A GENETICAL AND BIOCHEMICAL INVESTIGATION OF THE UPTAKE OF DNA BY MAMMALIAN CELLS IN CULTURE

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"The Mind is indeed restless, Arjuna: it is indeed hard to train. But by constant practice and by freedom from passions the Mind in truth can be trained".

Bhagavad Gita, 6, 35.

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Norman Lockhart

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ABBREVIATIONS

Each abbreviation is fully explained on its first appearance in the text, but they are all tabulated here for ease of reference.

AA; 8-azaadenine

AAR; 8-azaadenosine

AR, CR, GR, TR, UR; ribonucleosides

AdR, CdR, GdR, TdR, UdR; deoxyribonucleosides

AMP, CMP, GMP, TMP, UMP; deoxyribonucleotides

dAMP, dCMP, dGMP, dTMP, dUMP; deoxyribonucleotides

APRT; Adenine phosphoribosyl transferase

aCyt; arabinosyl cytosine, 1-β-D-arabinofuranosylcytosine

AzG; 8-azaguanine

AzH; 8-azahypoxanthine

AzT; 6-azathymine

BrdUrd; bromodeoxyuridine, 5-bromo-2'-deoxyuridine

dCK; deoxycytidine kinase

DEAE-dextran; diethylaminoethyl dextran

DNase; deoxyribonuclease

EC10; Eagle's plus 10% (v/v) calf serum

EFC10; Eagle's plus 10% (v/v) foetal bovine serum

FdUrd; 5-fluoro-2'-deoxyuridine

HGPRT; inosinic pyrophosphorylase; hypoxanthine-guanine phosphoribosyl transferase

μCi; microcurie

PBS; Dulbecco's phosphate-buffered saline

RNase; ribonuclease

SDS; sodium dodecylsulphate

SSC; saline-sodium citrate

TG; 6-thioguanine

TGR; 6-thioguanosine

TK; thymidine kinase

The system used in naming the mutant cell lines is explained in fig. 3.4 and in table 3.1.

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INTRODUCTION

Section 1.1: GENERAL INTRODUCTION

The first report of cell-to-cell transfer of genetic information was the paper by Griffith (1928) which demonstrated the rescue of the capsular polysaccharide type of a killed strain of Pneumococcus by a live strain of different polysaccharide type when the two were injected together into the same mouse. Griffith thought that the "transforming principle" might be the polysaccharide itself and never quite arrived at the idea of the transfer of genetic characteristics. Further work, by Dawson and Sia (1931) and Alloway (1933), showed that transformation could be effected by cell-free extracts of the donor strain and that the transforming principle did not have the characters of the capsular polysaccharide. It was not until ten years later that Avery, McLeod and McCarty (1944) showed that the transforming principle had all the properties of DNA, including being specifically inactivated by deoxyribonuclease (McCarty and Avery, 1946). They were at that time unable to exclude the possibility that the active substance might be a trace contaminant in their DNA preparations, but all subsequent work has been consistent with the idea that transformation is a process involving uptake of macromolecular DNA and its integration into the bacterial chromosome. The process has attracted a lot of attention, both as a subject for study in its own right and as a means for elucidating the genetic fine structure of the bacterial genome. An excellent review of the work on transformation is found in the second edition of Hayes' monograph (1969).

A second mechanism of genetic transfer was found by Lederberg and Zinder and their colleagues (1951, 1952) as a result of studies on the appearance of prototrophic recombinants of Salmonella typhimurium. They were able to demonstrate that the filterable particles mediating the appearance of these recombinants were inseparable from the particles of the phage, P22. This process is termed transduction and has now been demonstrated in a number of systems (e.g. Morse, 1954; Lontit, 1959; Morse, 1959; Coetzee

and Sacks, 1960; Thorne, 1961). It involves the inclusion of a fragment of the genome of the infected bacterium in the phage particles which are released after the lytic cycle. This inclusion can occur either by the erroneous excision of a temperate phage which has been induced to undergo a lytic cycle, or by the encapsidation of pieces of DNA which have been produced by fragmentation of the host genome. In the former case, the transducing phage particles contain a fragment of bacterial DNA covalently bound to the remnant of the phage genome, while in the latter they contain only bacterial DNA. In either case, cells infected with the transducing particles receive some of the genes of the original host bacterium and there is the chance of these genes being expressed and possibly integrated into the recipient chromosome. Hayes (1969) reviews the mechanism and the genetical uses and implications of both types of transduction.

The demonstration and elucidation of transformation and transduction in bacteria soon gave rise to the question of whether similar phenomena could be detected in eukaryotic cell systems such as mammalian cells in culture. That these investigations have not reached a state of knowledge equal to that of the work on bacteria is the result of a combination of conceptual and technical difficulties.

Firstly, eukaryotic cells are very much more difficult to handle than are bacteria, and this is reflected in the relatively recent development of reliable methods of culturing them. They are, of course, essentially an artificial microorganism in that they are not adapted to independent growth. For this reason, they are extremely fastidious in their growth requirements, both with respect to the prevailing conditions of temperature, pH and so on, and the substances which are supplied in the growth medium. This inability of cells to grow in simple, defined conditions produces a number of difficulties for genetical work. Firstly, the culture techniques dictated by the growth requirements of the cells make cloning an extremely laborious

operation in the majority of cases, and even now only those cell lines which will form colonies in agar suspension culture can be cloned in reasonable numbers. This is a considerable obstacle to genetic analysis. Secondly, the complex requirements for aminoacids and vitamins which cultured eukaryotic cells exhibit mean that a class of mutant, the auxotroph, which has been so useful in bacterial genetics, is not available for the analysis of mammalian cells. Some work has been done in this direction, however. For example, Puck and Kao (1967) and Kao and Puck (1968) selected nutritionally-deficient cell lines by incubating the cells in medium deficient in the particular nutrient but containing 5-bromodeoxyuridine (BrdUrd). Cells which could grow in the absence of the nutrient did so, and incorporated BrdUrd into their DNA, while the auxotrophic cells did not grow and thus did not incorporate BrdUrd. As BrdUrd has a strong photosensitising effect, subsequent exposure of the cells to strong light preferentially killed those cells which had incorporated BrdUrd and in this way selected in favour of the auxotrophs, which were then grown up in complete medium to give a population consisting of the progeny of those cells which had not grown in the absence of the nutrient. This contained a high proportion of auxotrophs, but also probably included cells unable to incorporate BrdUrd for quite different reasons, such as thymidine kinase deficiency, and cells which were in G_o throughout their exposure to BrdUrd. A start has been made on the use of these mutants and this will probably be extended in the future (Kao, Chasin and Puck, 1969).

The lack of well-defined mutants has in the past meant that the study of genetic phenomena in eukaryotic cells has in most cases been restricted to the use of differentiated functions or of whole-organism characters which were often poorly understood biochemically. The most frequently-used type of genetic marker not falling into either of these two categories has been resistance to drugs which are toxic to the parental cell line. Examples of

this are the 8-azaguanine-resistant mouse (Littlefield, 1963) and human (Albertini and DeMars, 1970) fibroblasts, and human (Szybalski et al., 1962) or hamster (Subak-Sharpe, 1965) cells in continuous lines resistant to one or more antimetabolites.

The use of cells resistant to cytotoxic agents presents its own difficulties in the study of transformation. The problem arises because, with few exceptions, there are no systems in which a small number of drug-sensitive cells can be selected out of a resistant population. The typical experimental plan has been dictated by this technical deficiency, and usually involves treating the sensitive cell population with DNA from resistant cells and then exposing it to the cytotoxic agent. Ideally, only those cells which have specifically transformed to drug resistance by the DNA treatment will grow in the presence of the drug, although this has not been found to be the case in practice (Fox et al., 1969). Resistance to a particular toxic agent may arise in a number of ways. The resistant cells may be cryptic, so that the drug cannot enter them, or they may be modified either in the cellular function which is the target of the drug or in that which is necessary for the conversion of the drug to a toxic form. The other possible mechanism - that the cells gain the ability to detoxify the drug - is not often found in practice. In the three most frequent mechanisms outlined above, the common factor is that the change to drug resistance involves the loss or modification of a cellular function. In genetic terms, this is a change towards a recessive character, and raises two problems for the selection of transformants. The first of these stems from the presence in the cell of homologous pairs of chromosomes. This means that each cellular function is potentially determined at two independent sites within the cell, the only exceptions to this being those genes which are carried on the nonhomologous parts of the sex chromosomes. In a selection experiment in which it is necessary to detect a recessive character arising from a transformation event, it is necessary to eliminate

the dominant allele from both of these chromosomal loci. In fact it is not sufficient simply to insert the exogenous genetic material into the recipient cell chromosomes, because such insertion will not be detected owing to the continuing presence and operation of the dominant genes. In more particular terms, if the acquisition of drug resistance involves the loss of an enzyme, a detectable transformation event is essentially the inactivation or loss of the DNA specifying that enzyme, and this must occur in the same cell in two chromosomes. This restriction greatly diminishes the probability of a detectable transformation event. The other drawback of this type of system is that, even if the dominant genes in question are totally lost, their gene products may be quite stable and by their continuing presence delay the appearance of phenotypically resistant cells. In many of the studies of DNA-mediated transformation which have been published and which have made use of this type of drug-resistance marker, there have been difficulties both in interpretation and reproducibility (e.g. Majumdar and Bose, 1968; Fox, Fox and Ayad, 1969), and some of these difficulties may be due to the phenomena discussed above. For example, Fox et al. (1969) found considerable numbers of colonies arising from untreated populations of sensitive cells, even in the presence of high concentrations of the cytotoxic agent. These same workers also found great variations in the results obtained when the same experiment was repeated. The number of colonies obtained when similar numbers of sensitiv cells were incubated in the presence of the drug varied between 3 and 251, while DNA-treated cells gave between 9 and 197. Statistical analysis of the ratios of control to DNA treated colonies for each experiment indicated that the DNA-treated cells gave a higher frequency of "resistant" cells, but with such variation the results cannot be regarded as satisfactory.

Studies on transformation towards a dominant character have largely been restricted to the use of cells which express a differentiated function, such as the production of haemoglobin (Kraus, 1961), or the induction of

melanin synthesis in amelanotic melanoma cells (Glick, 1962). This latter case and the closely-related induction of pigment in cells from albino mice (Ottolenghi-Nightingale, 1969), are inductions of a single enzyme, tyrosinase, and offer some advantages for transformation studies. More recently, there have been developed systems which exploit the fact that some analogues of nucleosides are not toxic to cells unless they are phosphorylated. In this situation, it is clear that a cell line which has lost the capacity to phosphorylate a particular analogue will no longer be sensitive to it, but will also as a consequence be unable to phosphorylate the corresponding normal nucleoside. The resistant cells will thus be totally reliant on the de novo synthesis of the nucleoside, and if this pathway is blocked, for example with a folic acid antagonist such as aminopterin, the cell will be unable to grow. If such inhibition is performed in the absence of the analogue and after treatment of the cells with DNA from analogue-sensitive cells, transformants in the particular enzyme deletion will be detectable because they will have acquired the ability to use the preformed nucleosides from the medium and will thus be able to grow and form colonies. This system, involving as it does the regaining of an activity which was previously totally absent, is not subject to the problems of diploidy and phenotypic delay outlined above. The cells used in the present work carry this type of marker and are discussed in more detail in section 3.1.

In addition to these technical difficulties, eukaryotic cells present considerable topological and enzymic barriers to the incoming DNA molecules. Lysosomes contain high levels of nuclease activity and it seems likely that a high proportion of the DNA entering a eukaryotic cell will be exposed to the risk of complete degradation to its component nucleotides. The topological difficulties arise from the size and complexity of the eukaryotic cell and from the need for the pure DNA to reach the sites within the cell where the enzymic apparatus for transcription and replication is available. This will

present a much greater difficulty in the mammalian than in the bacterial cell, particularly if the mesosome plays a role both in DNA uptake and in chromosome replication. That these do not constitute insuperable difficulties is shown by the infectivity of isolated viral DNA (Herriot, 1961; Colter and Ellem, 1961; Di Mayorca et al., 1959; Weil, 1961; Atanasiu et al., 1962; Gerber, 1961; Ito, 1960, 1961). The most efficient recovery of the original virus infectivity after the DNA extraction is of the order of 0.1% (Pagano, 1970) and various factors contribute to this loss, although it should be borne in mind that loss of infectivity does not necessarily mean that none of the viral genes can be expressed. In the inactivation of virus, for example by UV light, infectivity is the first function that is lost. In short, it is not clear how much of the loss of infectivity is the result of damage to the DNA during the extraction process and how much is due to the difficulty in penetrating into and functioning within the cell.

One final difficulty in transformation studies in eukaryotes is the great complexity of the genome. While the DNA complement of a bacterium is 10^9-10^{10} daltons, that of a mammalian cell is 10^{12}. This means that unless a protein-specifying sequence is highly-reiterated - and the evidence is against this - it will be represented in a randomly-sheared population of mammalian DNA at a frequency of 10^{-3}-10^{-2} of that which it would have in a similar preparation of bacterial DNA. Hence a cell would have to absorb 10^3 times as much mammalian DNA as bacterial to give the same probability that a particular protein-specifying sequence will be represented among the intake. Even more extreme is the comparison with viral DNA. While every viral molecule is potentially infectious, a mammalian DNA preparation of molecular weight 10^7 daltons will carry the gene under investigation on only one molecule in 10^5. This complexity of the eukaryotic cell genome means that extracellular DNA concentration becomes a limiting factor, particularly in view of the reported cytotoxicity of DNA at high concentrations (Floersheim,

1962; Smith, 1964; Smith and Cress, 1967; Glick and Goldberg, 1966; Glick and Salim, 1967a and b; Glick, 1967a) and the ease with which high molecular-weight DNA will precipitate.

Methods of detecting DNA-mediated transformation

If transformation is to be detected, some means must be available for the identification of a small number of genetically-altered cells in a cell population. That which has been most frequently used is the exposure of the cell population to conditions in which only the altered cells can grow and divide (e.g. Szybalski and Szybalska, 1962a). Any genetic change is manifested as the appearance of colonies of cells. This is the rigourous means of identifying cells which have undergone a heritable change, but not all the phenotypic changes induced by treatment with DNA need be of this type. The analogous phenomenon in bacteria would be abortive transformation, in which the fragment of DNA introduced by the transducing phage particle does not enter into a close association with the recipient cell chromosome and does not replicate as rapidly as the recipient cell. This means in the extreme case that only one of the progeny of each cell division will receive the genetic function (Hayes, 1969, p.96 et seq.). Were this to occur in the eukaryotic cells which were being examined for transformants, no colonies would arise even though the input DNA was biologically active. Detection of this type of phenomenon requires the examination of individual cells in the DNA-treated population, and this has been carried out in various systems either by examining the cells for a visible product which is not present in the untreated cells (Glick and Salim, 1967), or by devising a method by which individual cells can be assayed for the acquisition of a genetic function. It is this latter method which is used in the present investigation. The detection of small numbers of genetically-altered cells in the presence of a large number of unaltered cells is achieved by exploiting the inability of the mutant cells to incorporate certain exogenous nucleosides into their

nucleic acid. The cells are exposed to the tritium-labelled nucleoside for a period of some hours, and are then fixed, acid-washed and examined by autoradiography. Only cells which have acquired the ability to utilise the exogenous precursors will be radioactively labelled. This type of procedure is able to detect the linear inheritance of the input DNA fragment and is also capable of detecting biological activity in the situation where the absorbed DNA functions only temporarily and is then degraded or otherwise lost.

The sensitivity of these methods is potentially very high. In the selective system, the limiting factor is the total number of cells which are exposed to the selective conditions after DNA treatment, and the number can be very high indeed. In the direct examination of cells, the sensitivity is largely determined by the total number of cells which can be effectively scanned after DNA treatment. In this, time is a limiting factor and it is important that the difference between the two types of cell should be as wide as possible. Ideally, the difference should involve a simple presence/absence choice so that individual cells can be immediately and unequivocally classified. In the autoradiographic system used in the present work (see section 3.3b) the nonincorporating cells are nearly ideal in that they exhibit an extremely low background of silver grains, whereas the incorporating cells under the same conditions become very heavily labelled. This clear distinction means that large numbers of cells can be easily and quickly scanned under a relatively low-powered microscope. Details of this are given in sections 2.5 and 3.1b, and the experimental results are set out in sections 5 and 6.

One other method which has been used for the detection of the induction of a cellular product is the extraction of a DNA-treated cell population and detection of the induced product by electrophoresis. Kraus (1961) used this method to detect the induction of a different haemoglobin type in DNA-treated bone marrow cells. The disadvantage of this method is that it requires

the production of the substance in detectable quantities, and this may not be achieved if only a small proportion of cells are transformed.

The chances of finding transformants can be increased if a means can be found to increase either the amount of DNA that the cells absorb or the efficiency with which the DNA reaches the intracellular sites at which it can function. A number of methods have been employed to achieve one or other of these increases. The infectivity of isolated viral DNA can be more efficiently detected if the cells are subjected to hypotonic treatment before infection (see Warden, 1968) and is increased to an even greater extent by pretreatment of the cells with diethylaminoethyl-dextran (DEAE-dextran) or by simply infecting the cells in its presence (McCutchan and Pagano, 1968). A comparison of the effect of this polycationic substance and of some others on the infectivity of DNA was carried out by Warden (1968), who found that DEAE-dextran was the most effective. Its effect on the uptake and functioning of isolated mammalian cell DNA is investigated in the present work. Szybalski and Szybalska (1962a) found that transformants could only be detected in their system if spermine was present in the DNA solution with which the cells were treated. The mechanism of the enhancement of uptake and biological activity of DNA by these substances is not clear.

Section 1.2: GENETIC EFFECTS OF DNA

The most convincing demonstration that DNA molecules can enter eukaryotic cells and become integrated in their genetic apparatus would be to show the acquisition of a genetic character determined by the exogenous DNA. It was just such an observation that gave impetus to the work on DNA uptake by bacteria, and many attempts have been made to show that a similar phenomenon does occur in eukaryotic cells. That the evidence accumulated so far is not totally convincing in either direction is in large part a result of the difficulties outlined in section 1.1.

The difficulties in producing genetically-marked cells in culture has encouraged the use of whole-organism characters, such as the classical Drosophila markers (Fox and Yoon, 1965, 1966, 1967, 1970), similar markers in other insects (Nawa et al., 1971) and natural products of cells displaying differentiated functions (Kraus, 1961; Glick and Salim, 1967; Ottolenghi-Nightingale, 1969).

Fox and Yoon, in a series of papers (1965, 1966, 1967, 1970), describe experiments in which Drosophila melanogaster eggs were collected and treated with DNA from genetically-different strains of Drosophila. Sixteen characters which had been used in conventional Drosophila genetics were examined in the adults which developed from the DNA-treated eggs and in their progeny. The markers included pigmentation and abdominal chaetae morphology, and transformation was found in 11 of the 16 characters at frequencies of up to 3×10^{-2}. In the other 5 markers, no transformation was seen (Fox and Yoon, 1970). In all cases, the transformation was specific for the genotype of the strain from which the DNA had been extracted, but various peculiarities were found in the expression of the characters. No fully-transformed individuals were found, all transformed flies being genetic mosaics. This mosaicism was passed to the progeny of transformed flies with a high probability after the first two or three generations, during which the transmission and expression

of the acquired genetic character was uncertain. Even in established lines, the loss was at a frequency of 10^{-3} - 10^{-4} per generation. Several lines carried the exogenous DNA markers cryptically for two generations after DNA treatment before any mosaic flies appeared. To account for these results, the authors postulate an exosome model in which the exogenous DNA is retained but not integrated into the chromosomes. The model involves a copy-choice mechanism which allows the mosaicism, high frequency of gene-loss and phenotypic delay to be coherently explained, but the nature of the exosome and of its transcription, replication and transmission remains unclear.

