture

Animal cells in culture show certain stable characteristics. The appearance of the cells used in the present work allowed clear distinctions to be made among cell strains. The activities of some enzymes also showed distinct differences between cells strains. Similar results were described by Westfall et al. (1963) who found differences in the arginase and thiosulphate sulphurtransferase in different cloned strains of mouse fibroblasts. Westfall (1962) in a review of his own, Potter's, and other results also described differences in the activities of glucose-6-phosphatase, glutamate dehydrogenase, deoxycytidylate deaminase, catalase, and β-glucuronidase, and differences in glycogen storage in several different cell strains. Alkaline phosphatase activity has been shown to differ greatly between cell strains; (Konigsberg and Hitowsky, 1962; Cox and MacLeod, 1962), and Maio and DeCarli (1963) were able to isolate strains of human epithelial cells with characteristically high and low alkaline phosphatase activity.

Therefore, it seems likely that many distinct differences in enzyme activity between cells are retained in culture. This contrasts with the report of Lieberman and Ove (1958) to the effect that the enzyme activities of several human cell strains were similar. However, the bulk of the evidence suggests that characteristic enzyme profiles can often be ascribed to cells in culture.

It is also possible to establish characteristic differences between cultured cell strains by other criteria. For example, Parker (1932, 1935)

claimed that the growth characteristics of certain fibroblastic strains were different. Hull and Tritch (1962) were able to distinguish 22 different cell strains, of human, monkey, pig, rabbit, rat, and mouse origins, by using their susceptibility to virus infection, their malignant properties, and their preference for certain media.

Although the karyotypes of some cells are subject to such variation as to render them difficult to recognise, there are other cell strains which retain a stable chromosome pattern during prolonged culture (Clausen and Syverton, 1962; Yerganian and Leonard, 1961). However, the karyotype is more often unstable in culture, and this aspect will be discussed later. The experiments of Briggs and King (1952, 1957, 1960) suggested that the effects of differentiation on the nucleus are irreversible. The present findings suggest that some irreversible traits, established in cells during differentiation, are retained when the cells are transferred to culture.

This implies that differentiation in animal cells is not strictly analagous to enzyme adaptation in bacteria. Although the gene arrangement (i.e. the operon-regulator arrangement) may be similar, the permanence of differentiative changes implies irreversible induction of gene function, as distinct from reversible induction and repression controlled by the concentration of metabolites within the cell.

Dedifferentiation

When a tissue explant is grown as a primary culture, the cells which grow out from it to form a monolayer are generally of a few restricted

types. Thus explants of many different tissues show outgrowths of morphologically typical fibroblasts, epithelial cells, and macrophages (Willmer, 1935). As there is no apparent difference in the appearance of these outgrowing cells, no matter from which tissue they are derived, they may be unspecialised, or dedifferentiated derivatives of the specialised cells of the explant. The term dedifferentiation has been used to describe the loss of specialised functions and appearance in these cells, and implies a permanent reversal of differentiation. That such an irreversible process does occur may be questioned on several grounds.

Firstly, those cells which form the outgrowth are not necessarily the typical functional cells of the tissue from which the explant was taken. The outgrowth will be composed of those cells which (1) migrate fastest on a glass substrate, and (2) are best adapted to the artificial culture medium. These characteristics are not always to be found in the typical functional cells of the explant, and the selection of a minority cell type may result. Cells of this type will typify the outgrowth and, consequently, will not be representative of the original tissue. This type of selection has been described in liver cultures by Sato et al. (1960) and in cultures of spleen by Till et al. (1964).

The reason for the morphological similarity between cells migrating from different explants may berelated to the nature of the two-dimensional substrate, as distinct from the three-dimensional matrix *in vivo*. Weiss and Garber (1952) showed that the shape of cultured cells was determined

by the nature of the substrate in which they were grown.

Observations of this kind have led to the formulation of a hypothesis of differentiation suggesting that the form and function of cells is determined by the interaction of two processes: (1) irreversible differentiation, and (2) "reversible expressions which cells assume in response to different environments, and which have been termed 'modulation'" (Weiss, 1950). The investigations of cultured cells, discussed below, suggest that this is a correct interpretation.

Although cultured cell strains may show typical enzyme patterns, comparison of these cultures with the tissues from which they were derived shows that many characteristic enzymes are apparently lost. This has been attributed to dedifferentiation, as described above. However, it is more likely that enzymes characteristic of the original tissue are lost from cultured cell strains due to selection of those cells best suited to culture (Sato et al. 1960).

If one assumes that outgrowths from primary explants and established cultured cell strains can be identified with typical functional cells of the tissue from which they were derived, then the absence of the appropriate modulation may be the main factor producing loss of function. Cell cultures derived from liver may lack glucose-6-phosphatase, fructose-1,6-diphosphatase, and fructokinase (Fenske et al. 1957), and tryptophan pyrrolase, tyrosine aminotransferase, and histidase (Auerbach and Walker, 1959). Ebner et al. (1960, 1961) described the disappearance of many different enzymes from bovine mammary tissue in culture. Peppers et al. (1960) found that the catalase of a hepatoma strain was

reduced from the level found *in vivo*. Lieberman and Ove (1958) examined the activity of eight enzymes of rabbit kidney cortex in trypsinised suspensions and in cell cultures, and found that the activities of alkaline phosphatase, β-glucosidase, catalase, and thiosulphate sulphurtransferase were greatly reduced in culture.

Although comparison with the tissue of origin shows a reduction of enzyme activity in most cases, Auerbach and Walker (1959) found aspartate transcarbamylase activity in a liver strain comparable with the level in the animal, and Ferske et al. (1957) found that although some liver enzymes were lost, glucose-6-phosphate dehydrogenase was retained. Westfall et al. (1953, 1958) showed that some liver cell strains remain compatible with their origin by the continued presence of glycogen and a high arginase activity.

The term dedifferentiation would imply that all the properties characteristic of the tissue of origin have been lost by the cultured cell, and its metabolism has become comparable with that of early embryonic cells. The distinctive patterns of enzyme activity described above and in the cell strains used here, whether they may be correlated with the tissue of origin or not, suggest that these cells are not truly dedifferentiated.

Frequent examples of loss of function, other than enzyme activity, have been presented and may be pertinent to this discussion. In 1920, Champy described the apparent loss of secretory activity of prostate when cultured *in vitro*. In earlier work with the same tissue Champy (1914)

observed that secretory function was retained when the prostate tissue was incubated with fibroblastic cells. When Moling and Fischer (1922) grew epithelial and fibroblastic cells in mixed cultures, the epithelial cells could still organise themselves into tubules, even after several months in culture.

The possibility that one cell population can effect another by inducing differentiation, or maintaining a differentiated state, has been explored by Auerbach and Grobstein (1958) and Grobstein (1962). They showed that the passage of soluble material from one group of cells to another can produce limited differentiation. It is possible that such a process was responsible for the presentation of differentiation in the mixed cultures described above.

The absence of thyroid function in outgrowths whilst it is retained in the explant (Gonzalez et al. 1956; Oppenheimer et al. 1956) suggests that aggregation of cells may cause persistence of differentiation. Grobstein and Auilling (1955) found that differentiation of nervous tissue was not found in dispersed cultures but was in explants above a certain minimal size. Similarly, freshly isolated chondrocytes continue to synthesise mucopolysaccharides and chondroitin sulphate when incubated as pellets but do not as monolayer cultures (Stockdale et al. 1965).

It is possible that maintenance of tissue in suboptimal aggregations or as dispersed cells could lead to the leakage of specific inducers or metabolites required for the preservation of the stability of certain

enzymes (Burkhalter et al. 1957a) and this could influence the retention of differentiated characteristics. It is possible that aggregation also satisfies some mutual requirement of tissue cells for a property of continuous membranes (Moscona, 1962), or for an inducer substance passing from one cell to its neighbour (Auerbach and Grobstein, 1958; Grobstein, 1962).