Similar findings have been reported in papers by Nawa and co-workers (Caspari and Nawa,1965; Nawa and Yamada, 1968; Nawa et al., 1971). These studies were concerned with the genetic effect of exogenous DNA on various species of insect, including the silkworm (Bombyx mori). Genetic changes in wing-scale colour and eyecolour were observed after DNA extracted from wild-type flies had been injected into the haemocoel of mutant larvae. The Bombyx eye colour marker is a useful one for transformation studies because the white-eyed mutants have a single enzyme lesion and it is acquisition of this enzyme that is being sought (see section 1.1). Homozygous white-eyed (w_1/w_1) larvae were injected with 20µg. of DNA in the presence of 50µg./ml. of spermine (Szybalski and Szybalska, 1962a) and allowed to develop to maturity. They were screened for dark-eyed individuals and for the laying of pigmented eggs, these two characters being determined by the same gene. The adults were also analysed by cross breeding. The w_1/w_1 larvae which had been treated with +/+ DNA subsequently produced pigmented eggs at very low frequency. Out of 626 batches of eggs, totalling 180,000, only one batch had pigmented members, and even that batch had only 3 out of 250. The progeny of flies from unpigmented eggs from these batches, however, included dark-eyed individuals at a frequency of 1 in 3,000, a rate much higher than that found among untreated flies. These dark-eyed flies were backcrossed

with w_1/w_1 flies, and mostly behaved as +/+ homozygotes, with a few flies giving dark-eyed progeny at a rate rather higher than would be expected from $+/w_1$. The authors (Nawa et al., 1971) postulate a model involving an exosome and resembling that of Fox and Yoon (1970).

Three reports have appeared in which a differentiated function was used as a genetic marker. A brief article by Kraus (1961) reported the induction of β^a haemoglobin in β^s bone marrow cells after they had been treated with DNA extracted from β^a cells. No further details have been published. Glick and Salim (1967) found that the number of cells producing melanin in a culture of amelanotic melanoma cells was increased to 0.82% by treatment with melanoma cell DNA and spermine. This represents a three-fold increase over the untreated controls or cells treated with DNA from unpigmented cells. DNase treatment of the DNA abolished this genetic effect. Ottolenghi-Nightingale (1969) used a similar system. Homogenised tissue from albino mice was treated in vitro with DNA from pigmented mice and implanted into the necks of albinos. Tissue from the sites of implantation was examined 14 days later by microscopy and scored for the presence of melanin-producing cells. It was found that 15/483 DNA-treated explants had groups of pigmented cells, while none of the 421 saline- or DNased DNA-treated controls had any pigmented cells. The production of melanin is a useful marker for transformation studies because it is determined by acquisition of the activity of a single dominant gene, that for tyrosinase.

Podgajetskaya and her colleagues (1964) also worked with a system involving implantation of DNA-treated cells in whole animals but the genetic system was less well-defined. Cells from sarcolysine-sensitive transplantable tumours were injected subcutaneously into rats together with DNA from sarcolysine-resistant cells. When tumours developed, the rats were given a course of sarcolysine injections at 72 hour intervals, under which conditions most of the tumours disappeared completely. Native DNA and RNase-treated

DNA from resistant cells increases the frequency of resistant tumours to 17% compared with 1.2% in rats which were not treated with DNA. No such increase was found in rats which received DNase-treated or denatured DNA. This work is necessarily imprecise because of the nature of the experimental system and does not seem to have been followed up. Various other studies on genetic transformation in whole-animal systems have been reported but the results have been either negative or inconclusive (Benoit et al., 1958; Medawar, 1958; Bearn, 1959; Kok, 1959; Shoffner et al., 1961; Svoboda and Haskova, 1959; Tigyi et al., 1959). The likelihood of finding such in vivo transforming activity is maintained by the finding that Pneumococcus DNA is recoverable in biologically active form after intravenous injection into test animals (Bendich et al., 1965), although Gosse et al (1965) found that DNA injected intravenously or intraperitoneally into mice or rabbits was degraded to a "subbiological" state within a few minutes. Ito and Evans (1961) and Ito (1970) were able to demonstrate tumour induction by injection of isolated tumour DNA, but they did not conclusively demonstrate the absence of viral DNA in their preparations. Hewer and Meek (1958) injected herring sperm DNA into mice and found some tumours in the duodenal mucosa, but the phenomenon was not reproducible. Ledoux et al (1967) were able to detect injected Bacillus subtilis DNA by equilibrium density-gradient analysis of tissue extracts even after it had become undetectable in the bloodstream.

Most of the other work that has been published has been concerned with cultured cells and has used genetic markers involving purine or pyrimidine metabolism. Three groups of workers have reported transformations from sensitivity to resistance to base or nucleoside analogues (Bradley et al., 1962; Majumdar and Bose, 1968; Fox, Fox and Ayad, 1969). The mechanism of this resistance is discussed in section 3.3 and it is subject to the complications of diploidy and phenotypic delay discussed in section 1.1.

Bradley, Roosa and Law (1962) and Roosa (1966) used P388 cells and

treated them with DNA which had been extracted from 8-azaguanine resistant P388 cells (P388/AzG). They did indeed find that DNA-treated cells gave rise to clones which would grow in the presence of toxic concentrations of AzG but isolation of these clones and further testing showed that they were fully sensitive. The conclusion reached was that the release of nucleotides by degradation of the absorbed DNA antagonised the effect of the AzG sufficiently to allow the cells to go on growing as if they were resistant. The later publication (Roosa 1966) reported that specific transformation could be detected in an experimental system designed to avoid this artefact.

Majumdar and Bose (1964, 1968) selected, from primary human uvular carcinoma culture, a line of cells resistant to 6-azathymine (AzT). Wild-type cells exposed to DNA from this resistant line and subsequently grown in agar containing AzT produced a considerable number of colonies, which were scored as transformants, although no attempt was made to isolate and further characterise these clones. Unfortunately, too, the number of colonies formed in the control cultures was not only high, but very variable. Statistical analysis showed that DNA treatment significantly increased the mean number of colonies, but the result was marginal. Further experiments to find the optimum conditions for transformation were reported, but once again the only criterion of transformation is the formation of colonies in the presence of AzT. The frequency of "transformants" reaches as high as 4% of the total cell population.

Fox, Fox and Ayad (1969), following up their biochemical work on DNA uptake (Ayad and Fox, 1968, see section 1.3), investigated the transformation of P388F lymphoma cells to iododeoxyuridine-resistance. The work is similar in approach to that of Majumdar and Bose, and the results are somewhat the same, with high frequency of colony formation in the untreated control cultures. To interpret the results, the authors normalise the colony frequency in the controls to 1.00 for each individual experiment, and find

that treatment with DNA from resistant cells (P388/I20) increased the number of colonies to 1.96 ± 0.34, while DNA from wild-type cells gives a frequency of 1.10 ± 0.22 (the confidence limits show the 95% points). While the increase is significant at P = 0.05, the considerable variation in colony numbers makes the system less than satisfactory. For example, the figure of 1.96 ± 0.34 is made up of results varying from 0.16 to 4.28 and the number of colonies in the controls varies from 3 to 250. No isolation of clones for further examination was undertaken.

A different approach was taken by Szybalski and Szybalska (1962a) who exploited the aminopterin-sensitivity of cells which have become resistant to purine analogues as a result of losing a purine phosphoribosyltransferase (see sections 1.1 and 3.1). This selective system allows the investigation of transformation from aminopterin sensitivity to resistance, which is a change involving the acquisition of an enzyme activity. In addition, it is a well-defined system biochemically, and has a very low frequency of spontaneous reversion. The cells used by these investigators were the D98S cells of Berman and Stulberg (1956) and had been selected for resistance to 8-azaguanine (AzG) and 8-Azahypoxanthine (AzH) respectively (Szybalski and Smith, 1959; Szybalski et al., 1962). These cells were highly resistant to the analogues, showed a low reversion rate and were deficient in hypoxanthine-guanine phosphoribosyltransferase (HG-PRT) activity. The cells were treated with DNA isolated from D98S cells and $HGPRT^+$ cells were selected using hypoxanthine-aminopterin-thymidine (HAT) medium. 12-15 days after treatment, the colonies were fixed and counted. Transformants appeared at frequencies proportional to the DNA concentration up to 100µg./ml. in the presence of spermine at 50µg./ml.. In the absence of spermine, there was no detectable transformation. The role of DNA in these experiments was quite specific, the ability to transform being a property of only those DNA preparations which were derived from cells which had HGPRT activity, and

being totally destroyed by treatment with DNase. Further experiments described the pH-dependence of transformation and showed that transforming activity formed a band in caesium chloride gradients coincident with the main band of DNA. One of the resistant colonies was isolated and tested for HGPRT activity in vitro. This assay showed a level of activity comparable with that found in the donor cells. The maximum frequency of transformation was found at 100µg.DNA/ml. and at pH 7.0-7.5, at which point the level was 0.03% of the total number of cells exposed to DNA. The transformants could be grown through many generations without loss of enzyme activity, so that the DNA seems to be effecting a permanent genetic change. Unfortunately, these very clear results have not been confirmed or extended by subsequent work.

Section 1.3: BIOCHEMICAL STUDIES ON DNA UPTAKE

Uptake of DNA by eukaryotic cells has received a lot of attention in recent years. It has been the subject of a number of reviews, the most recent of which are those by Ledoux (1965), Glick (1969) and Bhargava and Shanmugam (1971).

Penetration of DNA label into eukaryotic cells has not been difficult to demonstrate. DNA labelled in vivo (Kay, 1961; Rabotti, 1962; Wilczok, 1962; Yoon and Sabo, 1964; Borum, 1965; Meizel and Kay, 1965) or in vitro (Wilczok, 1962; Mathias and Fischer, 1962; Gartler, 1959; Sung and Quastel, 1963; Meizel and Kay, 1965) has been used and one group used DNA labelled by the Wiltzbach procedure, which involves exchange of the ^{1}H in DNA with high specific-activity gaseous ^{3}H (Borenfreund et al., 1959; Borenfreund and Bendich, 1961). After exposure to the labelled DNA under various conditions, the cells were usually subjected to procedures designed to remove any DNA that is adsorbed to the surface of the cells, such as washing with iodoacetate (Meizel and Kay, 1965; Kay, 1966) or with a solution of unlabelled DNA (Mathias and Fischer, 1962). The uptake and intracellular location of the DNA label is then determined using autoradiography (Kraus, 1961; Borenfreund and Bendich, 1961; Wilczok, 1962; Tiesler and Heicke, 1968) or liquid scintillation counting of label in whole cells (Sirotnak and Hutchison, 1959; Gartler, 1959; Sung and Quastel, 1963; Hill and Huppert, 1970) or in isolated nuclei (Gartler, 1959; Kay, 1961; Schimizu et al., 1962; Meizel and Kay, 1965; Kay, 1966; Robins and Taylor, 1968).

A number of other techniques of less general interest have been used. Bensch and his colleagues (Bensch and King, 1961; Bensch et al., 1964, 1966) have prepared DNA-protein coacervates and have used DNA-specific stains and light microscopy to investigate their uptake. They also tagged the coacervates with colloidal gold for electron microscopy, or relied on their

relatively high electron density for identification. Cocito et al (1962), in a series of similar experiments, used complexes of DNA with methylated albumin. They found that these complexes were taken up in larger quantities than was DNA itself, and were not catabolised by the intracellular nucleases. The same workers show, however, that complexing with methylated albumin will inactivate the infectivity of viral DNA, so it appears that the complexes are very stable, and that the uptake of these complexes is more likely to give information on protein uptake. No genetical experiments using such complexes have been carried out. The process of particle uptake by cells in culture is well known, and has been observed for such diverse particles as virions and chloroplasts.

Two groups have made use of acriflavine dyes to investigate DNA uptake. Adams et al (1965) used the dyes as microscopic stains, while Roth and his colleagues (1968) examined the quantitative aspects of acriflavine binding. They found that the dyes were bound in quantities characteristic of each cell line, and that treatment of the cells with DNA increases the dye binding to a higher and stable level. In addition to this, the treated cells exhibited an increased resistance to SV40 infection. The authors claim that the increase in the amount of dye which is bound is a result of the uptake of exogenous DNA although they do not make any firm proposals about the nature of the underlying mechanism. In some experiments, the level of acriflavine-binding is increased by as much as 25% after DNA treatment.

The process of DNA entry into cells is still poorly understood. The microscopical investigations quoted above have shown that DNA (Adams et al., 1965) and DNA-protein complexes (Bensch and King, 1961; Bensch et al., 1964, 1966) are visible within pinocytotic vesicles. It seems likely, in view of the high level of nucleases in lysosomes, that these structures are DNA which is about to be catabolised, although the delayed breakdown brought

about by complexing with protein (Cocito et al., 1962) could promote DNA survival. The role of protein in the uptake of infectious viral DNA is not understood, but it is clear that there are internal virion proteins, for example in SV40 (Huebner and Lane, 1965) and Adenovirus (Russell and Knight, 1967), and there is the possibility that these proteins may play a role in the initiation of infection, whether by a nonspecific protecting effect or by specific regulatory or transcribing functions. Obviously only the first of these would be relevant to artificial DNA-protein complexes. There is some disagreement about the kinetics of entry of DNA label into cells. Reports have appeared suggesting that it is a rapid process, complete within a few minutes (Sirotnak and Hutchison, 1959; Glick, 1967; Ayad and Fox, 1968), while others (Robins and Taylor, 1968; Hill and Huppert, 1970) present data which suggests that uptake of DNA label continues for at least 24 hours. The energy requirements for uptake also remain unclear. Glick (1967) has reported a Q_{10} for the process of 3.2, while the Q_{10} determined by Mathias and Fischer (1962) was 1.1-1.4. There is evidence for two-stage uptake, but the claim that the second is energy-dependent was not substantiated. Meizel and Kay (1965) used fluoride and dinitrophenol as inhibitors of glycolysis and oxidative phosphorylation respectively, and found that the former inhibited, while the latter stimulated, uptake of DNA. The two together were inhibitory. The stimulation of DNA uptake by HeLa cells that was observed when ATP was added to the medium (Loni, 1966) has not been confirmed. The evidence presented by Loni (1966) was based on a crude classification of grain counts in autoradiographic experiments. Tiesler and Heicke (1968) have reported that the uptake of ^{3}H-DNA is diminished by azide and cyanide only to 75% of the control value in a 30 minutes uptake period, whereas it is increased by ATP, dinitrophenol and protamine. The amount of DNA taken in has been estimated as amounting to 2% of the total DNA content of the recipient cell (Glick, 1967). Denatured or partly

degraded DNA does not penetrate into cells as freely as do the native molecules (Sirotnak and Hutchison, 1959; Gartler, 1959; Sung and Quastel, 1963; Kay, 1966), and this correlates with the finding of Podgajetskaya et al (1964) that denatured DNA has no transforming activity in their system.

Biochemical approaches to the intactness of the absorbed DNA have been difficult. That macromolecular DNA does penetrate the cells is suggested by the lack of competition for uptake between ^{3}H-thymidine-labelled DNA and thymine thymidine or thymidylate (Gartler, 1959), although Hill and Huppert (1970) found in their density-labelling experiments that bromodeoxyuridine (BrdUrd) will diminish the amount of DNA label entering the cells if the label is carried as ^{3}H-thymidine, which is incorporated into DNA by the same pathway as is BrdUrd. The authors do not develop this point.

Another method which has been used in an effort to decide whether or not the absorbed DNA remains intact has been to label the donor cells with ^{14}C-formate, which will be mainly incorporated into the adenine and thymine moieties of the DNA. The ratio of the specific activities of these two bases has been found to vary according to whether the cells are labelled in vivo or in vitro (Rabotti, 1962), and this ratio can conveniently be determined by chromatography of the hydrolysis products of the labelled DNA. Rabotti (1962) used BDF_1T mouse tumour cells which were labelled with ^{14}C-formate either in vitro or while growing as a tumour on BDF_1 mice. DNA was extracted from these cells after labelling and was added to unlabelled BDF_1T cells growing either as tumours or in vitro. On reextraction and analysis of the labelled DNA, Rabotti found that the specific activity ratio stayed close to that found in the original input DNA. For example, ^{14}C-formate labelling in vitro gave an A/T ratio of 3.5, and, when this DNA was taken up by cells growing in vivo, a ratio of 5.3 was obtained, in contrast to the ratio of 0.9 found after ^{14}C-formate labelling of cells in vivo. A similar situation was found to hold for the treatment of cells in vitro with

DNA from cells labelled in vivo. Meizel and Kay (1965) carried out similar experiments with Ehrlich ascites cells and found that the adenine/thymine specific activity ratio was maintained throughout uptake, although there was some evidence of breakdown. The difficulty in interpreting such experiments is that there is a likelihood that DNA is partly degraded and reutilised for recipient cellular syntheses and is partly maintained as macromolecules. A constant specific activity ratio would be expected if the DNA all remained intact, but even partial breakdown would give rise to variations in the specific activity ratio according to the contributions made to the pools made by the degraded DNA, by the exogenous precursors and by the de novo pathways within the recipient cell. Total reutilisation of the bases released by the degradation of absorbed DNA would maintain a constant ratio, thus mimicking conservation of the input DNA. All these experiments have involved the uptake of isologous DNA, but uptake by cells of a widely differing base ratio would be more informative. Robins and Taylor (1968) found that the relative specific activities of the four bases were maintained during DNA uptake, while uptake of a hydrolysate of the same DNA demonstrated that thymidine was much more efficiently absorbed than were deoxyadenosine or deoxyguanosine. These experiments are all strongly suggestive of the uptake of intact DNA molecules, but certain assumptions about the intracellular handling of nucleotides have to be made in their interpretation, and the nearly precise maintainance of the specific activity ratios is in conflict with other reports which indicate that at least part of the input DNA is broken down (Bradley et al., 1962; Roosa, 1966; Meizel and Kay, 1965; Hill and Huppert, 1970).

Autoradiography has little information to offer on this point. The size of the DNA molecules that are used in uptake experiments is considerably less than that which would be required for the identification of complete DNA molecules, in the manner of Cairns (1962), by examination of the pattern

of silver grains in cells which have absorbed ^{3}H-DNA. What little work has been done has suggested that the DNA label passes rapidly to the cell nucleus (Tiesler and Heicke, 1968; Loni, 1966) and can be specifically removed from the fixed cells by treatment with DNase. It is not known how much of this label represents intact donor DNA and how much is the result of reutilisation of the bases.

One of the potentially most useful approaches to the investigation of DNA uptake is the use of density labelling so that donor and recipient DNA can be distinguished using equilibrium-density gradients, but it is an approach involving considerable difficulties of interpretation. The first use of bromodeoxyuridine labelling in this context was by Gartler (1960), who labelled DNA simultaneously with ^{14}C thymidine and bromodeoxyuridine. This investigator looked at the uptake of doubly-labelled DNA by L cells for periods of up to 2 hours at 37^{o}C. Unfortunately the uptake of DNA label was not sufficient to provide satisfactory count-rates on the caesium chloride gradients, and the published profiles show ^{14}C (donor DNA) peaks of only 10-12 counts/minute. The quantitative estimates of DNA uptake that the author shows must necessarily be imprecise, and there is the possibility that some components of the absorbed label were not detected.

Robins and Taylor (1968) used ^{14}C and BrdUrd-labelled DNA, which was prepared by labelling lymphoma cells with ^{14}C-formate for 65 hours and with BrdUrd for the final 24 hours. This labelling system means that the DNA preparation will consist of a major part which is labelled with BrdUrd in one strand and a minor part which is labelled with BrdUrd either in both strands or in neither. The authors state that as much as 7.5% of the total DNA is not BrdUrd-labelled. The density-gradient profiles obtained on reextracting DNA from cells which have been treated with this mixture are necessarily difficult to interpret, especially as the maximum ^{14}C count rate is around 100 counts/minute. The authors conclude that any integration of donor DNA is at a level of less than 0.4% of the recipient cell DNA

complement. What this figure in fact represents is the lower limit of sensitivity that the authors estimate the system possesses. One thing the radioactivity profiles do show is that some fully-labelled donor DNA is still present as intact, dense molecules even after uptake and reextraction.

A different labelling system was used by Ayad and Fox (1968) who worked with murine lymphoma cells labelled in vitro with ^{3}H-iododeoxyuridine (^{3}H-IdUrd). The cells were exposed to labelled ^{3}H-DNA for a few hours at which time the cells were washed and the total DNA extracted. Equilibrium-density gradients at pH7 showed that the reextracted DNA contained heavy (donor) DNA and light (recipient) DNA, both of which were detected by ultraviolet absorbance. There was also a peak of radioactivity at intermediate density, coinciding with a very small "shoulder" to the light peak on the optical density profile. This intermediate peak is not labelled with ^{14}C if uptake occurs in the presence of ^{14}C-thymidine, and density gradients of denatured DNA show a heavy, ^{3}H-labelled and a light, ^{14}C labelled component, with no radioactivity at intermediate density. The lack of ^{14}C labelling in the intermediate band in gradients of native DNA suggests that, if integration of the donor into the recipient DNA is occurring, it involves only recipient DNA which has been synthesised before uptake of the donor DNA began. There are two points of interpretation which need to be mentioned. Firstly, the authors point out that during the preparation of density-labelled DNA, the increase in cell numbers led them to expect two peaks of radioactivity on density gradients - one of DNA labelled in both strands and one of DNA labelled in only one. Their ^{3}H-IdUrd-DNA preparations showed only one band as such gradients. This was not further characterised, but most probably represents the hybrid (half-heavy) duplex. This, of course, calls into question the nature of the peak at intermediate density in the native DNA gradients, because the authors seem to regard this as a hybrid molecule. The second point of interpretation also deals with the native DNA gradient.

As the label is ^{3}H-IdUrd, the specific activity of the DNA is necessarily proportional to the density-shift away from the unlabelled position. If the ^{3}H profile is corrected by this criterion to show the amount of DNA rather than simply the count-rate, the intermediate "peak" becomes only a tail on the dense side of the recipient DNA peak. Despite such uncertainties, the paper does demonstrate survival of donor DNA and suggests that there is some form of interaction between donor and recipient DNA.

In two papers Ledoux and Huart (1967, 1969) report the remarkable finding that ^{3}H-labelled Bacillus subtilis DNA can pass through the endosperm of germinating barley seedlings and can be detected, by use of equilibrium-density gradients in DNA extracted from root cells. Not only was the bacterial DNA detectable but there was also a peak at a position between barley DNA and bacterial DNA. This intermediate material dissociated on sonication into two peaks coinciding with the bacterial and barley DNA, while it yielded only one peak on denaturation. If unlabelled bacterial DNA is supplied to a seedling, along with ^{3}H-thymidine, the label is incorporated into the material of intermediate density, while incorporation into barley DNA is depressed. Ledoux and Huart (1969) postulate a model involving end-to-end covalent joining of barley and bacterial DNA duplexes.

Hill and his coworkers (Hill and Huppert, 1970; Hill and Hillova, 1971) have investigated the fate of absorbed DNA by incubating cells simultaneously with ^{3}H-DNA and ^{14}C-BrdUrd. The period during which the cells were exposed to DNA was longer than had been previously used, being 24 hours compared with 30 minutes (Robins and Taylor, 1968) and 4 hours (Ayad and Fox, 1968) used in previous publications. This was because Hill et al. found that only a very small proportion of the donor DNA was taken up in the first few hours. After 24 hours, 22% of the donor DNA had been absorbed and was DNase resistant, so that it was probably intracellular (Schimizu, Koyama and Iwafuchi, 1962). Neutral caesium chloride gradient analysis of total recipient cell DNA after uptake showed considerable quantities of ^{3}H-label but no ^{14}C-label in the

light peak. This light peak represents intact donor ^{3}H-DNA, and is slightly denser than the unlabelled cell DNA, although this density-shift did not occur if the DNA was taken up by BrdUrd prelabelled cells in the absence of BrdUrd. Recycling the light region of the gradient resolved two separate bands. One corresponded to the donor DNA in density and was only ^{3}H-labelled, and the other had a density intermediate between donor and recipient and was both ^{3}H and ^{14}C labelled. Reconstruction experiments did not show this intermediate band, and Painter and Cleaver (1969) have shown that repair synthesis was not capable of producing such a large density-shift. Hill and Hillova (1971) carried this work further, using chick embryo cells treated with mouse DNA. They found that in the absence of DEAE-dextran all input ^{3}H-DNA activity was found in the more dense, recipient bands, while DNA absorbed in the presence of DEAE-dextran was in a broad band extending on the more dense side of unlabelled mouse DNA marker. The difference was also evident in sucrose sedimentation-velocity gradients, for DNA absorbed in the presence of DEAE-dextran did not cosediment with the donor DNA as it did after absorption in the absence of DEAE-dextran. While noting this increased persistence of donor DNA in DEAE-dextran-treated cells, the authors concentrated on untreated cells, mainly on the grounds of a slight lack of coincidence between density bands of ^{3}H and ^{14}C activity after labelling cells with ^{14}C-BUdR and ^{3}H-DNA. They did not find this displacement in cells labelled with ^{14}C-BUdR and ^{3}H-TdR. The difference was more marked after sonication and in alkaline equilibrium-density gradients, where there was evidence of the existence in the DNA of regions which were relatively rich in ^{3}H label and which the authors conclude are regions where intact donor ^{3}H-DNA molecules have been integrated into the recipient cell genome.

This and other work is further discussed in section 7.

Section 1.4: POLYOMA PSEUDOVIRIONS: NATURE AND UPTAKE

The relatively advanced state of bacterial as compared to eukaryotic cell genetics allowed the early demonstration of the encapsidation of host cell DNA in phage particles by means of the detection of the genetic changes which are produced when this host DNA is introduced into further bacterial cells by infection with the transducing particles (Zinder and Lederberg, 1952). In contrast, the demonstration of host DNA in the particles of animal viruses has been possible only by biochemical means, and has proved to be considerably more difficult. Only in the case of polyoma are there solid grounds for believing that such a process occurs, although there is also evidence that SV40 and shope papilloma behave similarly (Grady et al., 1970).

The early work on polyoma was the demonstration of a degree of sequence homology between the DNA extracted from polyoma virions and the DNA of the cell type in which the virus had been grown (Axelrod et al., 1964; Winocour, 1965). The reason for this homology was not clear but Winocour (1967) was able to show that extensive purification of the virus did not remove the component homologous to cell DNA, and that sedimentation-velocity gradients of DNA extracted from polyoma virus resolved a more slowly-sedimenting component in which the homology resided. Fractionation of heat-denatured polyoma DNA on methylated-albumen-kieselguhr columns produced an infectious DNA with no detectable homology to cellular material. In pursuing this line of work, Winocour (1968) showed that infection by polyoma virus of cells that had been prelabelled with ^{3}H-thymidine produced radioactive, haemagglutinating but noninfectious particles, in addition to the infectious particles. On extraction, the DNA from these noninfectious particles was found to have a buoyant density in caesium chloride of 1.702, a value identical to that of mouse cell DNA. It also sedimented with the "slow" component (component III) of the total polyoma DNA (Dulbecco and Vogt, 1963; Weil and Vinograd, 1963; Vinograd et al., 1965). Michel, Hirt

and Weil (1967) carried out similar experiments on labelling of the cell DNA, buoyant density and base sequence homology and obtained similar results. These experiments demonstrated conclusively that a proportion of the polyoma virions released after an infectious cycle contain a piece of cellular DNA and probably no polyoma DNA. Michel and his colleagues (1967) called these particles "pseudovirions" and this term has been generally adopted.

In any assessment of the potential genetic effects of the infection of cells with these pseudovirions, it is important to know whether the encapsidated cellular material represents a highly specific region of the cell genome or is drawn at random from the whole genome. Data presented by Winocour (1968) showed that pseudovirion DNA hybridised to cellular DNA on filters to the same extent as did whole cellular DNA. This suggests that most of the cell genome is represented. In the same paper, Winocour estimates that 16-21% of the total population of "full" (i.e. DNA-containing) polyoma virions released after an infectious cycle are pseudovirions, and that the molecular weight of the encapsidated DNA is 3×10^6 daltons, which is very close to that of infectious polyoma DNA. Thus, each pseudovirion carries a single piece of cellular DNA of a size sufficient to carry 6-8 "average" genes, and apparently representing most parts of the host-cell genome.

The mechanism by which polyoma pseudovirions are produced remains obscure. Reports have appeared (Ben Porat et al., 1966; Ben Porat and Kaplan, 1967) which demonstrate the degradation of cellular DNA, when it has replicated after polyoma infection, to pieces which elute from methylated-albumen-kieselguh columns at a point characteristic of DNA with a molecular weight equal to that of viral DNA. A later paper (Cheevers et al., 1970) suggested, however, that the abnormal cellular DNA is less homogeneous in molecular weight than is polyoma DNA. The two groups do, however, agree that the appearance of host cell DNA in the relatively small molecular weight range is linked with the onset of viral DNA replication. It is still not firmly established

whether this small molecular weight material arises by degradation of cellular DNA or is synthesised de novo, but evidence strongly suggests that its formation is linked with production of pseudovirions. The appearance of ^{3}H-DNA in pseudovirions after growth of polyoma in prelabelled cells indicates that some at least of the pseudovirions contain DNA which has been synthesised prior to infection (Winocour, 1968; Osterman et al., 1970).

It is probable that the genetic potential of polyoma pseudovirions has received wider attention than the literature suggests. Certainly their potential is widely appreciated (e.g. Winocour, 1968; Tatum, 1969). Hirt and his colleagues have attempted to demonstrate genetic changes induced by polyoma pseudovirions, but without success (Personal communication).

Two papers have been published by Aposhian and coworkers (Osterman et al., 1970; Qasba and Aposhian, 1971) on their investigations of the intracellular fate of radioactively labelled DNA from pseudovirions after infection of cells which are permissive for the replication of polyoma virus. The pseudovirions were isolated by making use of the slight difference in buoyant density between them and normal polyoma virions. The procedure involved repeated equilibrium centrifugation in caesium chloride, with the less dense portion of the virus band being retained at each stage. The final pseudovirion preparation had a particle-infectivity ratio of 10^{6}, although the particles were all "full" as assessed by electron microscopy. In the first of the papers, it was shown that, after infection, 6% of the absorbed virions were uncoated, the criterion for uncoating being the conversion of the DNA into a DNase-sensitive form. The second paper deals with the infection of mouse embryo cells with pure pseudovirions from a polyoma stock grown in mouse embryo cells. It is shown that the label from pseudovirion DNA becomes DNase-sensitive and is associated with the nuclear fraction of the cells. It is also demonstrated that the labelled material after infection will hybridise with mouse embryo DNA. The authors state

that this demonstrates that the labelled material is pseudovirion DNA, but there seems no reason to suppose that it is not mouse embryo DNA that has been synthesised using the breakdown products from degraded pseudovirion DNA. All that is shown is that the nuclear label is not polyoma virus DNA. Indeed, in further experiments, involving the infection of human embryo cells with polyoma pseudovirions, it is shown by sedimentation velocity experiments that the labelled nuclear material is very heterogeneous. The authors also claim that they demonstrate the intranuclear uncoating of pseudovirions in human cells, but they do this by means of sucrose velocity-gradients under conditions that could release the DNA from intact pseudovirions.

The situation at present is that there is no solid evidence that the DNA from polyoma pseudovirions can be introduced into cells in a form that could be genetically active. The demonstration of uncoating in permissive cells is not unexpected and to date the scanty biochemical work in nonpermissive cells has not been either stimulated or supported by any demonstration that the encapsidated mouse genes can in fact be functional.

— TWO —

TECHNIQUES

Section 2.1: CELL CULTURE METHODS

All cell lines were routinely grown in monolayer culture in 20 oz. medical flat bottles or in 80 oz. flint glass Winchester bottles on roller culture racks. Experimental cultures were in 4 or 6 oz. medical flats or in 3 or 5 cm. Nunclon disposable plastic petri dishes. Suspension cultures were grown in the type of vessel illustrated in fig. 2.1.

Glassware washing procedures

All glassware used in cell culture was immersed in 3% chloros immediately after use and left to soak overnight. They were then put in calgon-metasilicate solution. Winchester bottles were left in this solution overnight at room temperature before thorough rinsing in several changes of tap water and of deionised water. Other cell culture glassware was boiled for 20 minutes before rinsing. All glassware was sterilised by autoclaving at 15 p.s.i. for 15 minutes, checking this by means of Browne's sterility control tubes.

Coverslips for cell culture were prepared for use by boiling in 3% chloros and thoroughly rinsing in 3 changes of distilled water. They were then rinsed once in absolute alcohol and left to dry at room temperature. They were placed in petri dishes and wrapped in aluminium foil before being sterilised in a hot air oven.

Media

All cell cultures were grown in Eagle's medium in the Glasgow modification, with double the original concentrations of vitamins and amino acids. Medium was dispensed in 160 ml. aliquots and stored at $4^{\circ}C$. For BHK21/C13 or PyY cells, this defined medium was supplemented with 1/9 volume of calf serum, which was prepared from calf blood obtained from the local abattoir and sterilised by filtration through a millipore disc of 0.22μ pore size. The mutant cell lines grew rather better when foetal bovine serum was used although they did not have an absolute requirement for it. For example,

there was little difference in plating efficiency. Foetal bovine serum was bought from Flow Laboratories Ltd., Irvine or from Biocult Laboratories Ltd., Paisley.

For subculture, cells were detached from the substrate using a trypsin-versene solution made up by mixing 1 part of 0.25% Difco trypsin in tris buffer with 4 part of 0.02% EDTA disodium salt (British Drug Houses Ltd.) in phosphate-buffered saline, pH 7.3. The solutions are made up separately, the trypsin being filter-sterilised and stored at -20°C, and the versene being sterilised by autoclaving and stored at room temperature.

Other standard solutions:

Tris-saline; table 2.1.

Phosphate-buffered saline; table 2.1.

Saline; 9 gm./1. sodium chloride.

Preparation of cells for storage

Cells for long-term storage at low temperature (-80°C in a Revco freezer) were suspended using trypsin-versene and thoroughly washed with Eagles-10% calf serum (EC10).

After the final wash, the cell pellet was suspended in the following medium at about 2.10^6 cells/ml.

Eagles medium	75% by volume.
Calf serum	20%
Glycerol	5%

The cell suspension was placed in screwtopped vials in 1 ml. amounts and brought slowly to -80°C at which temperature they were stored. They were recovered by warming rapidly to 37°C and seeding the contents of one vial into 9 ml. of Eagles-10% calf serum in a 2 oz. medical flat. The medium was removed 12 hours later, the cells were rinsed in EFC10 or EC10, and incubation continued after addition of 10 ml. EC10 or EFC10.

The cell lines are fully described in section 3.

Table 2.1

TRIS-SALINE, pH7.4

Sodium chloride	8 gm/1.
Potassium chloride	0.38 gm/1.
Disodium hydrogen phosphate	0.10 gm/1.
Dextrose	1 gm/1.
Tris*	3 gm/1.

Phenol red 0.0015%; penicillen 1000 units/1.; streptomycin 0.1 gm/1.

Adjusted to pH7.4 with hydrochloric acid.

* = tris (hydroxymethyl) aminomethane.

PHOSPHATE-BUFFERED SALINE (PBS)

solution A;	
Sodium chloride	10 gm/1.
Potassium chloride	0.25 gm/1.
Disodium hydrogen phosphate	1.44 gm/1.
Potassium dihydrogen phosphate	0.25 gm/1.

pH7.2

solution B; calcium chloride	1 gm/1.
solution C; magnesium chloride	1 gm/1.

"Complete" PBS is A:B:C:8:1:1 by volume.

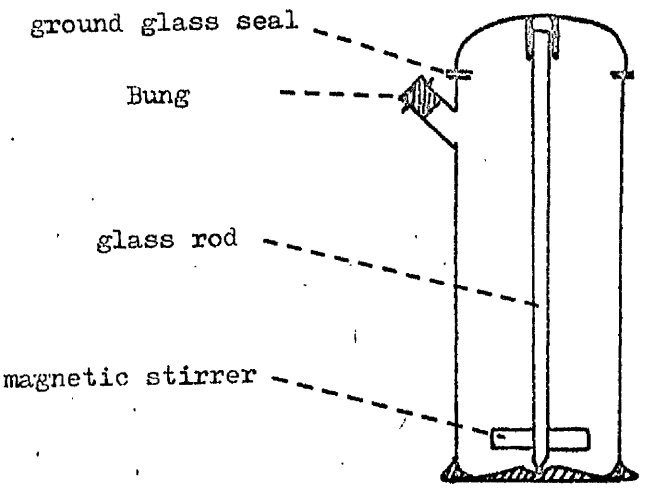


Fig. 2.1
Vessel used for cell suspension culture.

Table 2.2: Reagents for Mycoplasma stain.

0·6% (w/v) sodium citrate.

Carnoy's fixative; glacial acetic acid, 1 vol. in absolute ethanol, 3 vols..

2% Gurr's orcein in 60% glacial acetic acid.

Culture methods

Cells were routinely grown in medical flats or roller bottles which were fitted with screw tops. These were either plastic or metal with silicone liners so that the tops provided a gastight seal which meant that the atmosphere inside the bottle could initially be kept at a level of carbon dioxide sufficient to maintain the correct pH in the medium. The 20 oz. medical flats were seeded with 2-5 x 10^6 cells in 50 ml. Eagles-10% calf serum and contained 25-40 x 10^6 cells when confluent, the variation being between different cell types. The PyY/TG/CAR/BUDR cells needed to be seeded more thickly than this, and each 20 oz. bottle received about 8 x 10^6 cells. The 80 oz. roller culture bottles were inoculated with 20 x 10^6 cells in 200 ml. of medium and yielded 2-4 x 10^8 cells when confluent. All cell cultures were maintained at 37°C.

The transformed cell line, PyY, and its derivatives have the ability to form colonies when suspended in soft agar containing Eagle's medium and calf serum. The nontransformed line, BHK21/C13, does not have this ability. Agar suspension culture is a convenient method of cloning cells, and can also be used to isolate uninfected cell lines from mycoplasma-contaminated cultures. The procedure is as follows.

Basal agar is made up. It contains:-

1.3 x Eagles medium	4 volumes
calf serum	1 volume
distilled water	1 volume

This is held at 44°C and mixed with 1.5 volumes of 2.5% agar at 44°C. This medium has a final agar concentration of 0.5%. It is dispensed into 5 cm. petri dishes at 7.5 ml. per dish and allowed to solidify. The distilled water component can be made up to contain any antibiotics or selective agents to which the cells are to be exposed. The overlay medium, into which the cells are to be seeded, is made up in aliquots containing

2 ml. of 1.3 x Eagles medium and 0.5 ml. of 1.6% agar. This is maintained at 44°C in bijou bottles, to which is added the appropriate number of cells in 0.1 ml. of Eagles medium, and the contents are mixed and poured over the basal medium. For a 5 cm. dish, 10^3 cells is a convenient number because it usually gives a sufficient yield of well-dispersed colonies under nonselective conditions. Of course, the actual number of cells used in particular cultures varied according to the conditions under which the cells were to be grown.

After about a week at 37°C in an incubator with a humid, 5% carbon dioxide atmosphere, the colonies could be picked out of the agar using a finely-drawn glass pasteur pipette and ejected into petridishes containing growth medium. This procedure gives reliable cloning provided that the original cell inoculum was a single cell suspension and that the colonies are well dipersed.

Mycoplasma Contamination

The danger of the contamination of tissue cultures by Mycoplasma has been recognised in recent years (McPherson, 1966) and every effort was made to ensure that the cells used in the present study were Mycoplasma-free.

The method used for the detection of Mycoplasma infection was the aceto-orcein method of Fogh and Fogh (1964). In this, the cells are grown on coverslips in petridishes until they are nearly confluent. At this point, the medium is poured off and replaced with 3 ml. of 0.6% sodium citrate, which is slowly diluted with distilled water until the concentration is 0.45%. This slow dilution is necessary to avoid the bursting or detachment of the cells. After 10 minutes if this hypotonic treatment, 4 ml. of Carnoy's fixative (table 2.2) is added and the mixture immediately discarded, to be replaced with a further 2 ml. of Carnoy's fixative in which the cells are left for 10 minutes. The coverslips are then removed to dry petri-dishes, in which they are left to dry at room temperature before staining

for a few minutes with a drop of orcein. The stained coverslips are washed in ethanol and thoroughly dried. They are mounted in euparal and inspected under phase-contrast optics at magnifications of 400x and 1000x.

Using this technique, Mycoplasma are detected as darkly-stained granules scattered over the surface of the cells, which are stained a light purple.

In developing the technique, Fogh and Fogh found complete correlation between it and detection of Mycoplasma by the more laborious and slower culture methods. Over a number of years, it has been in use in this institute and has been found to give reliable and rapid detection of even very light infection.