During propagation of established cell strains, none of the above requirements are satisfied; leakage of metabolites and inducers is maximal and surface contact between cells is greatly reduced. Great care is usually taken to prevent the mixing of two different cell strains, eliminating any chance of the passage of inductor substances between one cell type and another. The lack of any one of these requirements may be sufficient to reduce the state of differentiation in cultured cells.

When cultured cells are compared with their tissue of origin, another considerable difference is evident. Most cell culture techniques have been aimed at maximum proliferation to give a greater yield of cells whereas tissue cells normally turn over at a much slower rate. Davidson (1955) found that acid mucopolysaccharide synthesis in proliferating cultures, was increased when cells were overcrowded. Although Foley et al. (1960) described a loss of pigment formation from malignant and normal cultured cells, Ebeling (1924) and Whittaker (1955) were able to demonstrate renewed accumulation of pigment when cell growth was inhibited.

Some differentiated features have been produced in cell culture of long established strains by inhibiting proliferation. When Goldberg

et al. (1963) maintained mouse embryo fibroblasts for 1 - 3 weeks without transfer they observed the production of collagen fibres, even after 100 generations in culture. Rose (1962) grew HeLa cells, ten years after their establishment, in a cellophane "sandwich" culture and obtained partial differentiation in the appearance of columnar epithelial-like cells, and Evans (1962) obtained development of acini in liver-cell cultures by a similar technique.

As all the cell strains used in the present survey of enzyme activity were propagated with maximum proliferation, it is possible that this may explain the absence of high activity of some enzymes. For example, although strain HEM cells could be correlated with their origin from liver by the production of glucose-6-phosphatase, the activity of arginase, another typical liver enzyme, was absent from these cells. Similarly, the other examples of loss of enzyme activity that were quoted above, may have been due to the proliferating nature of the cultures used.

Some stable differences between all strains remain, and may be correlated with the cell type. However, other specialised functions are lost. Therefore selection of cells atypical of their origin, and the absence of modulation are probably the more correct interpretation of alteration in cells during their establishment in culture.

Loss of specialised functions in cell culture may be due to the adaptive nature of some enzymes. The environment of cultured cells is devoid of many substances found in the animal but not essential for cell growth. Among these are hormones and some of the vitamins. It

is also very difficult to simulate the physico-chemical conditions that exist in the tissue-cell environment *in vivo* when the tissue is cultured. It is quite likely that the absence of enzyme inducers, chemical and physical, from culture media may be responsible for the low activity of some enzymes.

The presence of high arginase activity in ascites cells from peritoneal cavity of the mouse and the absence of arginase activity in the same cells in culture (see "Results"), suggest the lack of appropriate inducers in the culture medium. However, culture of ascites cells in ascitic fluid does not restore arginase activity to the level found in the animal, and hence the inducing factor may be labile or dependent on unidentified physical conditions in the peritoneal cavity.

Fluctuations in enzyme activity have been noted in cultured cells in the present survey. Since there are indications in the literature that some of these enzymes might be inducible *in vivo*, it is quite likely that one of the principal factors controlling the level of enzyme activities in cultured cells is the degree of induction produced by factors in the medium.

Whatever the cause of loss of specialised function in culture, any evidence that supports this process suggests that at least some part of differentiation may be reversible. If this is so, then enzyme adaptation may play a considerable part in the maintenance of differentiation. Thus the study of enzyme adaptation in cultured cells may provide

valuable information on part of the process of differentiation.

Reversible adaptation must first be distinguished from irreversible spontaneous variation. The amount of spontaneous variation occurring in animal cells in culture must therefore be determined.

Spontaneous irreversible alterations in cultured cells

Alterations in the individual chromosomes, and in the number of chromosomes, occurred spontaneously in the Y-5 Chinese hamster fibroblasts. As Harris (1967) has stressed the importance of a high protein supplement in preventing spontaneous alterations in culture, and different effects have been detected in the chromosomes of HeLa cells with different sera (Saksela and Saxen, 1962), the increased instability of the Y-5 strain may have resulted from the reduced supplement concentration.

Maintenance of Chinese hamster fibroblasts in other laboratories has produced a stable line, retaining the normal diploid karyotype (Yerganian and Leonard, 1961), and has also produced a strain exhibiting similar karyotypic instability to the Y-5 strain (Yu, 1965). This may mean that different cell strains of similar origins exhibit a degree of spontaneous variation that is characteristic of the strain. Alternatively, changes in culture conditions, such as the reduction of serum or other supplement may produce variation of the karyotype.

Chromosomal instability is by no means the only example of spontaneous variation in cultured cells. The appearance and growth potential of cultured cells may undergo a spontaneous alteration known as transformation. For example, Ruddle (1962) recorded spontaneous morphological changes in

pig kidney cells from a cloned strain, while Carter et al. (1961) and Ludovici et al. (1962) obtained strains with epithelial morphology from hitherto fibroblastic strains. In such established cultures, particularly where the strain has been cloned, transformation of the cell population implies that cell cultures are prone to spontaneous irreversible alterations.

The expected result of spontaneous variation would be a heterogeneous population of cells, unless the majority of the variants were lost by selection. There are, in fact, many examples of heterogeneity which indicate that, in spite of the effects of selection, many of the products of spontaneous variation are retained in cell cultures.

Ruddle (unpublished) was able to select a variant of the Y-5 Chinese hamster strain that grew in the absence of bactopeptone. During maintenance for the present work, this strain was adapted first to grow in 5% instead of 10% calf serum, and then to grow from an inoculum of 10^4 cells/ml. instead of 10^5/ml. In each case the surviving cells represented a minority of the total inoculum, implying selection of preadapted cells, rather than adaptation of the whole inoculum. Hence the Y-5 strain, although it had been cloned twice prior to this period of culture, had become heterogeneous in respect to these cultured requirements, and must have undergone spontaneous variation after cloning.

Heterogeneity of cloned cell lines has also been demonstrated by the ability of cell strains to produce variant strains resistant to metabolic analogues. Such were the examples of azaguanine resistance

in a human bone marrow strain (Szybalski, 1959), and 8-azaguanine, aminopterin, and deaminopurine resistance in pig kidney cells (Harris and Ruddle, 1960). It was possible to select a strain of the Y-5-11 cells capable of unimpaired growth in chloropurine, and this provides a further example of the heterogeneity of the population. In addition, the continued selection that occurred in successive generations suggests continued variation.

The heterogeneity of cultured cell strains may involve the activity of certain enzymes. The activity of specific enzymes in variant strains resistant to metabolic analogues has been shown to differ from the activity in the parent strain; e.g. reduced guanine monophosphate pyrophosphorylase has been found with 8-azaguanine resistance (Szybalski et al. 1961), low uridine kinase with 5-fluorouracil resistance (Skold et al. 1962), and increased dihydrofolate reduction with amethopterin resistance (Hakala and Ishihara, 1962).

In the present studies it was possible to demonstrate alkaline phosphatase deficient clones of strain Hela, and glucose-6-phosphate dehydrogenase and β-glucuronidase deficient clones of strain Y-5-11. These results are similar to those obtained by Maio and DeCarli (1962) with a strain of human epithelial cells. Differences in alkaline phosphatase activity between sublines of one strain have also been shown in Chang liver cells (Konigsberg and Hitowsky, 1962) and in several epithelial lines (Cox and Macleod, 1962).

Variations in drug sensitivity and enzyme activity may be the

result of chromosomal variations similar to those discussed above; however, no positive correlation has yet been proved.

It is evident from the present results, and from many examples in the literature, that spontaneous variation and resultant heterogeneity of culture populations is quite commonplace. Consequently, any discussion of variations in enzyme activity is subject to certain reservations, and only such fluctuations in activity as can be shown to be reversible may be accepted as possible examples of enzyme adaptation.

4.2. Enzyme adaptation in animal cells

The enzymic responses which have been construed as adaptive in animal cells *in vivo* have been produced by three principal types of stimuli (see Section 1) (Knox et al. 1956), (1) non-specific dietary changes, such as starvation and refeeding (Weber et al. 1962) or altered diet (Grossman et al. 1962; Schimke, 1961, 1962), (2) addition of specific substrates to the diet (Freedland and Harper, 1957, 1959; Fitch and Chaikoff, 1959; Knox, 1951; Lee, 1956; Lin and Knox, 1957), and (3) administration of various hormones (Weber et al. 1962; Salas et al. 1963; Shaw and Koen, 1963; Segal et al. 1962; Goldstein et al. 1962; Dietrich, 1954; Knox and Auerbach, 1957; Knox, 1951; Lin and Knox, 1957; Schor and Frieden, 1959; Halevy and Avivi, 1960; Hilf et al. 1962; Rosen et al. 1963; Greengard et al. 1963; Knox and Goswami, 1960).

Fluctuation of the activities of some enzymes in cultured cells

Variations in the activities of several enzymes were observed in the results of the present survey. It is possible that some of these were irreversible and due to the spontaneous permanent changes discussed above. However, as many of these variations were reversible, it seems more likely that they were due to enzyme adaptation. Further support for this assumption may be obtained from previous examples in the literature of fluctuation of some of these enzymes.

The twenty-fold variation in alkaline phosphatase activity in Hela

cells is compatible with previous claims of adaptation in this enzyme. Hormones (Cox and MacLeod, 1962), organic phosphate (Maio and DeCarli, 1963) and changes in osmolarity (Nitowsky et al. 1965) can all raise alkaline phosphatase activity, and it is repressed by cystine and cysteine (Cox and MacLeod, 1963). It is quite possible that there are many more unidentified fluctuations in culture media which could have caused alterations in the activity of this enzyme. The apparent lack of specificity reduces the usefulness of this enzyme as a tool for examining enzyme adaptation in animal cells, in spite of the substantial response it produces.

The spontaneous variation in glutamyl transferase activity in HLM and Y-5 cells in the present survey agrees with the adaptive nature of this enzyme suggested by DeMars (1968) and Paul and Pottrell (1968).

Glucose-6-phosphatase activity varied in HLM and glucose-6-phosphate dehydrogenase activity varied in HeLa. These enzymes were reported to respond to alterations in the hexose content of the diet of rats (Fitch and Chaikoff, 1959; Freedland and Harper, 1957, 1958), and may have varied in the present investigations due to alterations in hexose concentrations in the media at different times of assay. However, the results of Pottrell (1962) who showed that these enzymes are not readily induced by hexoses in cell culture, suggest that this is not the case.

Aspartate aminotransferase varied fifteen-fold in HLM cells and sixfold in L cells. These observations are compatible with the report of Fitch and Chaikoff (1959) that this enzyme was inducible in rat liver.

It is also possible that other aminotransferases are adaptive as the activities of tyrosine and alanine aminotransferases have been increased in rat liver by the administration of cortisone.

Fluctuation in arginase activity

Arginase activity fluctuated considerably in HeLa cells in culture, and in L5178Y and Landschutz cells grown in mice. Schimke (1962c, 1964) showed 2-8 fold increases in arginase activity in HeLa, KB liver, and other cell strains when grown in the presence of arginine. The demonstration of the lack of any signs of activation on mixing extracts from control cells and from cells incubated with excess arginine led Schimke to the conclusion that the increases were adaptive.

Large increases in arginase activity in the present results were found on inoculating cultured ascites cells into mice. These represented an increase from an undetectable level to 400 enzyme units per 10^6 cells. Considerable variations in arginase activity (up to tenfold) were also observed in cloned strains of BHK21 hamster fibroblasts. At no time was it possible to simulate these fluctuations in arginase activity by incubation of BHK21 fibroblasts or HeLa cells with excessive or low arginine concentrations, although small increases in activity were obtained on some occasions after incubation with excess arginine. Apparently the presence of substrate alone is insufficient to induce arginase activity in these cells.

The induction of arginase in rat liver has not been achieved by adding substrate (Schimke, 1963), although 2-8 fold increases in activity

have been obtained in cultured Hela cells (Schimke, 1962c, 1964). This suggests that other factors participate in arginase induction. Similarly, increases in arginase activity following cold shock and incubation in high pH, suggest that substrate induction does not act alone. It is interesting to note at this point that alkaline phosphatase activity can be altered by changing the osmotic pressure of the incubation medium (Nitowsky et al. 1963).

It is possible that the connection between different non-specific effects on enzyme activity is in a regulatory property of membrane transport. Arginase activity may be under substrate control, and in turn the concentration of substrate may be controlled by a specific transport mechanism in the cell membrane. The possibilities of such a system will be discussed later.

Another factor influencing the induction of arginase may be the level of the uninduced enzyme. Induction of alkaline phosphatase by hydrocortisone and organic monophosphates is only possible in cell strains with low alkaline phosphatase activity (Cox and MacLeod, 1962; Nitowsky and Hers, 1963; Baio and DeCarli, 1963). Similarly, although the induced levels of arginase in uncloned Hela cells and in clone Hela-S3 were the same, the uninduced levels were different (Schimke, 1962c). Consequently, as clone S3 had a lower level of uninduced arginase activity, the degree of induction obtained was greater.

Since attempts to lower the arginase activity by reduction of the arginine concentration in the medium were not successful in Hela cells,

it is possible that the lowest levels of activity that were recorded represent the uninduced level of activity in these cells. This level (between 5 and 20 µM urea liberated minute/10^6 cells) is comparable with the level of activity obtained by Schimke (1962c, 1964) in uncloned HeLa cells grown in 0.02 mM arginine. It is, however, higher than the level he recorded in HeLa-S3 cells grown in either 0.6 mM arginine (Eagle's medium) or in 0.02 mM arginine. It is possible that the strain of HeLa used in the present investigations was more similar to the uncloned strain used by Schimke than to clone HeLa S3. The lack of significant increases in enzyme activity in the presence of arginine may have been due to a high uninduced level.

However, Fottrell (1962) obtained 3-9-fold increases in arginase activity after incubation of the same strain of HeLa cells with excess arginine (10 mM). As this represents a considerable difference from the present results, some irreversible alteration in the genotype, or its mode of expression, may have occurred in the few months between the experiments of Fottrell and the present series. The possibilities of such a change have been mentioned earlier in this discussion. A similar type of change has been observed in the ability of acetylcholine to cause an increase in cholinesterase activity in HM cells (Paul and Burkhalter, unpublished). This ability was lost in the course of three months culture and may have been due to a similar effect.

It is also possible that a high basic level of arginase activity was produced by active concentration of arginine within the cells. If

arginine was actively transported when the concentration in the medium was less than 1 mM, then it may not have been possible to obtain a level of arginase activity uninfluenced by arginine. This would suggest once again that the control of uptake of substrate may influence the activity of the enzyme.

Owing to the prevalence of contamination of cell cultures by pleuropneumonia - like organisms (PPLO) (Barile et al. 1962) it is always important to exclude these as a cause of metabolic variations. However, it seems unlikely that they could have accounted for changes in arginase activity, since Schinke and Barile (1963) have shown that arginase is not involved in the breakdown of arginine in these organisms. Furthermore, cultures were frequently treated with kanamycin which kills PPLO.