Mycoplasma are also evident in autoradiographs of infected cells which had been exposed to tritiated thymidine. They give rise to labelling in the cytoplasm and at the periphery of the cells. Thymidine-5-^{3}H is a highly specific DNA label so that uninfected cells display only nuclear labelling. Autoradiographs of cells infected with Mycoplasma and exposed to other labelled nucleic acid precursors which are incorporated in the cellular RNA as well as into the DNA will not show the presence of the Mycoplasma-directed incorporation because this will be masked by the heavy cytoplasmic labelling. One interesting observation was that mutant cells exposed to a labelled nucleoside that they were unable to incorporate showed no cytoplasmic or nuclear label even when they were known to be infected with Mycoplasma. This could be because the synthesis of Mycoplasma components was taking place only at a level not detectable in the autoradiographs, or because the enzymes of the scavenger pathways are not encoded in the Mycoplasma genome. This observation was not extended.

The steps taken when a particular cell line was found to be infected with Mycoplasma depended on the supply of that cell line that was available. If the line was one of which a plentiful frozen stock was in store, the

infected cultures were simply discarded and replaced with a stock freshly recovered from the freezer. In the case of a cell line which was in short supply, or if the infection was very light, the agar suspension cloning method (p. 33) was used, with the inclusion of antibiotics in the basal agar, to allow the isolation of uninfected clones. Routine inclusion of antibiotics against Mycoplasma in the culture media was not thought to be wise, and it was found that it was not sufficient to simply grow the infected cells in the presence of the antibiotics for two or three passages. Such a procedure merely suppressed the infection. In the agar cultures the following antibiotics were used together:-

Kanamycin	500µg/ml
Novobiocin	50µg/ml
Aureomycin	5µg/ml

Colonies were picked and grown up for retesting. In all but the heaviest infections, there was little difficulty in obtaining uninfected cells.

Section 2.2: LIQUID SCINTILLATION COUNTING

2.2a: Equipment

Radioactivity assays were carried out using liquid scintillation counting techniques. Three counters were used in the course of the project. They were:-

1. Nuclear Chicago Mark 1.
2. Intertechnique model SL40.
3. Philips Liquid Scintillation Analyser, which was used in all the double label experiments.

2.2b: Scintillant systems

Two scintillation fluids were used. All the experimental work was carried out using a toluene-based fluid with the following composition:-

PPO (2,5-diphenyloxazole)	5 gms.
POPOP (p-bis-2-(5-phenyloxazolyl)-banzene)	0.3 gms.
Toluene	1 litre

PPO and POPOP were obtained from Koch-Light Ltd. and the toluene was Analar grade.

Toluene-based fluids have the disadvantage that they are not miscible with aqueous samples, so that assays in this fluid were carried out with the samples dried on filter paper discs. This technique is described fully below (section 2.2e).

The second scintillation fluid used was Bray's fluid. This was used in some of the early experiments and for determination of DNA specific activity throughout. Bray's fluid was made up to the following formula:-

PPO	4 gm.
POPOP	0.2 gm.
Naphthalene	60 gm.
Methanol	100 ml.
Ethane-diol	20 ml.

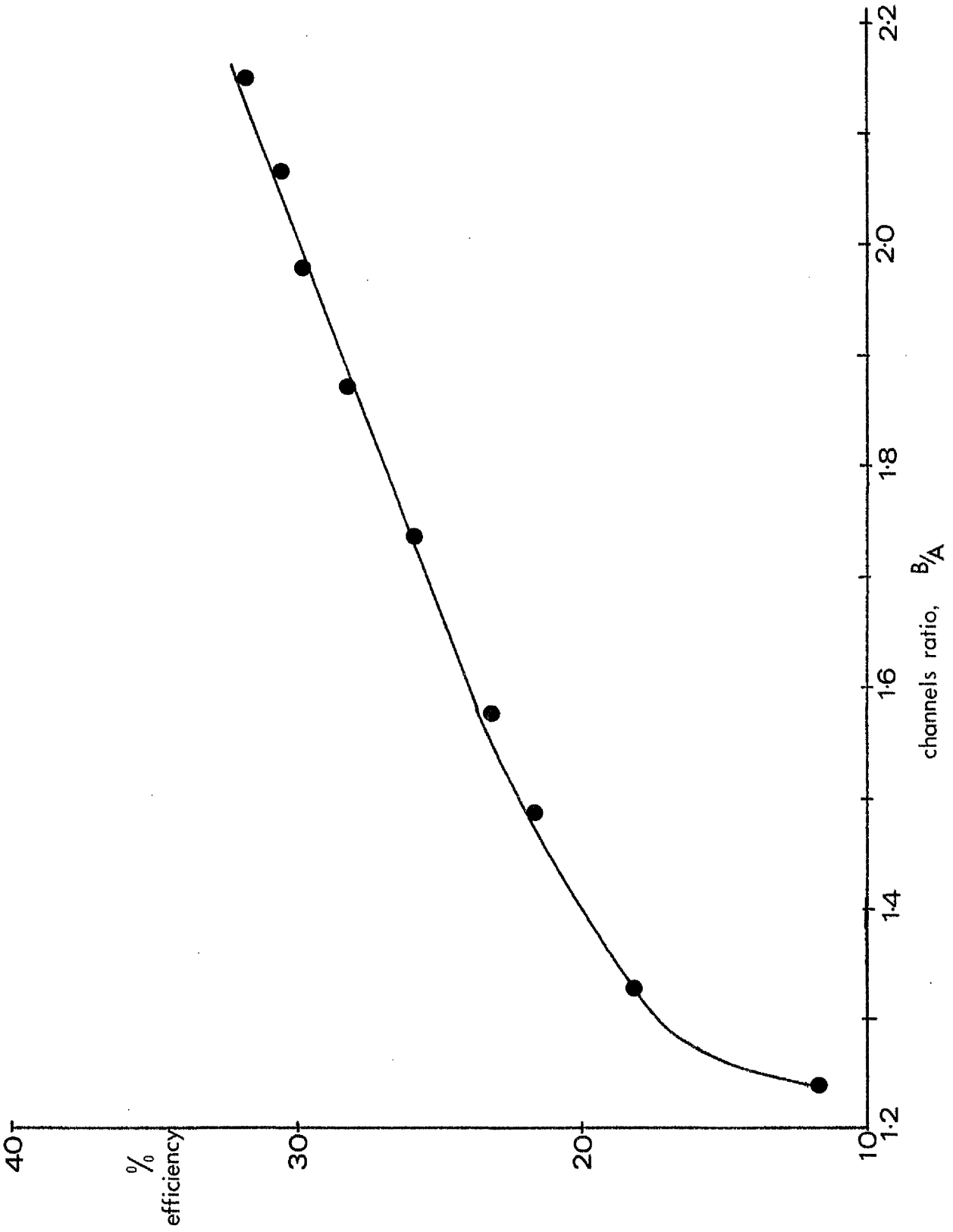


Fig.2.2 Quench correction curve. Water quench in Bray's scintillant.

Dioxane	880 ml.

The dioxane was obtained from Koch-Light Ltd., and was stored at room temperature in dark bottles under an atmosphere of pure nitrogen.

2.2c: Quench correction and efficiencies of ^{3}H detection

The specific activities of tritiated DNA preparations were determined using Bray's fluid. Quench correction was carried out using a channels ratio method with tritiated hexadecane (Radiochemical Centre, Amersham) of accurately-known specific activity as a standard. This method is not applicable to the system using disc-borne samples because of the heterogeneous nature of the disc system. This is discussed more fully below. In the channels ratio procedure, a series of vials were prepared containing 10 ml. of Bray's fluid and 50µl. of ^{3}H-hexadecane. The specific activity of the hexadecane had been determined by the manufacturer and the activity at the time of use was calculated from this figure plus the date at which it had been determined and a correction factor based on the decay curve of tritium.

The quenching material was water in volumes ranging from 0 to 2.0 ml. Channels A and B of the counter were set so that the ratio $\frac{\text{counts in channel B}}{\text{counts in channel A}}$ was approximately 2.2 for the unquenched sample. Channel C was set to the balance point for maximum tritium count-rate. The total count rate, from channel C, and the ratio of counts, B/A, was found over a 10 minute period for each of the set of water-quenched vials, and these values were plotted graphically to give a curve such as that shown in fig. 2.2.

If a sample of unknown absolute specific activity is counted using the same channel settings, the ratio B/A obtained for it may be fitted onto the curve to find the efficiency of counting, and the absolute activity in disintegrations per minute can be calculated from this and the total count-rate in channel C.

Table 2.3 Radioactive compounds.

All the radioisotope-labelled compounds were obtained from The Radiochemical Centre, Amersham.

Pyrimidines.

thymidine-methyl-^{3}H	TRK120	17-25 Ci/mmole
deoxycytidine-5-^{3}H	TRK211	13-16 Ci/mmole
uridine-5-^{3}H	TRK178	30 Ci/mmole
thymidine-2-^{14}C	CFA219	59 Ci/mmole

Purines.

hypoxanthine-^{3}H(G)	TRA 74	0.5 Ci/mmole
guanosine-^{3}H(G)	TRA 39	0.8-1.2 Ci/mmole
adenine-2,8-^{3}H	TRK 23	2.0-4.3 Ci/mmole
adenosine-^{3}H(G)	TRK 3	8.4 Ci/mmole

The specific activities of DNA-^{3}H preparations were determined as follows.

0.1 ml. of DNA-^{3}H solution of known concentration was mixed with 0.1 ml. of concentrated formic acid and held at 100^{o}C for 30 minutes in a small, tightly-closed vial. The hydrolysate was diluted to 5 ml. with distilled water, so that the concentration of formic acid (about 2.) is sufficiently small to prevent it from diminishing the counting efficiency. To vials containing 10 ml. of Bray's fluid was added 0.5 ml. of the diluted hydrolysate. A quench curve was constructed for each fresh batch of Bray's fluid and carried out in paralled with the 3_{H}-DNA determination. Values for the specific activity of ^{3}H-DNA preparations are found in section 4.1.

2.2d: Efficiency of counting in the disc sample system

Once its specific activity had been determined, a stock of ^{3}H-DNA could be used to find the efficiency of counting of toluene-based fluid with the sample dried onto a filter paper disc. This was done by applying 100µl. of ^{3}H-DNA to a disc, drying it thoroughly and counting at balance point settings for four minutes.

It was found that only a very small volume of scintillation fluid was needed to give near-maximum efficiency (fig. 2.3). This is in line with the experience of Davies (1966) and De Recondo and Fichot (1969). In all subsequent experiments 5 ml. of scintillant was used.

The lack of a convenient method for monitoring the counting efficiency of individual samples is a drawback of this system of radioactivity assay, so an attempt was made to define the limits within which constant efficiency can be assumed. Discs receiving ^{3}H-DNA alone gave reproducible counting efficiencies varying from 22.5-24% between different batches of fluid. De Recondo and Fichot (1969) reported that up to 500µg. of protein could be carried on the disc without giving rise to significant self-absorption, but in the present system discs loaded with DNA-^{3}H and varying amounts of

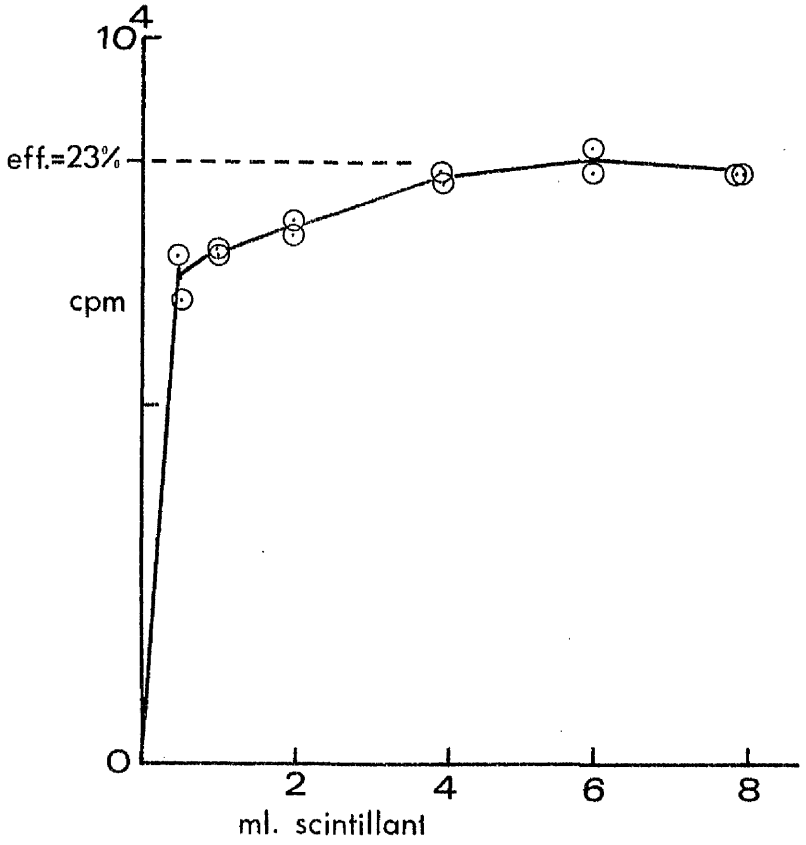


Fig 2.3 Effect of scintillant volume on count-rate of a ^{3}H-DNA sample dried onto a filter paper disc. Toluene-PPO-POPOP fluid.

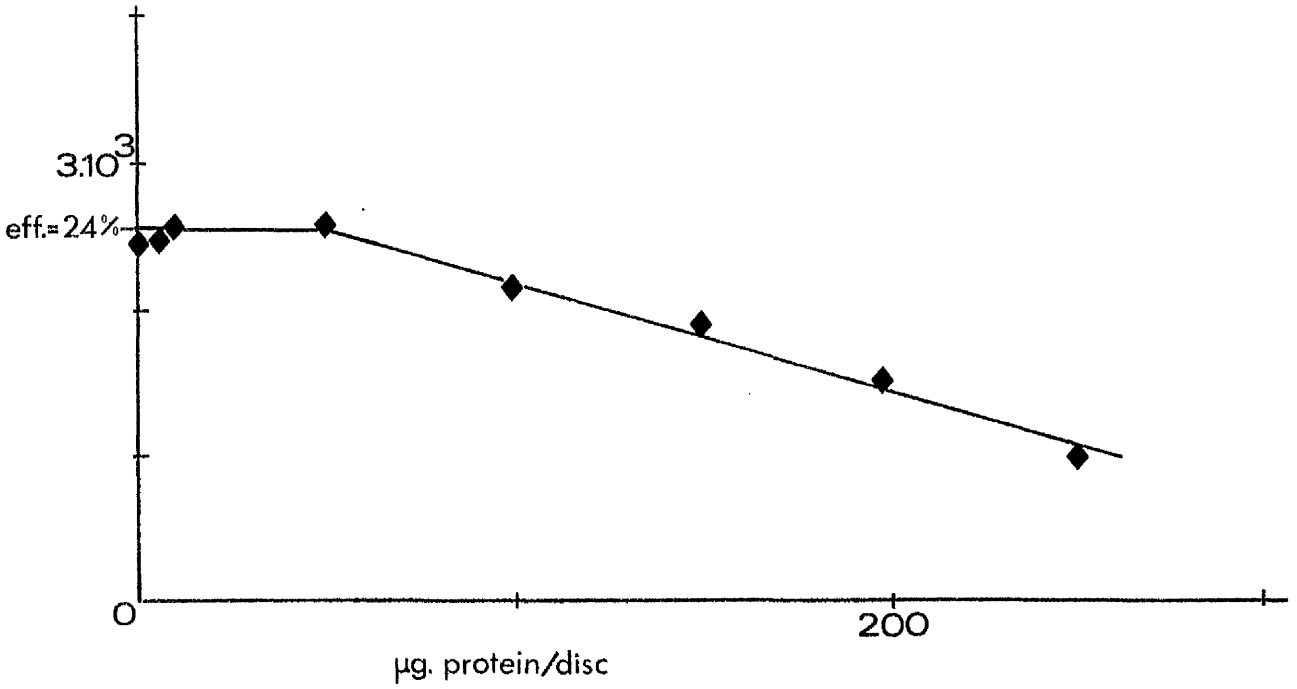


Fig.2.4 Effect of bovine plasma albumen on the count-rate of a ^{3}H-DNA sample dried onto the same disc. Toluene-PPO-POPOP.

bovine plasma albumen showed that 150µg. of protein was sufficient to lower the counting efficiency while 250µg. was enough to decrease the efficiency to 10% from the unquenched level of 24% (fig. 2.4). As the amount of protein on the discs under experimental conditions will certainly not exceed 10µg., the effects of self-absorption can be neglected. As a test of this, ^{3}H-DNA was dried onto discs with varying numbers of intact cells, and subjected to the standard Trichloracetic acid (TCA) and ether-washing procedure. Within the limits tested, that is between 0 and 2 x 10^{5} cells/disc, there was no detectable self-absorption. The actual figures were:-

^{3}H-DNA, 1µg./disc	no cells.	1.88×10^{3} cpm.*	efficiency = 22%.
	2×10^{5} cells	1.90×10^{3} cpm.*	efficiency = 22%.

* = mean of triplicate discs.

These experiments show that, while absolute activities cannot be reliably determined for disc-borne samples, no correction for counting efficiency is necessary in comparing samples in any particular experiment.

2.2e: Sample preparation routine

Whatman No. 1 filter papers of 2.4 cm. diameter were most conveniently handled by impaling them on steel pins set vertically one inch apart on an aluminium plate, each disc being identified by means of a number written on it in soft pencil. 50 or 100µl. samples were applied to each disc using an automatic pipette, and dried under an infra-red lamp. All discs were then washed twice in 5% trichloroacetic acid (TCA) at +4°C, once in ethanol at room temperature, and twice in diethyl ether at +4°C. After thorough drying under the infra-red lamp, the discs were placed in screwtop vials and counted under 5 ml. of toluene-PPO-POPOP scintillant.

In some of the experiments involving sucrose gradients, the level of radioactivity was so low that 100µl. was not a large enough aliquot to give satisfactory count rates, but instead of loading several discs with

aliquots from the same sample and counting them simultaneously in the same vial the fractions were adjusted to 5% TCA and left at +4°C for several hours. The precipitates were collected on millipore filter discs of 0.22μ or 0.45μ pore size by suction filtration and dried before washing in TCA and ether. The dried discs were assayed for radioactivity in the same way as the filter paper discs.

2.2f: Double label counting

This was always done using the Philips Liquid Scintillation Analyser, which has the computing facilities to enable it to calculate and print out the ^{3}H and ^{14}C count rates. All the calculations were based on the assumption that the counting efficiency did not vary from sample to sample. The evidence to justify this assumption has been discussed in section 2.2d.

Three channels of the Philips LSA were used. Channel 1 was the ^{14}C detector and channel 3 the ^{3}H detector, while channel 2 was used in the computation of the ^{14}C spillover into the ^{3}H channel. The counter was previously calibrated to give balance point counting of single isotope samples under the same conditions of quenching as prevailed in the experimental samples. The ^{14}C channel discriminators were set to totally exclude ^{3}H and the proportion of ^{14}C found in the ^{3}H channel was found by repeated counting of a disc loaded with ^{14}C alone. The settings of the various parameters are shown in table 2.4. The setting of channel 3 was such as to give maximum ^{3}H efficiency, as determined by repeated counting of a ^{3}H sample, but with as narrow a window as possible.

In carrying out the necessary calculations, the counter makes use of three computer boards, each of which carries out the same form of calculation. This is calculation mode 4 (see Philips manual), and is in effect the application of a quadratic equation which is intended to be fitted to a quench correction curve for the assay of samples of varying quench, and is of the form $px^2+qx+r=y$. In conditions of uniform quench and when the

Table 2.4 Philips LSA settings for $^{14}C/^{3}H$ dual label counting on discs.

Programme board	calculation mode 4.
Computer board 1	p = 0 q = 0 r = +1
	background = 10 cpm.
	N = 0 next board = 2
Computer board 2	p = 0 q = 0 r = +0.612
	background = 0
	N = 0 next board = 3
Computer board 3	p = 0 q = 0 r = +1
	background = 35 cpm.
	N = 0 next board = 3

CHANNEL	ATTENUATOR	DISCRIMINATORS
1	3.0	2.4-10.0
2	3.0	2.8-10.0
3	1.2	0.4- 6.0

calculation of disintegrations per minute is not required, these coefficients are set as in table 2.4. The outline of the calculation is as follows.

Computer board 1: Takes the ^{14}C count rate, corrects it for background, and feeds it to the print out. Channel 1 = ^{14}C cpm.

Computer board 2: Takes the ^{14}C count rate and, using the set value of r (table 2.3), calculates the amount of ^{14}C which will be found in the third channel. This value is fed to the third computer board.