Fluctuations in the activity of arginase in clones of the BHK21 hamster strain have not been accounted for by the present results. It is apparent, however, that arginine alone, or with citrulline, is insufficient to induce arginase. This leads to the suggestion that, in common with HeLa cells, some extra factor is required for the induction or arginase. As the arginase activity of Landschutz cells increases rapidly on inoculation into mice, it is possible that this, or a similar factor, is found in the peritoneal cavity of the mouse. The factor may be hormonal or physico-chemical and its effect may be upon arginase induction directly or on the activity of arginine transport.

Comparison of enzyme adaptation in bacteria and in animal cells

During enzyme induction in bacteria increased protein synthesis occurs within a few minutes of the addition of inducer. In the case of β-galactosidase induction in E. coli the rate of enzyme synthesis, as a fraction of total protein synthesis ("differential rate of synthesis"- Monod et al. 1962), is maximal within 3-4 minutes of the addition of a β-galactoside (Monod et al. 1962). The initiation of enzyme synthesis in animal cells takes considerably longer, and the differential rate of synthesis does not become maximal for a period of several hours to several days. For example, Cox and MacLeod (1962) recorded little significant increase in alkaline phosphatase activity of HeLa cells until 24 hours after addition of prednisolone, and the activity did not reach a maximum until 90 hours after addition of prednisolone. Adaptation of glutamyl transferase activity in HeLa cells appears to be more rapid, but again a lag phase of 2-5 hours was implied when the increased activity of this enzyme was followed after substitution of glutamic acid for glutamine in the culture medium (Paul and Pottrell, 1963). In this case maximum enzyme activity was reached by 48 hours.

Differences in enzyme adaptation between bacteria and animal cells may be related to the existence of organotypic homeostasis in animals. The bacterial cell is in direct contact with its environment, whereas the animal cell is protected from changes in the external environment. Hence, although the metabolism of the bacterium must respond immediately to any environmental change, the primary reactions of an animal are

behavioural, and it is possible that only persistent changes in the environment will cause adaptation of enzymes.

Enzyme adaptation, as distinct from the continued synthesis of all enzymes at a high level, represents a considerable economic saving to the bacterium. In animal cells economy is produced by tissue organisation; specific tissues are responsible for the synthesis of certain enzymes, and no cell is required to synthesise all the enzymes that the animal might require.

Although organotypic homeostasis may have eliminated the necessity for such rapid and profound adaptive changes in enzyme activity as have been recorded in bacteria, fluctuations in the concentration of metabolites in the internal environment still occur, and enzyme adaptation may still be required to cope with these. Tissue cells may respond to alterations in their immediate environment by substrate induction or product repression; in addition such adaptive responses may be coordinated by the action of hormones.

Hence the synthesis of enzymes in animal cells may be under a tripartite control. (1) The enzyme is either present or absent as a result of the particular path that differentiation has followed in the cell. (2) The amount of enzyme synthesised may be governed by the concentration of substrate or product in the extracellular fluid. (3) The amount of enzyme synthesised in different tissues may be under the coordinating control of the endocrine system.

Physico-chemical fluctuations in the environment may act as stimuli

for enzyme adaptation. The resultant changes in enzyme activity may, in turn, restore the physico-chemical variation to the optimal metabolic level. Hence, the function of the responses of enzyme activity to physico-chemical variations may be as homeostatic feedbacks. If this is so, enzyme adaptation may be sensitive to changes such as differing osmotic pressure, as with alkaline phosphatase (Nitowsky et al. 1963), and differing pH, as found in the present study with arginase. Alternatively, such physico-chemical effects may only represent non-specific interference with the control mechanism.

These diverse stimuli which affect the activity of certain enzymes may act directly on the regulation of synthesis of the enzyme. Alternatively, they may regulate enzyme synthesis indirectly by altering the uptake of specific substrates which may act as inducers.

4.3. Membrane permeability as a regulator of metabolism

The entry of metabolites into bacteria has been shown to be controlled by specific mechanisms known as "permease systems" (Cohn and Monod, 1957). The β-galactoside permease system of E. coli can concentrate β-galactosides to 100 times the concentration found in the internal medium by a catalytic process that may involve the formation of a temporary complex between the permease system and the β-galactoside. There is a protein component of this system known as "β-galactoside permease", and the structural gene directing the synthesis of the permease maps in the same operon as β-galactosidase. Both proteins are induced by β-galactosides.

By the use of uncoupling agents it has been shown that the system is energy dependent, and inhibition with parachloromercuribenzoate indicates that sulphydryl groups participate in the permease function.

A Na+- and azide-sensitive system has been demonstrated in E. coli as being responsible for a 500-fold concentration of valine (Cohen and Rickenberg, 1956). Phenylalanine and methionine are also concentrated in E. coli, but by a stereospecifically distinct system from valine (Cohen and Monod, 1957). The active transport of amino acids is reversible and is abolished by the substitution of other groups for the α-amino or carboxyl groups of the amino acids.

The existence of permease systems in bacteria has proved to be important in the study of enzyme adaptation. The demonstration of permease-like systems in animal cells may therefore be relevant to the study of enzyme adaptation in animal cells.

For this reason it is interesting to find that some specific transport mechanisms have been described in animal cells. For example, inorganic ions, sugars, and amino acids have all been shown to be actively transported against a concentration gradient. Since several sugars may compete for the same mechanism, and different amino acids also compete with each other for entry, the control of uptake may not have the degree of specificity that has been observed in bacteria. There are, however, specific mechanisms for transporting different groups of compounds; e.g. acidic, basic, and neutral amino acids are transported by different stereospecific mechanisms (Hokin and Hokin, 1963).

Results from the present work suggest that a specific system may exist to promote the uptake of arginine and other basic amino acids in cultured Hela cells.

The uptake of arginine in cultured Hela cells

The uptake of arginine in Hela cells appears to be mediated by a specific transport mechanism. The activity of this mechanism may influence the intracellular concentration of arginine and may be responsible for the variable results in different attempts to induce arginase by substrate.

The kinetics of arginine uptake suggest that specific sites in the membrane catalyse the process. Competition by glutamine, lysine, ornithine and citrulline suggests that the transport mechanism is shared by other basic amino acids, and is not specific for arginine. Since the rate of arginine uptake is reduced in the presence of mercuric chloride and other

inhibitors of sulphydryl enzymes, it is possible that sulphydryl groups are involved in the active site.

The inhibitory effects of sodium fluoride and ouabain may have been exerted mainly upon ATPase. This suggests that some energy may be required by the process, and, since a respiratory inhibitor (KCN) and uncoupling agents had no effect on uptake, the energy requirement may have been satisfied by glycolysis. Christensen et al. (1952b) found that uptake of glycine in duck erythrocytes was unaffected by anoxia, cyanide, dinitrophenol, fluoride, cupric ion or phloretin phosphate and Christensen (1961a) suggested that glycolysis could supply the energy for the active transport of amino acids. It seems likely, therefore, that there are specific sites mediating arginine transport in HeLa cells and that the operative mechanism may derive energy from glycolysis. If energy is required then active transport may operate. Little, if any, active transport occurred in the presence of 1 mM arginine, but there are indications that active transport may have occurred more strongly in the presence of lower concentrations of arginine. Schmike (1964) found that a 2-3-fold concentration of arginine occurred in HeLa cells at an extracellular arginine concentration of 0.02 mM while no concentration occurred at 2 mM. The earlier work of Pies and Eagle (1958) and Eagle (1959) with HeLa cells, and that of Christensen et al. (1952a) with Ehrlich's ascites cells, suggested that active transport of arginine occurred; this has also been demonstrated in kidney cortex slices (Rosenberg et al. 1962).