Computer board 3: Takes the ^{14}C value from board 2 and subtracts it from the detected ^{3}H count rate. The result is corrected for background and fed to the print out.

Channel 3 = ^{3}H cpm.

Section 2.3: EXTRACTION OF DNA

The method used routinely was a modification of that described by Marmur (1961). Confluent monolayers of BHK21/C13 or PyY cells were grown in 80 oz. roller culture bottles, and each such bottle contained 2-3 x 10^8 cells. This number of cells should yield 2.0-2.5 mg. of DNA. The reagents are described in table 2.5.

Extraction Procedure

1. Medium was removed from the bottles and the cell monolayers were washed in isotonic saline. To each bottle was added 0.2 or 0.4 ml. of 20% SDS and 10 ml. of EDTA-saline. The bottles were swirled so that the cells were thoroughly rinsed in the SDS solution while still attached to the glass. This procedure was adopted because of the difficulty in ensuring that all the cells in a pellet are lysed. The formation of clumps of cells can result in incomplete lysis and consequent reduction in the yield of DNA. The cells in monolayers are not protected in this way and the addition of SDS usually produced rapid lysis, which was visible as a considerable increase in the viscosity of the liquid in the bottles and the detachment of all cellular material from the glass.

2. The lysate was poured into a conical flask and the culture bottles rinsed once in EDTA-saline, the rinsings being added to the same flask. The pooled cell lysate was heated to 60°C in a water bath for 20 minutes.

3. The lysate was cooled to room temperature and the SDS concentration adjusted to 0.1-0.2% by addition of EDTA-saline. Pronase was added to a final concentration of 50µg/ml and the mixture incubated at 37°C for 30 minutes. This step was added to the procedure of Marmur (1961) because the heavy protein interface material in the first chloroform extraction often appeared to trap a considerable amount of DNA. Pronase digestion improves DNA yields by reducing the quantity of material that is precipitable at the interface.

Table 2.5 Reagents for Marmur extraction.

SDS: 20% (w/v) Analar sodium dodecylsulphate in distilled water.

EDTA-saline: 0.1M Analar EDTA (disodium salt)
0.15M sodium chloride pH 7.8

Ribonuclease: Sigma pancreatic ribonuclease. Stored at -20°C as solution in distilled water at 2 mg/ml. boiled before use.

Chloroform-isoamyl alcohol: 24:1 (v/v).

SSC: 0.15M sodium chloride
0.015M sodium citrate

4. After cooling, 1/4 volume of 5M. sodium perchlorate was added to bring the salt concentration to approximately molar.

5. The first deproteinisation step was a gentle shaking at 4°C with 1 volume of chloroform-isoamyl alcohol for 15 minutes. The mixture formed two layers on standing, the lower layer being organic. After centrifugation of the mixture at 10,000 rpm in the 10 x 50 fixed-angle rotor of an MSE18 centrifuge, the upper (aqueous) layer was collected using a wide-bore, hooked pasteur pipette, and the nucleic acid was precipitated by addition of 2 volumes of absolute alcohol at -10°C. The precipitate was collected by spooling on a glass rod, and redissolved by gentle shaking overnight in SSC/10 at 4°C. The salt concentration was then adjusted to SSC by the addition of 1/9 volume of 10 x SSC.

6. Ribonuclease was added to a final concentration of 50µg/ml and the solution was incubated at 37°C for 15 minutes.

7. The deproteinisation step (no. 5) was repeated twice.

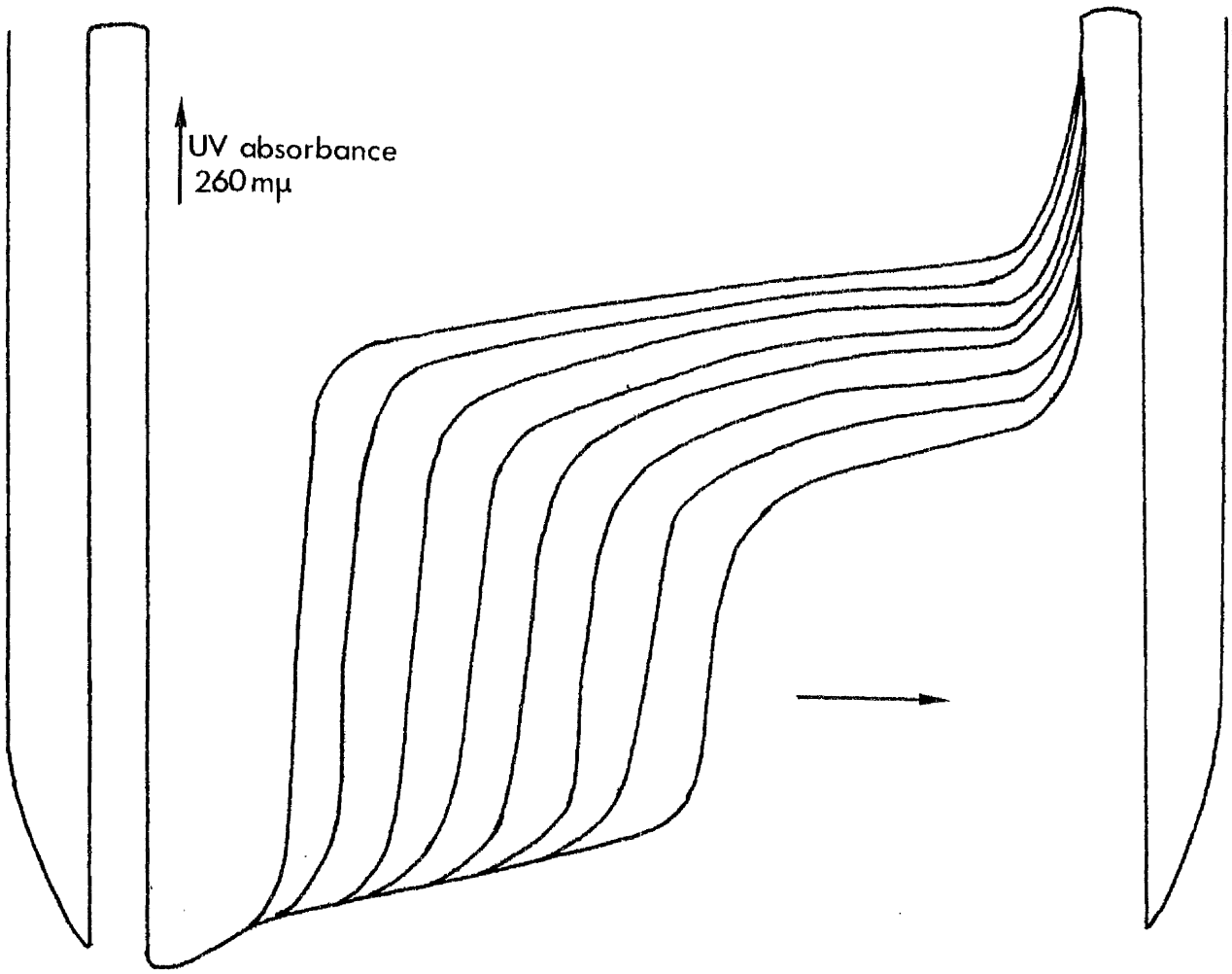
8. The solution in SSC was diluted and its ultraviolet absorption spectrum was read in a Perkin-Elmer recording spectrophotometer. The UV spectrum was used as an index of:-

i. DNA concentration; using an equivalence of 1 O.D. unit to 40µg of DNA. The stock solution was diluted to give an absorption of approximately 1 unit, and the concentration of the stock calculated from this and the dilution factor.

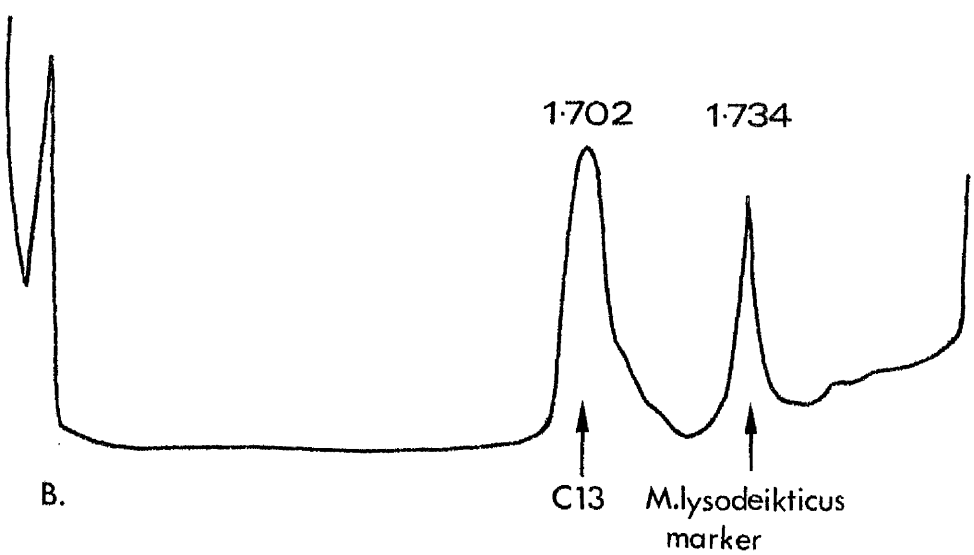
ii. DNA purity; by calculating the ratio $E_{260}:E_{280}$, which has a value of about 1.90 for pure DNA. Protein contamination will increase the relative E_{280}.

On the basis of this latter criterion, one more deproteinisation step was usually necessary. Each bottle yielded about 2 mg. of DNA, although bottles in which the cells were approaching senescence gave very much less than this.

The typical DNA preparation from this procedure had a sedimentation coefficient in the 22-28S range (fig. 2.5a), which corresponds to a molecular weight of 12-16 x 10^6 daltons. These values were obtained using a Spinco model E analytical ultracentrifuge at 44,770 rpm, using UV optics with automatic exposures at 4 minute intervals. The samples were 40µg/ml DNA in SSC. Analytical caesium chloride gradients with a starting density of 1.710 gm.cm.$^{-3}$ were brought to equilibrium at 44,770 rpm for 19 hours and showed a peak at ρ = 1.702 gm.cm.$^{-3}$ (fig. 2.5b), relative to Micrococcus lysodeikticus DNA (ρ = 1.734 gm.cm.$^{-3}$).



A.



B.

Fig.2.5 Analytical ultracentrifuge analysis of Marmur-extracted DNA.
A. Sedimentation-velocity; 44,770 rpm in Spinco model E. Optical density traces of negatives from UV absorbance; exposures at 4 min. intervals.
B. Equilibrium-density; 44,770 rpm for 19hr. in caesium chloride of starting density 1·710. UV absorbance profile.

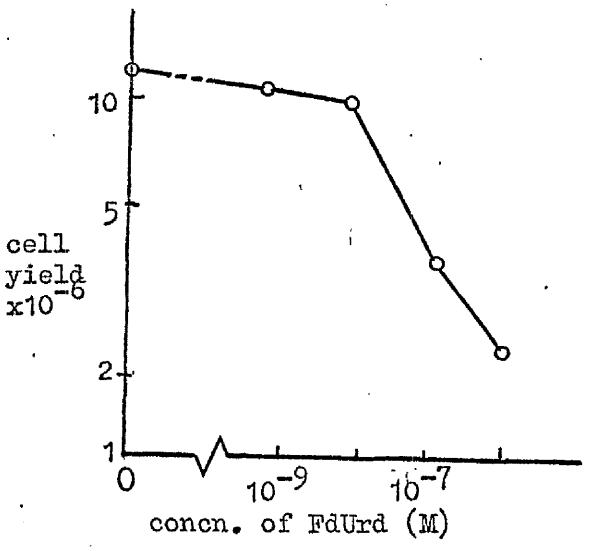


Fig. 2.6; Toxicity of FdUrd for PyY cells. 5 days growth in EC10 with the indicated concentrations of FdUrd. Total cell yield found by counting cells suspended in a known volume of medium.

Section 2.4: PREPARATION OF LABELLED DNA

Tritiated DNA was prepared by labelling cells over a period of days with thymidine-methyl-^{3}H (Radiochemical Centre, Amersham; see table 2.3). Two factors are relevant to the preparation of high specific activity DNA by this method.

1. Exogenous ^{3}H-thymidine is likely to be diluted with unlabelled thymidylate produced by the following pathway:-

$$CDP \longrightarrow dCDP \rightleftharpoons dCMP \rightleftharpoons dUMP \xrightarrow{\text{thymidylate synthetase}} dTMP$$

The final step, carried on by thymidylate synthetase, is inhibited by fluorodeoxyuridine (FdUrd). Such inhibition should lead to a higher degree of dependence on the exogenous thymidine, and hence to an increase in its inforporation into DNA.

2. Tritiated thymidine has itself a marked cytotoxic effect when it is present at high specific activity (Rauth, 1968).

FdUrd is an extremely toxic analogue, and produces inhibition of cell growth even at very low concentrations (fig. 2.6). This toxic effect can be reversed by the addition of thymidine, which is the substance of which the cells are starved as a result of the inhibition of thymidylate synthetase by FdUrd. The reversal is a direct result of the alleviation of this deficiency, and does not reflect any diminution of the enzyme inhibition. The concentration of thymidine required to allow cells to grow in the presence of toxic concentrations of FdUrd was determined by growing cells in EC10 in the presence of 10^{-7}M FdUrd, and with the addition of various concentrations of thymidine. After 4 days, the total number of cells on each plate was determined. The results are shown in fig. 2.7, from which it can be seen that 5-10µg/ml (20-40µM) thymidine is required for the cells to grow at the same rate as the controls (no FdUrd). Preliminary experiments also revealed that the BHK21 and PyY cell lines were subject to considerable inhibition of growth by the ^{3}H-thymidine (Amersham code number, TRK120; specific

Fig 2.7; Reversal of the toxicity of FdUrd by thymidine in the medium. Cells were inoculated into petri dishes at $5x10^4$ cells per dish and grown for five days in the thymidine concentrations shown on the graph. The medium was EC10. Cell yields were found by counting the total number of cells per dish, and are shown $x10^{-6}$.

No FdUrd △

10^{-7}M FdUrd ▲

Fig. 2.8; Reversal of the cytotoxic effect of ^{3}H-thymidine by thymidine in the medium. Details are as in fig. 2.7.

Control; no ^{3}H-TdR ○

2μCi/ml ^{3}H-TdR ●

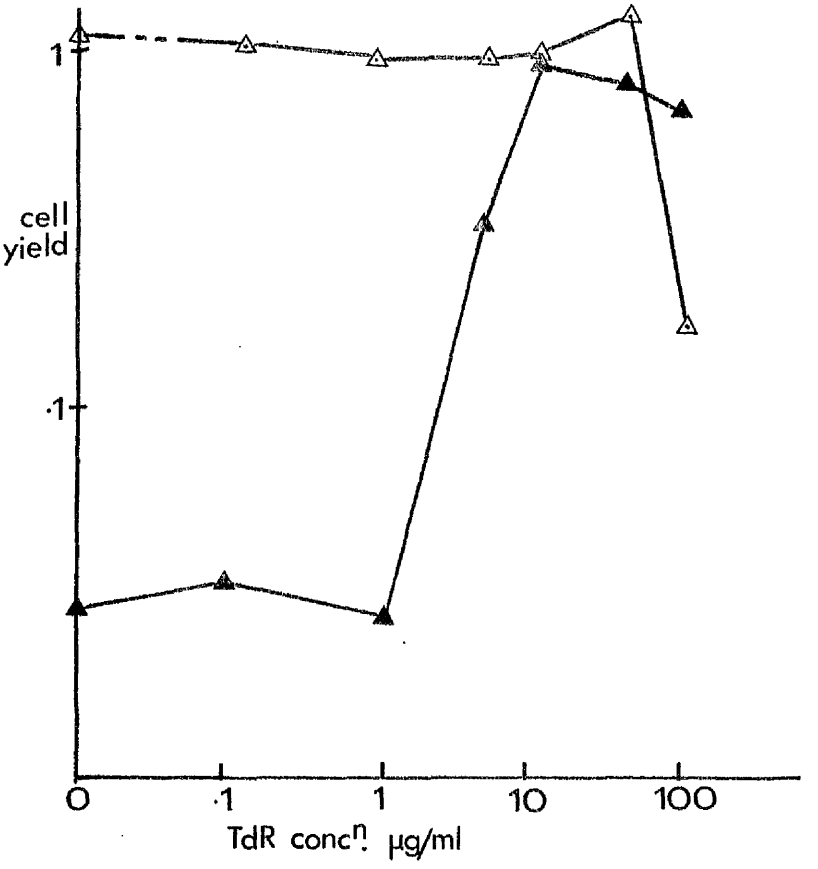

1
cell
yield
·1
0
·1
1
10
100
TdR concⁿ. µg/ml

Fig. 2.7

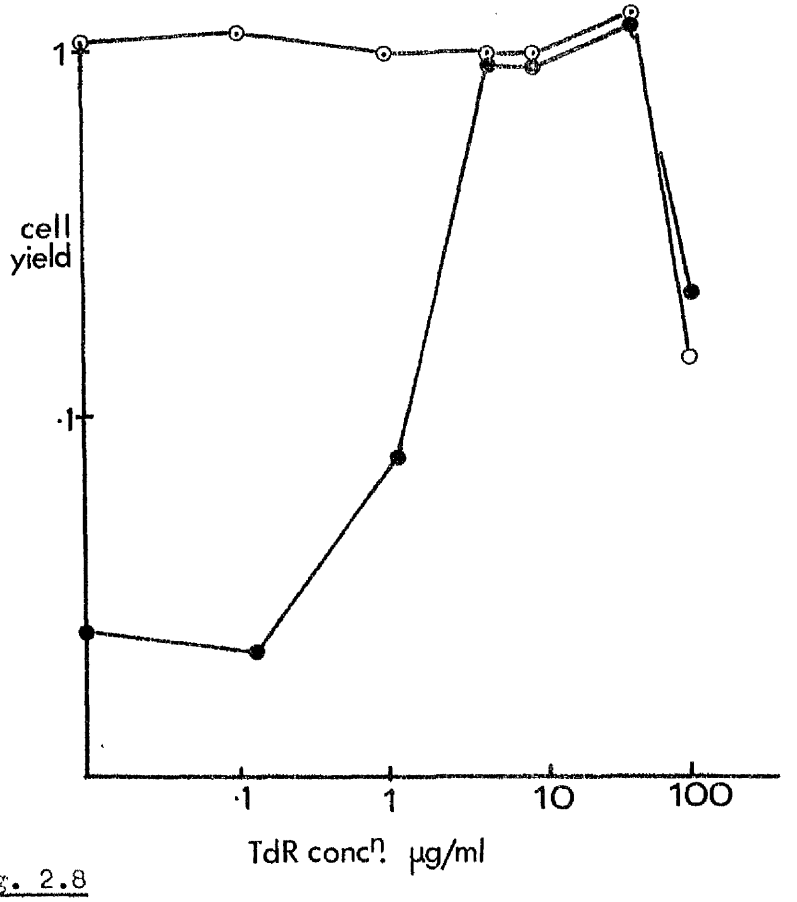

1
cell
yield
·1
·1
1
10
100
TdR concⁿ. µg/ml

Fig. 2.8

activity 17-25 Ci/mmole), the effect being antagonised by unlabelled thymidine (fig. 2.8). This toxic effect is dependent on the specific activity of the exogenous nucleoside, as is shown in several experiments on the subject.

Firstly, higher concentrations of the labelled nucleoside require proportionately more unlabelled nucleoside in the medium to prevent the toxic effect. This was shown using ^{3}H-thymidine at concentrations between 0.5 and 5.0μCi/ml, in which range the thymidine concentration which is needed to allow the cells to grow properly is proportional to the concentration of the label (fig. 2.9; table 2.6). ^{3}H-deoxycytidine (Amersham code number TRK211; specific activity 13.6 Ci/mmole) also exhibits this cytotoxic effect, and in this case the addition of deoxycytidine to the medium will allow cell growth (table 2.6). This antagonistic effect is quite specific. Thymidine will not reverse ^{3}H-deoxycytidine toxicity, nor deoxycytidine that of ^{3}H-thymidine.