Since Johnstone (1959) claimed that arginine was not actively concentrated in Ehrlich's ascites cells, and Christensen et al. (1952a) found that the degree of concentration varied in different media, the active transport of arginine may only occur under specific conditions. One possible controlling condition has already been mentioned above, i.e. the extracellular concentration of arginine. Differing degrees of concentration in different concentrations of arginine suggest that the process is under some sort of feedback control, and gradually ceases to be active as the arginine concentration is raised. Thus the absence of active transport in Johnstone's experiments (1959) may be due to the fact that his incubations were carried out at a concentration of 15 mM arginine in the suspension medium. On the other hand, the active concentration described by Christensen et al. (1952a) occurred in a medium containing 2.2 - 6.0 mM arginine.

Certain hormones may be necessary to produce active concentration of arginine at levels higher than 0.1 mM. However, hydrocortisone, insulin, and stilbestrol had no significant effect on uptake of arginine in HeLa cells in the present experiments. Arginine transport in these cells may, however, respond to different hormones which were not examined. Noall (1957) showed that different hormones were required to stimulate the uptake of α-aminoisobutyric acid (AIB) in rat liver and rat uterus. Glucocorticoids, pituitary growth hormone, insulin and oestradiol have all been reported to stimulate amino acid uptake (Christensen, 1961a).

The effect of the regulation of metabolite uptake on enzyme induction

The existence of specific mechanisms for the uptake of metabolites by animal cells means that there may be some form of metabolic regulation at the cell membrane. For instance, it may be necessary to achieve induction of the arginine transport mechanism before arginase is substantially induced. This would be similar to the requirement for β-galactoside transport in β-galactosidase induction in E. coli (Monod et al. 1962). It is possible that arginine transport does not function actively in cultured HeLa cells at the 10 mM concentration of arginine used in the medium in experiments to try to induce arginase. It is also possible that, while active transport is occurring at lower concentrations of arginine in the medium, it is not possible to obtain a basic, uninduced level of arginase activity. Hence arginase activity may be maintained at about the same level in different extracellular concentrations of arginine by the differing activity of the transport mechanism.

4.4 RNA synthesis in animal cells.

Since Volkin and Astrachan (1956) described the appearance of a specific RNA in T2-infected E. coli, the presence of an intrinsic RNA directing protein synthesis in both infected and uninfected bacteria has been established (Gros et al. 1961; Brenner, Jacob and Meselson, 1961; Spiegelman, 1961; Riley and Pardee, 1962) and this fraction has been called messenger RNA. Its activity has been seen to increase during β-galactosidase induction in E. coli (Gros et al. 1961) and it is almost certainly the fraction involved in the regulatory mechanisms postulated by Jacob and Monod (1961).

The presence of rapidly synthesised RNA in mammalian cell nuclei and evidence of its hybridisation with homologous DNA (Scherrer et al. 1963; Mason et al. 1963) has led to the supposition that a messenger fraction may exist in mammalian cells. Sedimentation analysis of this fraction implied that it is larger than bacterial messenger, as it has a sedimentation value of 35 - 45 S whereas the sedimentation value of bacterial messenger RNA is 8 - 12 S (Monod et al. 1963).

The rapidly-labelled RNA fraction in animal cells

The sequential appearance of labelled RNA in nucleus nucleolus and cytoplasm during incubation with radioactive precursors of RNA raises the question of the precursor relationships of these fractions. If the messenger RNA theory is to be upheld in animal cells then the RNA label that appears in the cytoplasm must be proved to have been derived from the nucleus, and in particular from DNA.

The typical pattern of behaviour of labelled RNA on autoradiography of mammalian cells incubated in the presence of a labelled RNA precursor is as follows (Goldstein and Micou, 1959 a and b; Amano and Leblond, 1960). Label is distributed generally over the whole nucleus in the first 30 minutes and quickly accumulates over the nucleolus within the first hour. It is only at 2 - 4 hours that label eventually appears in the cytoplasm. The kinetics of appearance and disappearance of label obey the kinetics of a precursor relationship between nuclear and cytoplasmic RNA (Singh and Koppelman, 1963) and between nucleolar and cytoplasmic RNA (Amano and Leblond, 1960). However, Harris (1963) claimed that only a small proportion of the nuclear label appears in the cytoplasm, and this amount can appear during mitosis, in the absence of the nucleolus and of RNA synthesis on the chromatin.

Ablation of the nucleolus with a U.V. microbeam reduces the cytoplasmic label by 70% and Perry et al. (1961) concluded from this that much of the cytoplasmic RNA was derived from the nucleolus. Whether the nucleolus synthesises cytoplasmic RNA or not it certainly appears to be involved in any possible movement of RNA from the chromatin to the cytoplasm.

Autoradiographs in the present series have shown that radioactivity may accumulate slowly in the cytoplasm of L'S' cells when 0.1 mM 8-azaguanine or 0.1 µg./ml. actinomycin D are used to inhibit further RNA synthesis after a brief pulse. The rate of appearance of label in the cytoplasm is compatible with a precursor relationship similar to that described by Amano and Leblond (1960), Goldstein and Micou (1959), and

many others, but the failure of either of these drugs to inhibit RNA synthesis completely in the concentrations used suggests that independent synthesis could be proceeding in the cytoplasm as predicted by Harris and LaCour (1963). This conclusion must be accepted with caution, however, since terminal addition of cytidine to existing sRNA has been shown to occur in the presence of actinomycin (Merits, 1963) even at concentrations of 100 µg./ml. (Paul and Struthers, unpublished). This implies that incorporation of label into cytoplasmic RNA may represent terminal additions to sRNA and the association of this labelled sRNA with ribosomes; there may be no true synthesis.

The appearance of labile and stable fractions in the cytoplasm continues even when 0.1 µg./ml. actinomycin is added before the isotope and is only abolished when the actinomycin concentration is raised to 100 µg./ml. (Paul and Struthers, 1963). Under these conditions the rapidly labelled, labile fraction does not appear in the cytoplasm even if isotope is supplied for 10 minutes before the drug. As label does not appear in the nucleolus in the presence of 100 µg./ml. actinomycin, RNA is either degraded before it reaches the nucleolus, or else actinomycin interferes with the nucleolar step of the transport of the rapidly labelled fraction to the cytoplasm.

Sedimentation analysis of the rapidly-labelled material in L'S' mouse fibroblasts gives results corresponding more closely with those of Scherrer and Darnell (1962), Scherrer et al. (1963), Tamaoki and Mueller (1963), and Eason et al. (1963) who all report that this material sediments

approximately in the 35 - 45 S position, than it does to the reports of Hiatt (1962) and Spörn and Dingman (1963) who demonstrated the rapidly labelled material in positions ranging from 6 - 30 S. Spörn and Dingman also report rapidly labelled material sedimenting below the 40 S position and it may be that the material with a sedimentation value of 6 S that they observe is a degradation product.

Mason et al. (1963) and Scherrer et al. (1963) have obtained hybridization between the 35 - 45 S sedimenting rapidly-labelled material and homologous DNA. The latter, Perry (1962) and Tamaoki and Mueller (1963) claim that this material becomes associated with, or is a precursor of, ribosomal RNA. The results of Scherrer et al. (1963), Tamaoki and Mueller (1963), and Perry (1962) depend on the complete inhibition of RNA synthesis after a pulse in the presence of labelled precursors by 5 µg./ml. actinomycin or less. As the present results, and those of Paul and Struthers (1963), have shown that synthesis may proceed for a considerable time in the presence of this amount of drug, the incorporation into ribosomal RNA that Scherrer et al. and Tamaoki and Mueller found may have been de novo synthesis in the ribosomal fractions and not the incorporation of an undegraded rapidly-labelled fraction.