Table 2.6

Cells	^{3}H-thymidine spec. act^{y}. Ci/mmole	concn. μCi/ml	concn. μM	TdR concn. (μM) at 50% cell growth	final spec. act^{y}. Ci/mmole
PyY	23.7	0.5	0.021	4.4	1.08
PyY	23.7	2.0	0.084	17.8	1.07
BHK21	17.4	2.0	0.114	16.9	0.97
	^{3}H-deoxycytidine			CdR	
PyY	13.8	2.0			0.2-1.7

That the toxic effect of tritium-labelled compounds depends on their incorporation into the cells was shown by making use of the inability of the mutant cell line PyY/TG/CAR/BUDR, which have been selected for, among other things, resistance to BrdUrd and have been shown to be totally lacking in thymidine kinase (TK) activity (Mrs. L. Edwards, personal communication).

Fig. 2.9; Reversal of the toxicity of ^{3}H-thymidine by the addition of unlabelled thymidine to the medium.

PyY or PyY/TG/CAR/BUDR cells were grown for 5 days in the presence of the concentrations of labelled and unlabelled thymidine shown in the diagram. Total cell numbers were determined by counting.

PyY/TG/CAR/BUDR yields ◇

PyY; no ^{3}H-thymidine △

PyY; 0·5 or 2·0μCi/ml ▲

Also shown are the conditions which allow cell growth to 50% of the maximum yield.

Thymidine μM	Concentration of ^{3}H-thymidine. 0μCi/ml	0.5μCi/ml	2.0μCi/ml
0	1.48×10^{6}	1.72×10^{6}	1.96×10^{6}
0.5	-	-	-
2	-	-	-
10	1.49	1.99	1.97
50	2.23	-	-
100	1.98	2.09	1.82

Total cell yields for PyY/TG/CAR/BUDR. In the presence of tritiated thymidine.

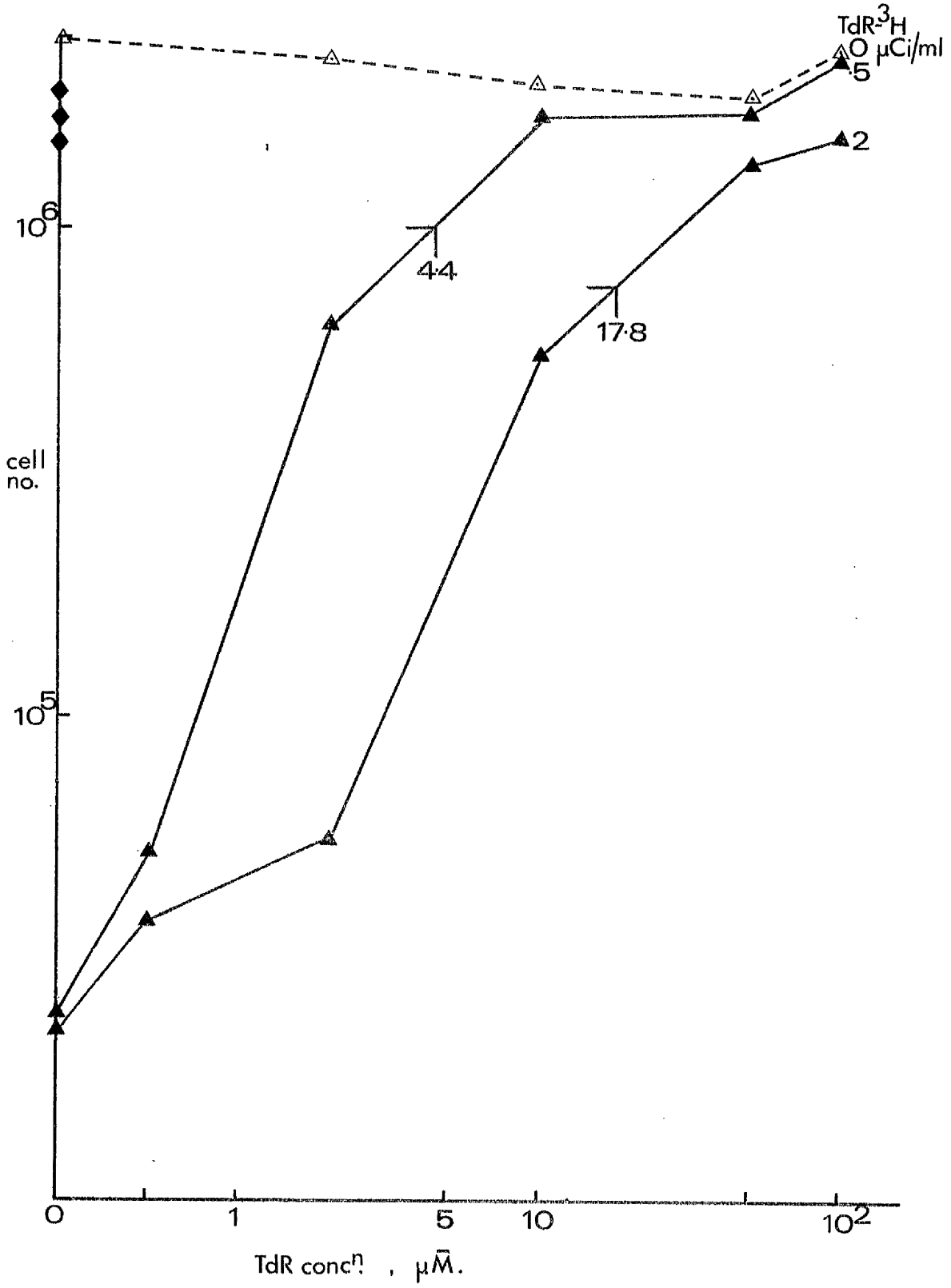


Fig. 2.9

These cells are unable to incorporate thymidine into their nucleic acids (see sections 3.1, 3.2 and 3.3c). Fig. 2.9 shows a comparison of the effect of labelled thymidine on PyY and on this TK^- cell line in the presence of various concentrations of unlabelled thymidine. It is evident from these results that the TK^- cells are very little effected, while the PyY cells show the typical reduction in cell growth at high specific activity. There is also the typical reversal of the thymidine-^3H toxicity by the unlabelled nucleoside. The mechanism of this toxicity is not known, but it seems probable that the cellular DNA is damaged more and more by progressive increases in specific activity until a stage is reached at which the DNA can no longer be repaired. This is a phenomenon which is not considered in many publications in which thymidine-^3H of high specific activity is used. Rauth (1968) found that the doubling time of cells was lengthened by ^3H-thymidine and that their plating efficiency was lowered. These effects were detectable when the specific activity was as low as 0.05 Ci/mmole, and were very marked at 0.5 Ci/mmole. In cells lines of differing ability to incorporate thymidine, he found that the effect of the labelled compound was proportional to the amount of radioactivity incorporated into the cells. These results were not presented in detail, but they seem to be in good general agreement with the work described here.

These experiments make it clear that the PyY cells require 20μM thymidine at a specific activity of not more than 0.5 Ci/mmole if labelling is to be carried out in the presence of FdUrd. In order to assess the effect of FdUrd on the specific activity of the cellular DNA, PyY cells were grown in the presence or absence of FdUrd, with the addition of labelled thymidine at various concentrations and specific activities. After 4-5 days growth, the number of cells produced from a fixed inoculum under these different conditions was found. One such experiment gave the results set out in fig. 2.10. At levels of thymidine concentration at which reasonable

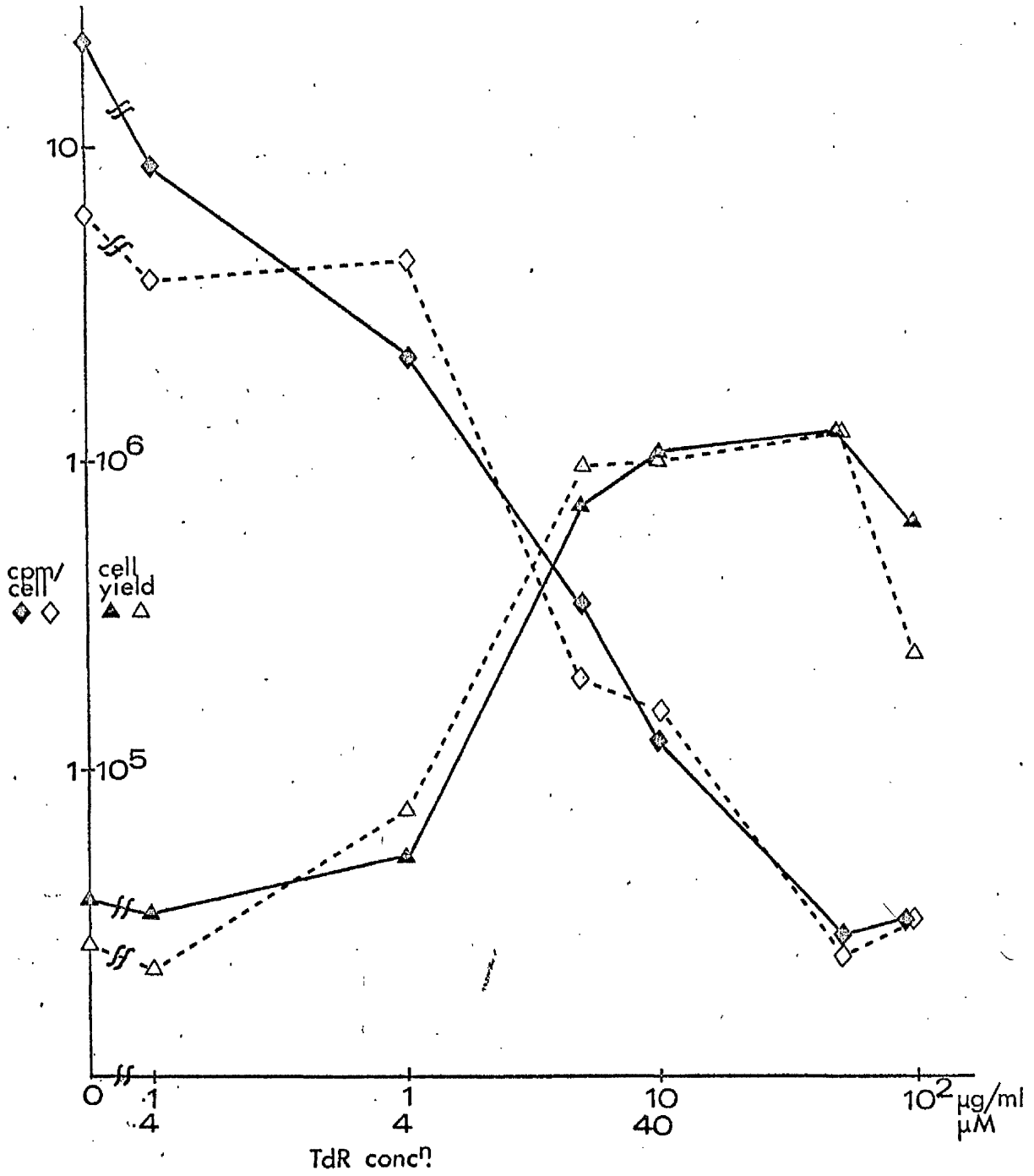


Fig. 2.10; Effect of different concentrations of unlabelled thymidine on the incorporation of ^{3}H-thymidine and on cell growth in the presence and absence of 10^{-7}M FdUrd. 2µCi of ^{3}H-thymidine/ml; 4 days growth.

dotted lines; no FdUrd

solid lines; 10^{-7}M FdUrd

Radioactivity assayed on filter paper discs.

cell growth was found, FdUrd made no difference to the amount of thymidine-^{3}H that was incorporated. At lower concentrations of thymidine, there was slightly increased incorporation in the presence of FdUrd, but the cell yield was greatly reduced. It seems that, in these cells, the restrictions imposed on DNA labelling by radiation damage are much more important than dilution of the labelled compound by the product of de novo synthesis. This would mean that inhibition of the thymidylate-synthesising pathway would only marginally increase the incorporation of the exogenous label.

The method adopted for the routine production of ^{3}H-thymidine-labelled DNA was to inoculate 80 oz. roller culture bottles with 20-25 x 10^6 PyY or BHK21 cells in 200 ml of EC10. To each bottle was added 100µCi of thymidine-methyl-^{3}H (TRK120; 17-25 Ci/mmole). The specific activity was adjusted to about 0.8 Ci/mmole by the addition of 0.1 ml of unlabelled thymidine solution of an appropriate concentration. For example, if the labelled compound has a specific activity of 20 Ci/mmole and it is added at 0.5µCi/ml, its concentration is 0.025µM, so that to reduce its specific activity by a factor of 25 (i.e. to 0.8 Ci/mmole) the final concentration of unlabelled thymidine must be 0.6µM. This means that 0.1 ml of 1.2 mM thymidine should be added. The cells were incubated at 37°C after adjusting the atmosphere in the bottle to 5% carbon dioxide. After 4-5 days, the cell sheets were confluent, and the DNA-^{3}H was extracted by the Marmur method described in section 2.3.

The DNA-^{3}H preparations were stored at -20°C in SSC at concentrations of 0.2-1.0 mg/ml. The specific activities were determined as described in section 2.2c, and lay in the range 2.5-8.0 x 10^4 dpm/µg (11-36 µCi/mg).

Density-Labelled DNA

Previous publications dealing with the entry of density-labelled DNA into mammalian cells have been complicated by a variety of factors (Ledoux and Huart, 1967, 1969; Ledoux et al., 1967; Ayad and Fox, 1968; Robins and Taylor, 1968). Preparation of density and radioisotope-labelled DNA was undertaken with the following points in mind.

1. A density-labelled population of DNA extracted directly from cells will consist of a mixture of molecules labelled in both strands and those labelled in one strand or neither. The relative proportions of these three molecular types will depend on the labelling conditions and more particularly on the number of replicative cycles undergone by the DNA during the labelling period.

2. Labelling with BrdUrd renders the DNA more sensitive to shearing forces and to light. (Aoki et al., 1966; Boyce, 1966; Rasmussen and Painter, 1966; Rauth, 1967; Cleaver, 1967, 1968; Hutchinson and Hales, 1970).

3. BrdUrd replaces thymidine in DNA and hence will compete with it for incorporation.

The labelling method was to incubate the cells in the presence of BrdUrd and ^{3}H-deoxycytidine and to extract the labelled DNA with the minimum of manipulation or exposure to light. Caesium chloride gradients were then used to separate out the fully-labelled DNA molecules.

Deoxycytidine-5-^{3}H (Radiochemical Centre, Amersham; TRK211) shows a pattern of incorporation in autoradiographs similar to that of ^{3}H-thymidine. Reference to the pyrimidine utilisation pathways shown in fig. 3.2 shows that exogenous deoxycytidine is either incorporated into DNA or converted to thymidine, in which case the 5-H atom is replaced with a methyl group. This means that such conversion of deoxycytidine-5-^{3}H will produce unlabelled thymidine. In short, the label will only be incorporated into

DNA cytosine, and there will be no competition with BrdUrd. Tritiated deoxycytidine also shows a specific-activity-dependent toxic effect (see table 2.6), and unlabelled deoxycytidine was always added to the medium. The full labelling and extraction procedure is laid out below.

80 oz. roller culture bottles were inoculated with 20×10^6 cells in the following medium.

Eagle's / 10% calf serum	200 ml.
BrdUrd (see fig. 2.11)	5 µg/ml.
deoxycytidine-5-^{3}H (TRK211)	0.5 µCi/ml.
deoxycytidine	2 µM approx.

The cells were incubated at 37°C for 4-5 days in bottles wrapped in aluminium foil. Even with the exclusion of light, the cells at the end of this period differed markedly in morphology from those grown in the absence of BrdUrd. The BHK21/C13 cells had lost their parallel orientation and individual cells were more spread on the glass than normally (see Stellwagen and Tomkins, 1971).

1. Cells were rinsed in EDTA-saline and lysed using 20% SDS. The lysate was heated to 60°C for 20 minutes, and cooled. SDS concentration was adjusted to 0.1-0.2%.

2. Pronase was added to a final concentration of 50 µg/ml, and the lysate incubated at 37°C for 20 minutes.

3. Sodium perchlorate was added to a final concentration of 1M, and the solution gently shaken with 1 volume of chloroform/isoamyl alcohol at 4°C for 15 minutes. The mixture was spun at 10,000 rpm in the 10 x 50 fixed-angle rotor of an MSE18 centrifuge. The upper (aqueous) layer was collected.

4. Nucleic acids were precipitated from the aqueous layer by addition of 2 volumes of absolute ethanol at -10°C. The precipitate was collected by spooling on a glass rod, and redissolved in SSC/10 at 4°C.

The precipitated nucleic acid differed in texture from that from cells not labelled with BrdUrd in that it was less obviously fibrous and showed much less adhesion, both to itself and to glass.

5. The solution was adjusted to SSCx1 and subjected to RNase treatment at 50µg RNase/ml for 15 minutes at 37°C. It was then dialysed against SSC for 2-3 days in darkness at 4°C.

6. Solid caesium chloride was added to give a refractive index of 1.401-1.402, and the preparation was spun in the titanium-50 rotor of a Spinco model L preparative ultracentrifuge, at 40,000 rpm for 3 days. Details of the density-gradient technique are given in section 2.6. Fractions were collected and the radioactivity assayed by drying 10µl aliquots of each fraction onto filter paper discs, and counting under toluene-PPO-POPOP as described in section 2.2d. Ultraviolet absorption was monitored either by reading the optical density of individual fractions or by fractionating through a UVicord so as to obtain a continuous absorption profile. The radioactivity and UV absorption profiles of one such gradient are shown in fig. 2.12. The peak fractions (shown by the arrows on the horizontal axis of fig. 2.12) were taken and dialysed exhaustively against SSC in order to remove the caesium chloride. Fig. 2.14 shows the UV absorption spectrum of a BrdUrd-labelled DNA preparation and displays the relative increase in E_{280} which is characteristic of BrdUrd-labelled molecules. Buoyant density measurements were carried out in the analytical ultracentrifuge and by measuring the refractive index of fractions from preparative gradients. The fully-labelled DNA had a buoyant density of 1.746 and the hybrid material one of 1.728.

Attempts to analyse the BrdUrd-labelled molecules on alkaline caesium chloride gradients were unsuccessful. In the experiment shown in fig. 2.13, duplicate BrdUrd-labelled samples were placed in caesium chloride gradients

Fig. 2.11;Cytotoxicity of bromodeoxyuridine. PyY cells grown in Eagle's-10% calf serum for 4 days in the presence of BrdUrd.

Fig. 2.12; Caesium chloride equilibrium-density gradient seperation of the three classes of molecule in a preparation of DNA labelled with BrdUrd and ^{3}H-deoxycytidine. The DNA was extracted by lysis of the cells with SDS, followed by treatment with pronase, a gentle deproteinisation, and treatment with RNase. The solution was dialysed against SSC and adjusted to a measured refractive index of 1·402 with solid caesium chloride. It was centrifuged for 3 days at 40,000rpm in a titanium-50 fixed-angle rotor. Fractions were collected by piercing the tube and UV absorbance was determined using a Perkin-Elmer spectrophotometer. Radioactivity was assayed by drying 50ul aliquots of each fraction onto filter paper discs, and counting under toluene-PPO-POPOP after TCA- and ether-washing.