The significance of inhibition of the appearance of rapidly-labelled cytoplasmic RNA by actinomycin D.

The most significant finding in the present experiments with actinomycin D is the apparent blocking of the appearance of labelled RNA in the cytoplasm by high concentrations of this drug. Reich (1963) has suggested that actinomycin may cause degradation of the rapidly formed

RNA in the nucleus. If this is so then RNA synthesised in the absence of actinomycin, could be degraded before it reaches the nucleolus or cytoplasm.

If rapidly-formed RNA is not degraded by actinomycin then the drug may block some process involved in the transport of rapidly-formed RNA to the cytoplasm. Electron microscope studies by Journey and Goldstein (1961) revealed that actinomycin reduced the osmophilia of the nucleolus and caused its fragmentation. Perry (1963) was able to show that the accumulation of label in the nucleolus was more sensitive to actinomycin than RNA synthesis on the chromatin. It is possible that the failure of label to appear in the nucleolus and cytoplasm of actinomycin-treated cells in the present series is the result of drug-induced malfunction of the nucleolus.

The use of actinomycin has not provided unequivocal evidence for movement of RNA to the cytoplasm nor has it greatly enhanced our knowledge as to the identity of the messenger fraction. It does indicate, however, that if the rapidly-labelled fraction can be equated with messenger, then the nucleolus is probably involved in its transport to the cytoplasm.

Actinomycin-resistant RNA synthesis

The results of Perry (1963) imply that the nucleolus is involved in the synthesis of ribosomal RNA, but suggest that this synthesis is more sensitive to actinomycin than synthesis on chromatin. Burdon (1963) claimed that RNA synthesis in the nucleolus was actinomycin resistant, and sucrose density gradient analysis of RNA synthesised in the presence

of actinomycin in the present work, showed that the rapidly-labelled peak was most sensitive, and the soluble least sensitive. Incorporation of activity into ribosomal RNA was always more resistant to actinomycin than incorporation into the rapidly-labelled fraction. On one occasion there was considerable ribosomal RNA synthesis in the presence of actinomycin when no synthesis occurred at all in the rapidly labelled fraction.

The incorporation into soluble RNA during actinomycin inhibition has been shown to be due to terminal addition of cytidine rather than formation of new polymers (Merits, 1963) (Paul and Struthers, personal communication). Paul and Struthers (1963) found that when cells were incubated with tracer, and then with 100 µg./ml. actinomycin with tracer still present, incorporation continued in the cytoplasmic ribosomes, as well as in the nucleus. A variable amount of ribosomal RNA may be synthesised in the presence of actinomycin. If this is so then either the movement of the rapidly-labelled fraction to the cytoplasm is independent of ribosomal RNA and inhibited by actinomycin, or attachment of rapidly-labelled RNA to the ribosomes is an integral part of the transport process and is sensitive to actinomycin. In either case, inhibition by actinomycin suggests that RNA synthesis may be involved in the movement of rapidly-labelled RNA to the cytoplasm.

Metabolic regulation and RNA synthesis in animal cells

The findings of Harris et al (1963) that most of the rapidly-labelled nuclear RNA does not reach the cytoplasm, has led him to suggest that the turnover of RNA within the nucleus is involved largely in gene

regulation as the repressor substance of Jacob and Monod (1961). Many other reports have also shown that when labelled RNA accumulates in the cytoplasm it is not proportional to the loss of label from the nucleus. This is evident from the experiment in the present series using low concentrations of actinomycin when a labile peak was obtained in the cytoplasm at two hours, but with a much lower activity than would be predicted from that lost from the nucleus. This has also been found by Paul and Struthers (personal communication). It is therefore quite possible that the apparent surplus turnover of RNA is involved in a regulatory function.

The rapidly-labelled fraction almost certainly accumulates in the nucleolus shortly after its formation. This has been claimed by Goldstein and Micou (1960) and Amano and Leblond (1960) and was also observed in the autoradiographic studies reported here (Fig. 3.42). The existence of ribosomal RNA in the nucleolus is suggested by the results of Sibatani et al. (1962) and is confirmed by reports of Georgiev and Chentsov (1962) who reported ribosomal-like structures in the nucleolus. Perry (1960) claims that 70% of cytoplasmic RNA originates in the nucleolus and concludes that it may be synthesised there (Perry, 1963). Burdon (1963) has also shown that the incorporation of uridylate residues into polyuridylic acid proceeds in the nucleolus. He suggested that this may be related to synthesis of part of the ribosomal molecules in the nucleolus.

If the movement of messenger RNA to the cytoplasm is dependent on

RNA synthesis in the nucleolus, this presents a possible site for regulation of the function of messenger RNA. Hence, the nucleolus may be involved in metabolic regulation. Growth hormone, which, it is claimed, can reverse the inhibitory effect of actinomycin D (Korner, 1963), causes an increase in the activity of RNA in the nucleolus (Talwar et al. 1962). Hence some hormonal regulation of metabolism may take place at the nucleolus.

The current hypothesis that proteins are synthesised on polysomes (Goodman and Rich, 1962; Zimmerman, 1963; Wettstein et al. 1963;; Staehelin et al. 1963; Noll et al. 1963) implies that ribosomes are not attached to messenger RNA until protein synthesis starts in the cytoplasm. It might also be suggested that, although the attachment of messenger RNA to ribosomes occurs mainly in the cytoplasm, the initial attachment of one or more ribosomes occurs in the nucleolus, and is necessary for the stabilisation of messenger RNA and its movement to the cytoplasm.

Alternatively, the movement of messenger RNA to the cytoplasm may depend on a different synthetic process which is sensitive to actinomycin D, and does not involve ribosomal RNA directly.

The suggestion that many ribosomes may use a single messenger RNA molecule in the course of protein synthesis in mammalian cells (see above) implies that the RNA templates are relatively stable. This is also implied by the persistence of haemoglobin synthesis in anucleate erythrocytes, and by the continued synthesis of protein in actinomycin-treated cells at a level of 5 - 10% of normal (Reich, 1963). However,

the rate of decay of the rapidly-labelled peak of activity in the nucleus of actinomycin-treated cells, suggests that this fraction is not stable. Even when cells are not treated with actinomycin, the bulk of the nuclear activity disappears without appearing in the cytoplasm (Harris et al. 1963). Since the bulk of the nuclear rapidly-labelled RNA is not stable, although RNA templates in the cytoplasm may be, then the movement of messenger RNA may be dependent on a process of stabilization.

This process may be actinomycin-sensitive. It may also be sensitive to other factors, e.g. hormones, inducers, and physico-chemical conditions, involved in metabolic regulation. If this process were subject to metabolic regulation, it is possible that only the RNA templates that are required for protein synthesis at a given time are stabilized, and move to the cytoplasm. Among these would be templates for constitutive or induced enzyme synthesis. It is possible that some repression of enzyme synthesis occurs by inhibition of movement of the appropriate templates to the cytoplasm. If these templates are not stabilised they may be degraded in the nucleus. Thus the degradation of rapidly-labelled RNA in presence of actinomycin D (Reich, 1963) may be due to the prevention of its stabilisation and movement to the cytoplasm.

Several modifications of the metabolic regulatory mechanism may have occurred during evolution of the animal cell. The indications of irreversible non-genetic changes in gene expression during differentiation suggest that the function of the genes is not directly analogous to the reversible regulation proposed by Jacob and Monod (1961) in bacteria.

Also, hormones appear to play a greater part than substrate in the induction of mammalian enzymes. Two developments may have contributed to this modification of the regulatory mechanism.

(1) The presence of the nucleolus and/or a specific synthetic process as a necessary step in the movement of messenger RNA to the cytoplasm may present an additional site of regulation of the function of messenger RNA.

(2) The physical state of the chromatin may affect the ability of the DNA to prime the synthesis of messengers.

The first possibility has already been discussed. Evidence for the second possibility comes chiefly from studies on Drosophila and amphibian oocytes. In the former local distensions of the chromosomes known as "puffs" have been shown to incorporate RNA precursors at a greater rate than neighbouring regions of the chromosome (Fujita and Takamoto, 1963). At the interband regions of the chromosomes, where staining shows that the chromatin is less condensed, incorporation of RNA precursors also proceeds at a higher rate (Fujita and Takamoto, 1963). In the latter, Gall and Callan (1962) have demonstrated a high rate of RNA precursor incorporation in loops of the lampbrush chromosomes of amphibian oocytes.

These, and other reports, led to the suggestion that the physical nature of chromatin particularly the degree of condensation, may influence its ability to initiate RNA synthesis. However, as many of these specialised alterations of chromatin structure are reversible, these phenomena do not provide an explanation of the irreversible changes in

gene function found during differentiation. It is possible that both reversible and irreversible changes in the function of chromatin are produced by DNA-histones. However, the evidence for this is contradictory, and the only positive reports so far have been obtained with DNA of pea seedlings (Bonner and Huang, 1963; Bonner et al. 1963).

4.5. General Conclusions

Cultured cells are subject to spontaneous variation which may produce a heterogeneous population of cells even when cultures are derived from single cells. As a result of this variation the synthesis of various enzymes may be altered irreversibly. Thus fluctuation of enzyme activity in cultured cells may be subject to erroneous interpretation.

Although such variation does occur, certain features of cultured cells remain relatively stable, and allow different cell strains to be distinguished. These features include the appearance of the cells and the activities of certain enzymes. The presence of such stable features suggests that cultured cells are not completely dedifferentiated, although many of the specialised functions of tissues are lost during the establishment of a cultured cell strain.

Reversible fluctuations in the activity of some enzymes in cultured cells suggests that they may be adaptive, and the absence of the appropriate inducers in culture media may cause the loss of the activity of these and other enzymes. Experiments with arginase suggest that the substrate alone is incapable of producing fluctuations in enzyme activity comparable with those that have been observed to occur spontaneously in golden hamster fibroblasts in culture, and in ascites cells when inoculated into mice.

An investigation of the uptake of arginine in HeLa cells has suggested that a specific transport mechanism may be involved. This mechanism may concentrate arginase more than threefold at low levels of

arginine in the medium (0.01 mM), but does not appear to do so at the normal culture level (0.6 mM). The induction of such a process may be necessary before arginase can be induced by substrate.

Metabolic regulation in animal cells may involve a messenger RNA analogous with that described in bacteria; and this fraction of RNA may be identifiable with the rapidly-labelled RNA of animal cells. However, the movement of this fraction to the cytoplasm has not been demonstrated in these experiments. It is possible that such movement may be inhibited by actinomycin D. This suggests that an extra actinomycin sensitive stage is involved in the movement of rapidly-labelled RNA to the cytoplasm. This stage may be located at the nucleolus, and may provide an opportunity for the regulation of messenger RNA function.

Evidence of irreversible, non-genetic changes in gene expression, the nature of the stimuli that may cause enzyme adaptation, and the failure to demonstrate the direct movement of messenger RNA to the cytoplasm, suggest that metabolic regulation in animal cells may not be entirely comparable with the mechanism described by Jacob and Monod (1961) in bacteria.

S U M M A R Y

5. Summary

1. The level of activity of 12 enzymes was compared in 5 cultured cell strains. The results were correlated with the morphological characteristics of the cells. This led to the conclusion that cell strains can be distinguished from each other both by their appearance and by the activities of certain enzymes. Hence cultured cells do not appear to become entirely dedifferentiated. The absence of some specialised functions in cultured cells as compared with the tissue of origin is discussed.

2. The occurrence of spontaneous irreversible variation was examined in cultured cells prior to an investigation of enzyme adaptation as a cause of fluctuation of enzyme activity. The results of studies of the karyotype of Y-5 Chinese hamster cells showed that these cells were subject to considerable spontaneous genetic variation.

3. Evidence of heterogeneity within cell strains with respect to enzyme activity, resistance to metabolic analogues, and growth in certain culture conditions also suggests that some spontaneous variation occurred. These examples of heterogeneity may have been due either to genetic variation or to permanent changes in phenotypic expression comparable to those occurring during differentiation and carcinogenesis. These latter changes may not involve mutations. The importance of spontaneous variation in the interpretation of fluctuations of enzyme activity is discussed.

4. Reversible fluctuations in the activity of certain enzymes were noted in several cell strains. Thiosulphate sulphurtransferase, glucose-6-phosphate dehydrogenase, alkaline phosphatase, glucose-6-phosphatase, β-glucuronidase, aspartate aminotransferase, glutamyl transferase, and arginase activity fluctuated three- to ten-fold in different cell strains.

5. The arginase activity of BHK21 hamster cells and HeLa cells showed considerable fluctuation between assays. In Landschutz and L5178Y cells cultured in vitro no arginase activity could be measured, whereas high and variable amounts were found when these cells were grown as ascites tumours in mice. As previous reports had suggested that arginase may be adaptive in several cultured cell strains, a more detailed investigation was made of the factors influencing arginase activity in culture. At no time was an increase in activity produced comparable with either the spontaneous variations in BHK21 cells or with the differences observed between Landschutz and L5178Y ascites cells grown in culture and in the animal.

6. Incubation in the presence of excess arginine and citrulline did not give reproducible induction of arginase activity in these studies. Increased activity during incubation at a high pH and after cold shock suggested that some unidentified factor may have a greater effect on arginase activity than substrate. This was also suggested by increases in arginase activity of cultured ascites cells after growth as ascites tumours.

The significance of the present and previous results are discussed in relation to enzyme induction in bacteria.

7. The entry of arginine to the cell was considered as a possible factor influencing the induction of arginase. The results of uptake experiments indicated the existence of specific sites for the transport of arginine and other basic amino acids into HeLa cells. These sites appear to involve sulphydryl groups and evidence is presented to show that the transport mechanism derives energy from glycolysis rather than from respiration.

8. Arginine may be concentrated in HeLa cells in the presence of a low extracellular concentration of arginine (0.01 mM). The implications of such a system for the uptake of arginine is discussed, with special reference to arginase induction.

9. Labelling experiments were undertaken to study the intracellular movement of RNA in an attempt to confirm the existence of a messenger fraction in animal cells, which might be involved in enzyme inductions. No conclusive evidence was found for the movement of rapidly labelled RNA from the nucleus to the cytoplasm in cultured mouse fibroblasts.

10. Actinomycin D was used in an attempt to follow the movement of labelled RNA in the absence of further incorporation. When a concentration of actinomycin of 0.1 µg./ml. was used for 15 minutes autoradiography of the labelled cells showed that this dose did not inhibit incorporation of ^{3}H-uridine. 15 minutes' incubation in a concentration of 100 µg./ml. inhibited 75% of all incorporation of ^{3}H-uridine.

Sucrose gradients of RNA from cells treated with 5 µg./ml. actinomycin for 15 minutes showed that incorporation of ^{3}H-uridine continued in all fractions. When cells were treated with 100 µg./ml. actinomycin incorporation was greatly reduced and restricted to ribosomal and soluble RNA.

11. The incorporation which was found in soluble RNA may have been due to terminal addition of cytidine. Incorporation in RNA sedimenting at other regions of sucrose gradients may have been due to true synthesis of a fraction of RNA resistant to actinomycin.