Peaks of UV absorbance are shown: ▼

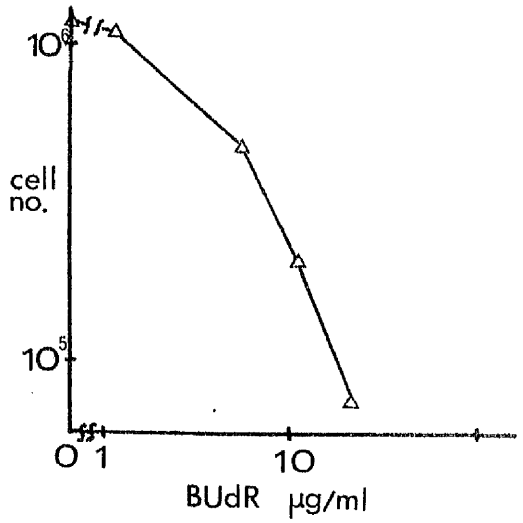


Fig. 2.11

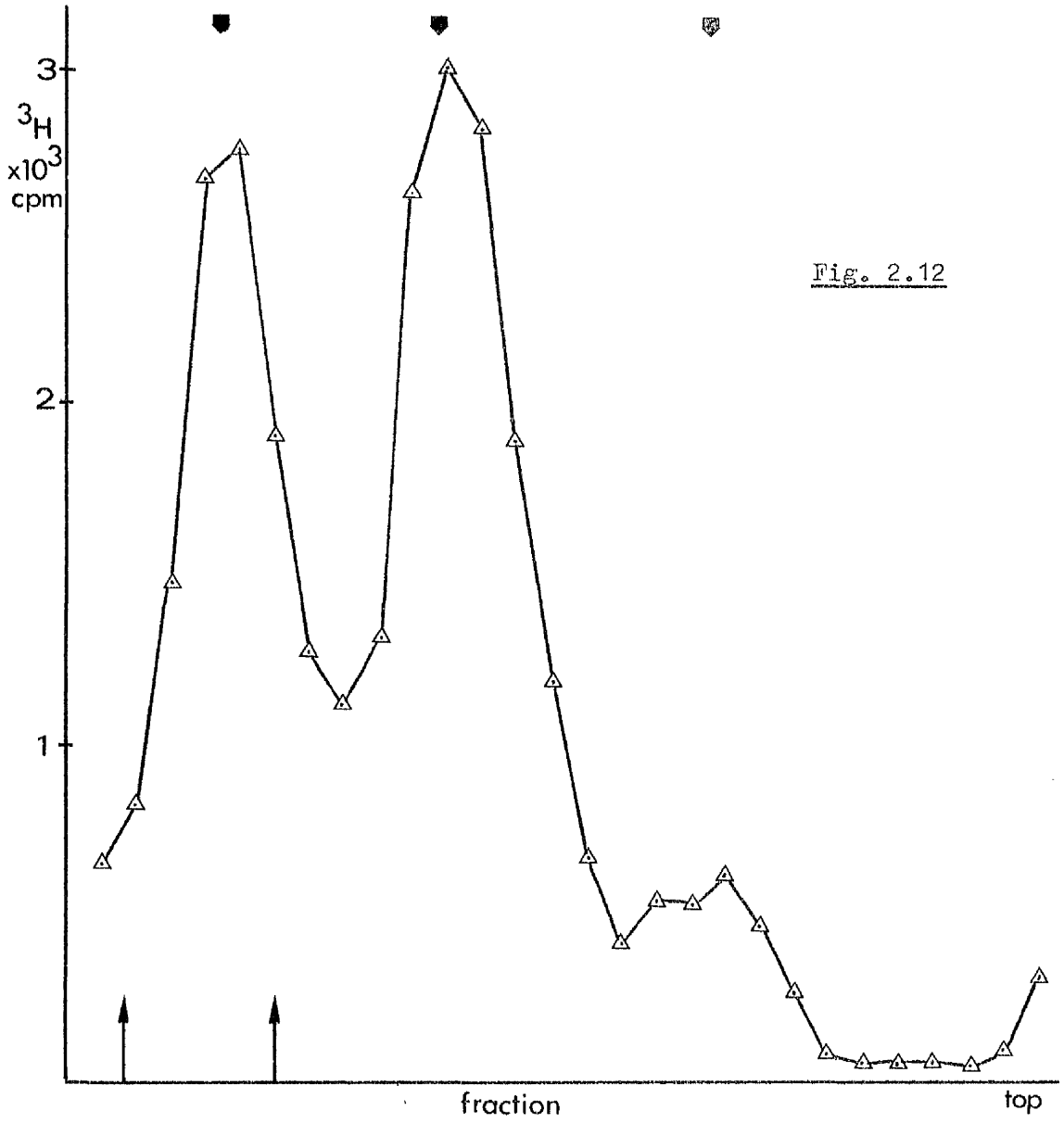


Fig. 2.12

Fig. 2.13; Analytical caesium chloride equilibrium-density gradient analysis of ^{3}H-CdR/BrdUrd-labelled DNA. Each sample was 1µg of DNA in 1ml of SSC centrifuged for 19 hours at 44;770rpm. Absorbance profile at 260mµ is shown.

A. at pH 7

B. at pH 12

Fig. 2.14; UV absorbance spectrum of ^{3}H-CdR/BrdUrd-labelled DNA purified by marmur extraction and banding in caesium chloride gradients. This spectrum is that of the pooled and dialysed heavy peak material. $E_{260}:E_{280}$= 1·77.

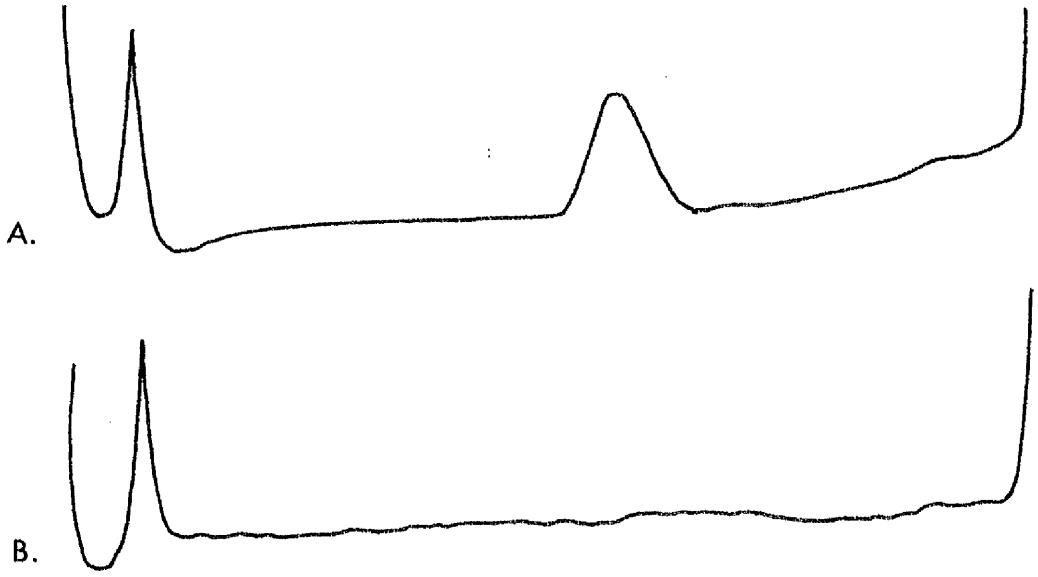


Fig. 2.13

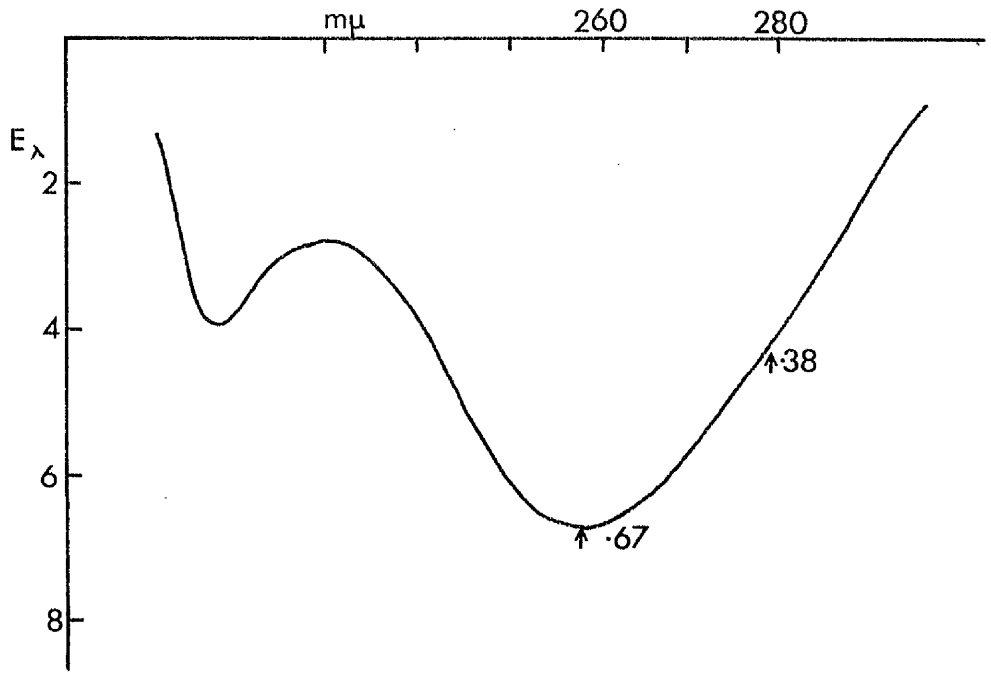


Fig.2.14

at pH 7.0 (fig. 2.13A) and pH 12.0 (fig. 2.13B) and centrifuged simultaneously in a Spinco model E. In preparative gradients, the same phenomenon was found - the BrdUrd-labelled DNA did not form bands in alkaline caesium chloride gradients. The most likely explanation of this observation is that the BrdUrd-labelled molecules are extensively damaged with single-strand breakages, arising either from damage by incorporated radioisotope, or from the effect of light on the photosensitised molecule, so that the molecules in denatured preparations are to small to for discernible bands in equilibrium density gradients. The use of such damaged DNA would not ne a reliable means of investigating DNA uptake by cells, so the use of density-label in the experimental work was restricted to the uptake of ^{3}H-DNA by cells labelled with ^{14}C thymidine and BrdUrd. That is, BrdUrd was used to label the recipient rather than the donor molecules.

Section 2.5: AUTORADIOGRAPHY

The procedure employed throughout was a stripping film technique using Kodak AR10. This has a grain of 0.2μ and tritium β-emissions have a maximum range of 2μ in the emulsion. The factors influencing the resolution of autoradiographs are discussed in Schultze (1969) and no detailed treatment will be given here. With AR10 layed over fixed cellular material labelled with tritium, the position of a disintegration can be fixed within 1μ.

Procedure 1. Preliminary

Cells for autoradiography were grown on coverslips which had been washed with hot hypochlorite solution as described in section 2.1. Microscope slides were washed in absolute alcohol, dried, and dipped in a solution containing 0.5% (w/v) gelatine and 0.05% (w/v) chrome alum. The slides were drained and allowed to dry in air. The gelatine film helps the adhesion of the photographic emulsion.

2. Fixing and washing

After labelling, the coverslips were either washed in the petri-dishes or removed into racks for transfer to beakers containing the successive washes. The detailed fixing and washing procedure was as follows.

1. Medium discarded, and the cells rinsed briefly once in PBS at room temperature.
2. Fixed in formal-saline for 4-6 hours at +4°C. Formal-saline is:-

sodium chloride	5 gm./l.
sodium sulphate	15 gm./l.
formaldehyde	4%

3. Transferred to 5% trichloroacetic acid (TCA) at 4°C and left for 2 hours.
4. Washed in a second lot of 5% TCA at 4°C for 2 hours.

5. Washed in two changes of distilled water at 4°C for 30 minutes each.

6. Rinsed once in absolute ethanol thoroughly at room temperature and left to dry in air at room temperature.

The thoroughly dry coverslips were mounted, cells upward, on the prepared microscope slides, using Depex mountant.

3. Application of the emulsion

AR10 film was stripped off the glass plate in pieces of suitable size by cutting the emulsion layer with a sharp scalpel and slowly peeling the pieces off the glass plate to avoid generating static, which would fog the film. The pieces were floated, sensitive side downward, on dustfree distilled water at 20°C, and left for a minute or so to spread. Unless it is allowed to do this, the film will not lie uniformly over the cells throughout the processing of the slides. The emulsion was picked up by bringing the slide up from under the water so that the film draped uniformly over the coverslips. The slides were allowed to drain and dry for at least a few minutes before being placed in a lightproof, airtight box containing some dehydrated silica gel. All handling of the emulsion was done in a darkroom lit only by a 25W. bulb behind a Kodak "Wratten" series 1 filter.

The slides were stored at 4°C during the exposure period, with one change of silica gel after 24-36 hours. Latent-image fading was not a problem, so no replacement of the air in the box was necessary. Exposure times varied from sample to sample, and were determined by trial development of some of the slides after about 2 weeks. The remainder were then left for as long as seemed necessary. Actual exposure times were between 2 and 6 weeks.

4. Development

Before developing, the slides were left at room temperature overnight to allow them to warm up so that the developer was not cooled.

Kodak D19 was used, at 20°C for 4 minutes, after which the slides were rinsed and fixed in Amfix for 3 minutes at 20°C. After thorough rinsing, the slides were partially dried and stained in a 1:50 dilution of Giemsa stain (Institute of Virology standard methods book; from "Practical Haematology", Dacia, 1956) in phosphate-buffered distilled water. After staining and rinsing, the slides were dried at room temperature and the excess emulsion was removed. Each coverslip was overlaid with another coverslip mounted in Depex.

The slides were examined at 100x and 1000x (oil immersion).

Section 2.6: CAESIUM CHLORIDE DENSITY GRADIENTS

The DNA sample for buoyant-density analysis was diluted to 4 ml. in the appropriate buffer, and solid caesium chloride was added and dissolved at room temperature. The amount of caesium chloride was calculated from the following equations (Vinograd, 1963).

$$W = 137.48 - 138.11 \quad \frac{1}{\rho}$$

$$\rho = 10.8610\eta - 13.4974$$

Where W = % caesium chloride (w/v)

ρ = density (gm. cm.$^{-3}$)

η = refractive index.

The density chosen was such that the DNA sample would be expected to come to equilibrium close to the centre of the gradient. The precise weight of caesium chloride according to the first equation was added and dissolved and the refractive index was checked using an Abbé refractometer. Any adjustments required by the second equation were made.

The final solutions were transferred to Beckman polyallomer tubes, and the tube filled with liquid paraffin and capped. There were some variations in the conditions of centrifugation, and these are described with the experiments in section 4. The standard conditions were 40,000 rpm for 3 days in a titanium-50 fixed-angle rotor of a Spinco model L ultracentrifuge.

Fractionation was by dripping under gravity in the early experiments. The tube was punctured at the bottom in the MSE apparatus designed for that purpose. Dripping was regulated by adjusting the entry of air into the top of the tube. Later, the apparatus in fig. 2.15 was used. Air was pumped in at 40 ml./hr. by the LKB pump, so that the gradient was fractionated in about 8 minutes at a uniform rate. Fractions were collected by hand.

Section 2.7: SUCROSE GRADIENTS

Sucrose gradients were used for the determination of the size of molecule with which the label derived from the input DNA was associated after absorption by the cells.

The gradients were formed using an MSE gradient making apparatus and an LKB peristaltic pump fitted with an 80 ml./hr. gear. All the gradients were made in cellulose nitrate tubes of the SW.25 or SW25.2 rotors for Model L or L-2 ultracentrifuges. The composition of these gradients was 10-30% sucrose in 1M sodium chloride, 0.3M sodium hydroxide, and 0.01M EDTA. Each gradient was loaded with 5-8 x 10^6 cells in 0.2 ml of saline, which was introduced into a layer of 0.4 ml of M sodium chloride, 0.3M sodium hydroxide and 5% (v/v) Decon, a detergent which effectively disrupts the cell and releases the DNA gently onto the top of the gradient. In order to ensure that the cells were totally and efficiently lysed it was necessary to introduce them into the top layer as a single-cell suspension, and the loaded gradients were left for a few minutes before centrifugation was begun. These were alkaline gradients (pH 12) so that the DNA released from the disrupted cells will be in its single-stranded form in the course of the sedimentation. Centrifugation was carried out at 5^oC for $4\frac{1}{2}$ hours at 22000 rpm in the swing-out heads mentioned above. The gradients were fractionated in the apparatus shown in fig. 2.15, with the LKB pump fitted with an 80 ml/hr gear. Fractions were collected and adjusted to 10% TCA. They were left at 4^oC for several hours. The precipitates were collected by suction filtration through 2.4 cm. Millipore discs of pore size 0.22μ, and were washed by washing through with cold 5% TCA. Radioactivity was assayed by immersing the discs, after two ether washes and thorough drying, in toluene-PPO-POPOP scintillation fluid.

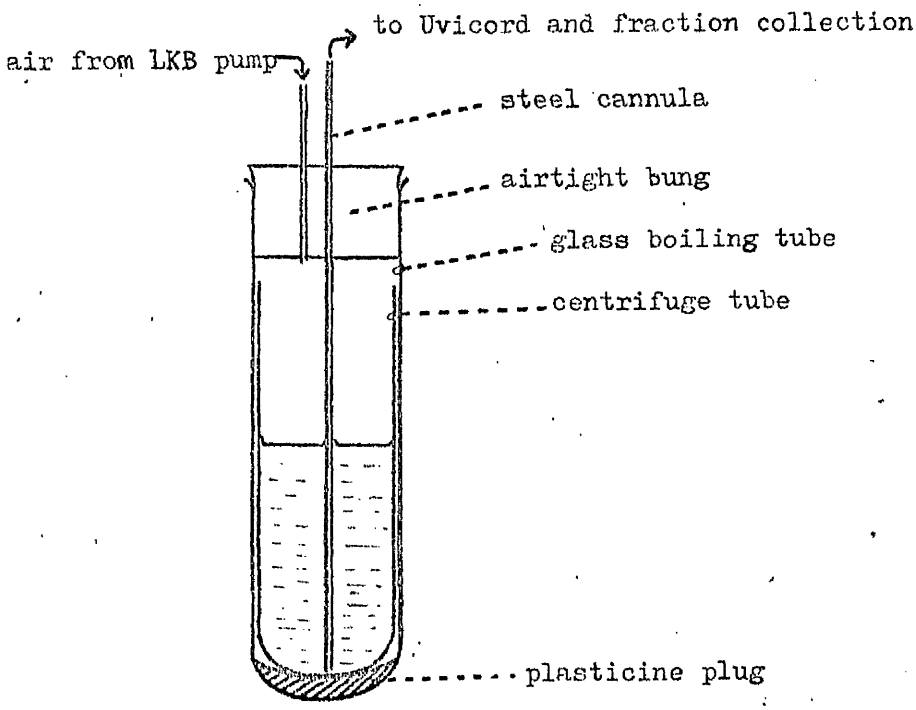


Fig. 2.15 Apparatus used for the fractionation of gradients by air displacement.

Section 2.8: PRODUCTION OF POLYOMA VIRUS

Polyoma virus was produced by the standard method of the Institute of Virology. Secondary mouse embryo cells in confluent monolayers were infected with 8 x 10^3 haemagglutinating doses per 80 oz. roller culture bottle. The virus was adsorbed for 2 hours at 37°C, after which the cells were incubated in 200 ml. of Eagles medium supplemented with 10% tryptose phosphate broth and 10% calf serum at 37°C for 7 days with a medium change at 2 days. The cells were harvested by shaking the bottles to detach the cells from the glass, and were left in the culture medium at 4°C for a few hours to allow the virus in the medium to adsorb to the cells. The cell suspension was checked for sterility by plating a sample onto blood agar and incubating at 37°C overnight. After this sterility check, the cell suspension was frozen and thawed three times, and the pellet from a centrifugation at 2000 rpm was collected. This pellet was subjected to successive receptor destroying enzyme (RDE) and tris extractions, with the supernatants being retained at each step and frozen at -20°C. The supernatants were thawed at the end of the extraction and assayed for haemagglutination using washed guinea pig erythrocytes. Those with HA titre above 10^3 were pooled, and spun at 23,000 rpm in an SW.25 rotor of a Spinco model L. The pellet was dispersed in tris by stirring and sonication. The virus was further purified by banding in neutral caesium chloride equilibrium-density gradients spun at 30,000 rpm for 18 hours in an SW.39 rotor. The virus bands were visible by virtue of their scattering of light under intense illumination. Two bands were visible, one being the "empty" particles which are deficient in DNA and are less dense than the complete, infectious virus, which forms the lower of the two bands and was collected by piercing the tube. The collected material was diluted in tris buffer and pelleted in the SW.25 head as described above. The pellet was resuspended in tris and stored in the dark at 4°C. Fuller details of the method are set out in the Institute of

Virology standard methods book.

The virus suspension on which most of the experiments were carried out was examined by Dr. E. A. C. Follett under the electron microscope, and was found to have about 4×10^{13} particles/ml., of which 98% were "full".