No rapidly labelled material was found in the nucleolus or cytoplasm of cells treated with actinomycin. The possibility of a block at the nucleolus of movement of rapidly-labelled RNA to the cytoplasm is considered and its implications discussed.

A B B R E V I A T I O N S

G L O S S A R Y of C Y T O L O G I C A L T E R M S

and

A P P E N D I X

A B B R E V I A T I O N S

DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
RNAse	Ribonuclease
ATP	Adenosine triphosphate
AIB	α-aminoisobutyric acid
IMPPase	inosinic acid pyrophosphorylase
NAD, $NADH_2$	nicotinamide adenine dinucleotide, oxidised and reduced (DPN)
NADP, $NADPH_2$	nicotinamide adenine dinucleotide phosphate, oxidised and reduced (TPN)
ATP	adenosine triphosphate

GLOSSARY OF CYTOLOGICAL TERMS

Karyotype	chromosome complement.
Diploid	each chromosome paired as found in most tissues in the animal.
Tetraploid	twice the diploid number of chromosomes.
Metacentric	centromere equidistant from either end of the chromosome.
Telocentric	centromere at one end of chromosome.

Nomenclature of cell strains

Cell strains are denoted by a series of letters and/or numbers referring to the origin of the strain; e.g. HLM, HeLa, Y-5 etc. Numbers following the strain designation indicate the clone number(s), if the strain has been cloned; e.g. Y-5-10-1-11-3 (contracted to Y-(6)3 for convenience) is clone number 3 of the Y-5 strain, and has been cloned a total of 6 times.

HANK'S BALANCED SALT SOLUTION (1949)

	Concentration (gm./litre)
NaCl	8.00
KCl	0.40
$CaCl_2$	0.14
$MgSO_4 \cdot 7H_2O$	0.10
$MgCl_2 \cdot 6H_2O$	0.10
$Na_2HPO_4 \cdot 2H_2O$	0.060
KH_2PO_4	0.060
Phenol red	0.02
$NaHCO_3$	0.35

Tris citrate-buffered balanced salt solution (Paul 1962, unpublished)

X10 BSS	65 ml.
0.2M Tris	80 ml.
0.1 M Citric acid	50 ml.
25% $MgCl_2$	10 ml.
Water to	1 litre

Used in same proportion as balanced salt solution (BSS) (Hanks & Wallace, 1949).

EAGLE'S MEDIUM

	Concentration	
	mg./litre	mM
L-arginine	17.4	0.1
L-cystine	6.0	0.05
L-histidine	3.2	0.02
L-isoleucine	26.2	0.2
L-leucine	13.1	0.1
L-lysine	18.2	0.1
L-methionine	7.5	0.05
L-phenylalanine	8.3	0.05
L-threonine	11.9	0.1
L-tryptophan	2.0	0.01
L-tyrosine	18.0	0.1
L-valine	11.7	0.1
L-glutamine	146.0	1.0
Choline	1.0	
Nicotinic acid	1.0	
Pantothenic acid	1.0	
Pyridoxal	1.0	
Riboflavine	0.1	
Thiamine	1.0	
Biotin	1.0	
Folic acid	1.0	
Glucose	2000	
NaCl	8000	
KCl	400	
$CaCl_2$	140	
$MgSO_4 \cdot 7H2O$	100	
$MgCl2 \cdot 6H_2O$	100	
$Na_2HPO_4 \cdot 2H_2O$	60	
KH_2PO_4	60	
$NaHCO_3$	350	
Phenol red	20	
Penicillin	0.5	

WAYMOUTH'S MEDIUM MB/752/1

	MG./litre	mM
NaCl	6000	103
KCl	150	2.0
$CaCl_2 \cdot 2H_2O$	120	0.82
$MgCl_2 \cdot 6H_2O$	240	1.18
$MgSO_4 \cdot 7H_2O$	200	0.81
Na_2HPO_4	300	2.11
KH_2PO_4	80	0.59
$NaHCO_3$	2240	26.7
Glucose	5000	27.8
Ascorbic acid	17.5	0.1
Choline HCl	250	1.8
Cysteine HCl	90	0.57
Glutathione	15	0.05
Hypoxanthine	25	0.18
Glutamine	350	2.38
Thiamine HCl	10	0.03
Ca pantothenate	1.0	0.003
Riboflavin	1.0	0.003
Pyridoxin HCl	1.0	0.003
Folic acid	0.4	0.008
Biotin	0.02	0.0008
m-inositol $\cdot 2H_2O$	1.0	0.005
Nicotinamide	1.0	0.008
Vitamin B_{12}	0.2	0.00015
L-cystine	15	0.06
Glycine	50	0.66
L-phenylalanine	50	0.30
L-glutamic acid	150	1.02
L-aspartic acid	60	0.46
L-tyrosine	40	0.22
L-lysine HCl	240	1.42
L-proline	50	0.44
L-methionine	50	0.34
L-threonine	75	0.64
L-valine	65	0.55
L-isoleucine	25	0.19
L-leucine	50	0.38
L-tryptophan	40	0.20
L-arginine HCl	75	0.36
L-histidine HCl	150	0.80
NaOH	to pH 7.4	2.5

CULTURE MEDIUM FOR L5178Y LYMPHOBLASTS

	Concentration (gm./l.)
NaCl	8.0
KCl	0.4
$MgCl_2 \cdot 6H_2O$	0.1
Na_2HPO_4	0.06
$NaH_2PO_4 \cdot H_2O$	0.067
Glucose	1.0
$NaHCO_3$	1.0
5% acid hydrolysed casein + tryptophan, 0.5 mg./ml.	5 ml./l.
	(mg./l.)
Glycine	20
Cystine	7.5
Histidine	20
Glutamine	200
Thiamine HCl	1.0
Nicotinamide	0.5
Ca-pantothenate	0.5
Pyridoxal HCl	0.5
D-ribose	0.5
Riboflavin	0.5
Choline chloride	1.5
i.inositol	1.5
Biotin	0.01
Ascorbic acid	1.6
Glutathione (reduced)	1.5
Serum	2-10%
Peptone	0.6%
Folic acid	10 mg./l.
Penicillin	100 units/ml.
Streptomycin	0.050 mg./ml.
Phenol red	10 mg./l.
$CaCl_2 \cdot H_2O$	0.183 gm./l.

Special medium as used for culture of Chinese hamster fibroblasts, strain V-5-11, during selection experiments with metabolic analogues

	gm./l.	mM
Histidine HCl	75.2	0.36
Isoleucine	150	1.15
Leucine	100	0.76
Lysine	240	1.64
Methionine	15	0.10
Phenylalanine	50	0.30
Proline	25	0.22
Serine	50	0.48
Tryptophan	40	0.20
Valine	25	0.21
Glutamine	350	2.40
Threonine	7.5	0.06
Cysteine	25	0.21
Arginine	7.5	0.08
Tyrosine	10	0.06
Glucose	2000	11.11

Vitamins as in Waymouth's medium.

Medium made up in Hanks' balanced salt solution.

Trypsin-citrate solution as used in routine transfer of cell cultures, and during the harvesting of experimental samples for enzyme determinations

Difco Trypsin 1:250	1.25 gm.
Sodium Citrate	1.48 gm.
Sodium Chloride	3.00 gm.
Water to	500 ml.

Adjust to pH 7.8.

Sterilize by filtration through millipore filter GS 0.22μ.

Suspension fluid for counting cells by the Coulter electronic counter

Sodium Chloride	7 gm.
Citric Acid	10.5 gm.
Mercuric Chloride	1 gm.
Water to	1 litre

Filter through sintered glass filter to remove particulate impurities.

A C K N O W L E D G E M E N T S

A C K N O W L E D G E M E N T S

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