- THREE -

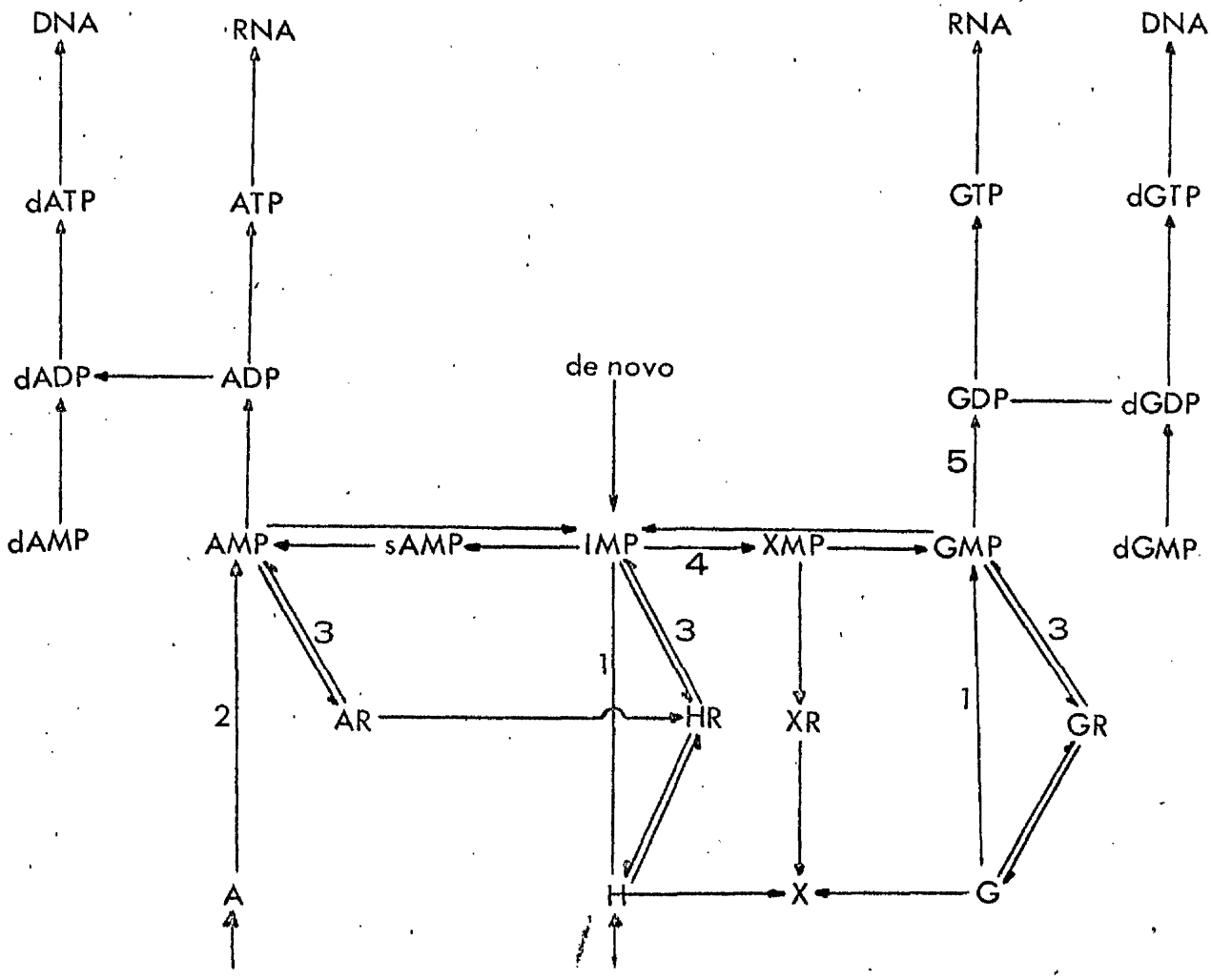
THE CELL LINES

Section 3: THE CELL LINES

3.1a: The genetic system

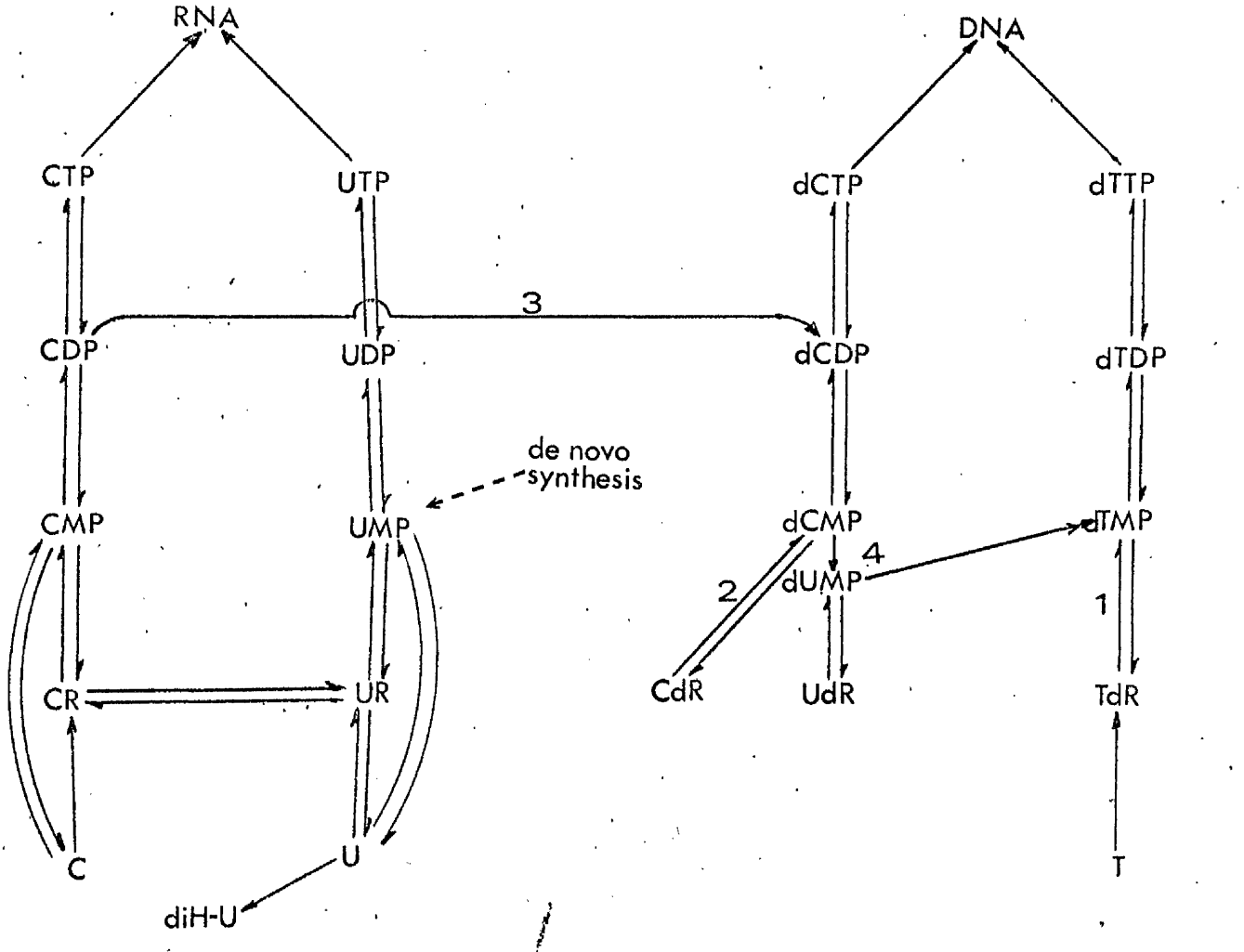
The purine and pyrimidine salvage pathways, which are concerned with the utilisation of preformed nucleosides taken in by the cells (Murray, 1971; Murray et al., 1970), offer a versatile system for genetic studies. The usefulness of this system stems from the fact that many nucleoside analogues will only exert a cytotoxic effect when they have been phosphorylated by cellular enzymes. The pathways are set out in figures 3.1 and 3.2 in some detail, and it may be seen that the first step in the utilisation of exogenous nucleosides for nucleic acid synthesis is phosphorylation. A number of cell lines selected for their resistance to one or other nucleoside analogue have subsequently been shown to have lost the enzyme activity necessary for this phosphorylation step (Szybalski et al., 1962; Littlefield, 1963, 1964; Subak-Sharpe, 1965, 1969; Friedmann et al., 1969; Ayad and Fox, 1968). Table 3.1 shows the selective agents used in this laboratory, and the enzyme activities which are missing from the resistant cells. In this way, cells are selected for deficiencies in particular enzymic activities by exposing them to analogues at toxic concentrations. The corollary of the enzyme deficiency in resistant cell lines is that these cells become dependent on the de novo synthetic pathways for their supply of nucleic acid precursors. This permits the design of a system in which there is selection in favour of those cells which regain or retain the enzyme activity. For example, a cell line which is deficient in hypoxanthine-guanine phosphoribosyl transferase (HG-PRT) activity is unable to use hypoxanthine in the medium as a purine source. If such cells are grown in medium which contains hypoxanthine as the sole purine and in conditions in which the de novo synthesis of purines is inhibited, for example by the addition of aminopterin, they will be unable to grow, as a result of the loss of both of their sources of purines. Only those cells which have sufficient HG-PRT activity to make use of the hypoxanthine in the medium will survive. In practice,

Fig. 3.1 PURINE SALVAGE PATHWAYS.



1. Hypoxanthine-guanine phosphoribosyltransferase.
2. Adenine phosphoribosyltransferase.
3. Kinase(s).
4. IMP dehydrogenase.
5. GMP kinase.

Fig. 3.2 PYRIMIDINE SALVAGE PATHWAYS



1. Thymidine kinase
2. Deoxycytidine kinase.
3. CDP-reductase.
4. Thymidylate synthetase.

Table 3.1 Summary of the biochemical genetic markers carried by the cell lines used in the present work.

SELECTIVE AGENT**	ENZYME DEFICIENCY	SYMBOL	BASES etc. NOT INCORPORATED
8-azaadenine, AA	APRT	/AA	adenine
8-azaadenosine, AAR+	permease?*	/AAR	adenosine
6-thioguanine, TG	~~HPP~~ HGPRT	/TG	hypoxanthine, guanine
6-thioguanosine, TGR	permease?*	/TGR	inosine, guanosine
cytosine arabinoside aCyt.	dCK	/CAR	deoxycytidine
bromodeoxyuridine, BrdUrd.	TK	/BUDR	thymidine

* see section 3.3c.

+ or tubercidin. See fig. 3.3 and section 3.1b.

** The structures of these analogues are given in fig. 3.3.

it is also necessary to add thymidine to the medium in order to relieve the thymidine deficiency caused by the inhibition of thymidylate synthetase by aminopterin. The medium usually employed is that of Littlefield (1964, 1966) or a variation of it (Szybalski and Szybalska, 1962a). It contains hypoxanthine, aminopterin and thymidine and is termed HAT medium. The importance of this in the context of DNA-mediated transformation is that it allows selection of a small number of HG-PRT$^+$ cells out of a large population of HG-PRT$^-$. This is more fully discussed in the introduction (section 1.2).

Further use can be made of the inability of analogue-resistant cells to use exogenous nucleosides for nucleic acid synthesis. If a population of cells is incubated in the presence of a tritiated nucleoside and the cells are subsequently fixed and exhaustively acid-washed, tritium label which has been incorporated into the acid-insoluble cellular components will be detectable by autoradiography. Individual cells which possess the enzyme activity will be distinguished from those which do not because only the former will take the labelled compound into acid-insoluble (polynucleotide) material, so that only they will appear labelled in the developed autoradiographs. This technique allows the detection of small numbers of HG-PRT$^+$ cells in the presence of a large population of HG-PRT$^-$ cells. It is also applicable to the other markers described, being useful in the detection of small numbers of APRT$^+$, dCK$^+$ and TK$^+$ cells.

3.1b: Mechanism of action of the analogues

6-thioguanine: This compound (fig. 3.3) is converted to 6-thio-GMP by HG-PRT. 6-thio-GMP is the active metabolite and is a poor substrate for further conversion to 6-thio-GDP. It appears to have at least three sites of action. Firstly, 6-thio-GMP has been reported to inhibit PRPP-amidotransferase, which is involved in the de novo synthesis of purines. Competitive inhibition of GMP kinase has also been reported but the primary

mechanism is almost certainly the inhibition of IMP-dehydrogenase. This inhibition is not reversible and the analogue seems to be covalently bound to the enzyme, possibly by the formation of disulphide bonds. The enzymes affected are indicated in fig. 3.1.

6-thioguanosine: The cytotoxic effect of this analogue is identical to that of 6-TG because it too is converted to 6-thio-GMP, which is the active metabolite.

8-azaadenine: This is phosphorylated to 8-aza-AMP by APRT. Its mode of action is not as well known as is that of the previous type of analogue, but the primary action seems to be an inhibition of the conversion of IMP to AMP (see fig. 3.1) by 8-aza-AMP.

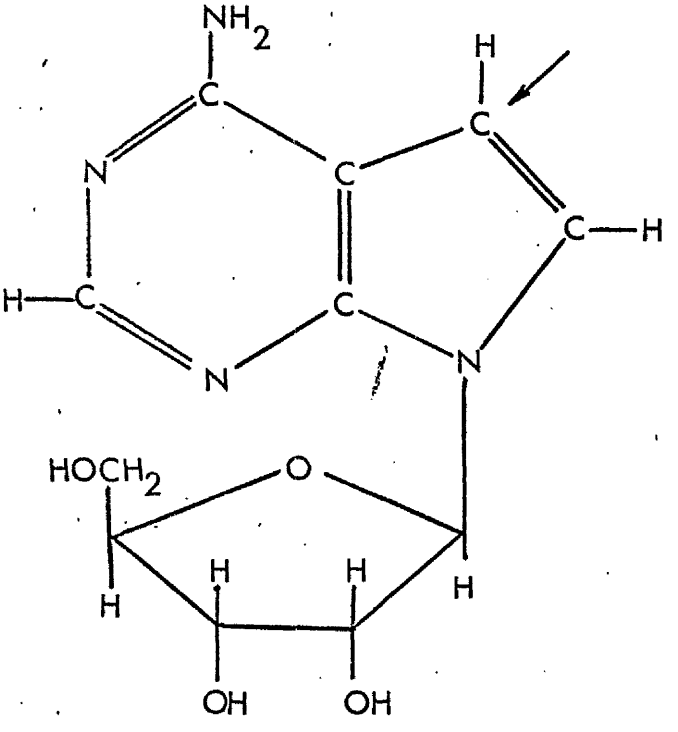
8-azaadenosine is converted to the same compound, 8-aza-AMP, and its action is therefore the same as that of AA.

Tubercidin: This antibiotic is 7-deazaadenosine (4-amino-7β-D-ribofuranosyl-pyrrolo-(2,3-d) pyrimidine; fig. 3.3). It is a close structural analogue of adenosine, and has been used as an alternative to 8-AAR in the selection of resistant cells. Its metabolism resembles that of AR, but this does not imply that its inhibitory mechanism is identical. In fact it is further phosphorylated to the triphosphate and is incorporated into DNA and RNA. The effects are only imperfectly understood at a molecular level, but it is clear that the analogue has an adverse effect on DNA replication and transcription and on protein synthesis. The evidence relating to the various suggested mechanisms is reviewed in Roy-Burman (1970).

Cytosine arabinoside (aCyt): This pyrimidine analogue (fig. 3.3) is phosphorylated by deoxycytidine kinase (fig. 3.2) to form ara-CMP which is further converted to the higher phosphates, ara-CDP and ara-CTP. The incorporation of ara-CTP into DNA is very low (Chu and Fischer, 1968, 1968a; Furth and Cohen, 1967) and the inhibition of CDP reductase, which had been considered sufficient to account for the cytotoxicity (e.g. Kimball et al.,

Fig. 3.3; Structural formulae of the purine and pyrimidine analogues used in the selection of the resistant cell lines described in sections 3.2 and 3.3.

The arrows indicate the points at which the molecules differ from the normal metabolites.



Tubercidin

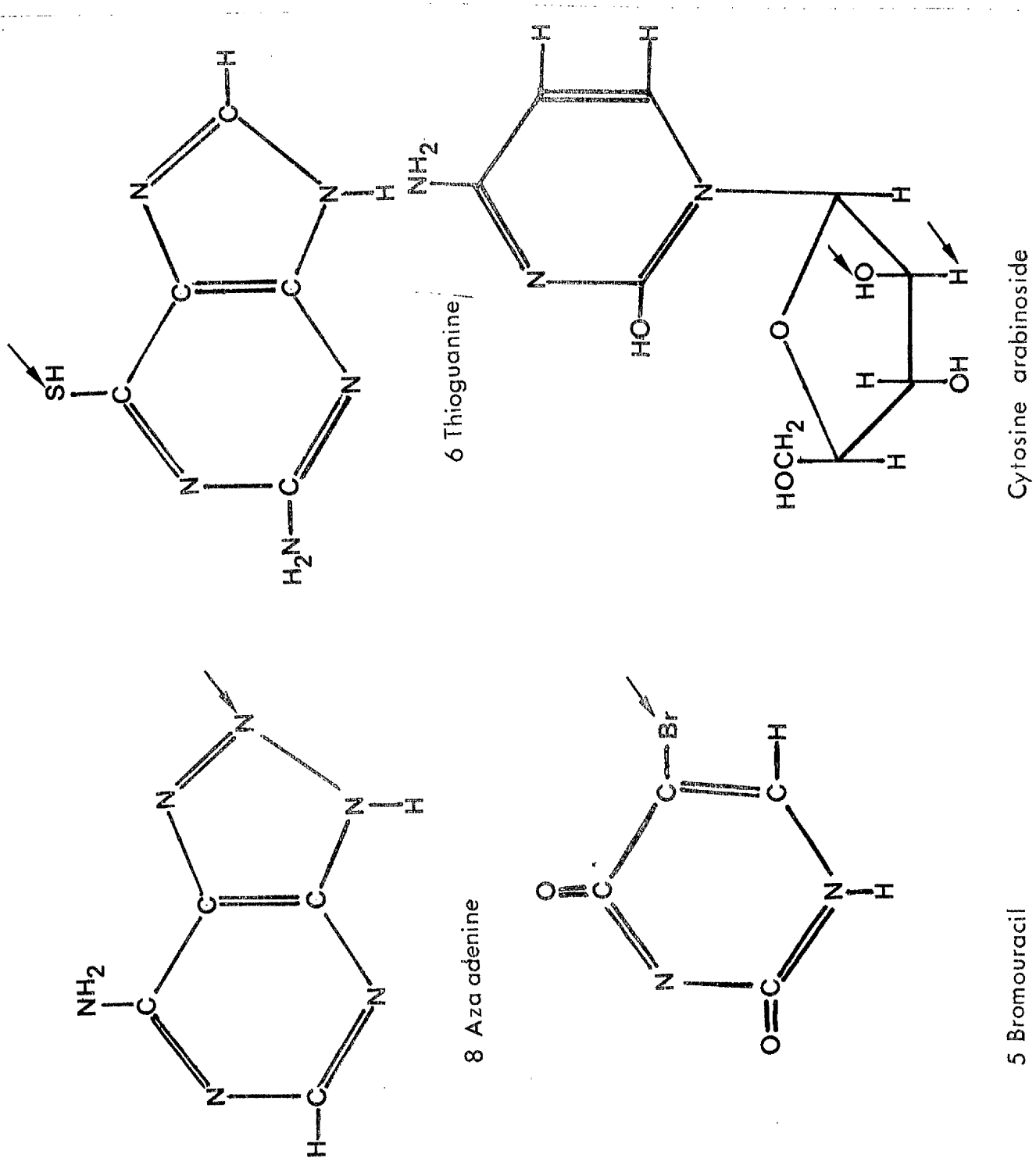


Fig. 3.3 Structural formulae of the analogues.

1966), has been shown to be of small importance (Kaplan et al., 1968). The primary mode of action seems to be an inhibition of DNA polymerase, competitive with dCTP (Furth and Cohen, 1967; Momparler, 1969).

5-bromodeoxyuridine: Incorporation of this analogue into DNA has led to its use as a density label, and gives rise to the mutagenic and radiosensitising effect. It is a close analogue of thymidine, because the Br atom has a van der Waals radius very close to that of CH_3-, and it is handled by the same enzymes. It therefore replaces thymidine in DNA. The lethal and mutagenic effects both arise from base mismatching, which can occur during DNA replication or transcription, producing base changes in the DNA or mismatching during mRNA synthesis with the consequent non-functional protein.

Section 3.2: ORIGIN AND DERIVATION OF THE CELL LINES

Nomenclature

The system of naming used routinely is to append to the name of the parental line, an abbreviation of the analogue to which the cell is resistant (see table 3.1). Thus a line of PyY cells which have been selected for thioguanine resistance become PyY/TG. The system is cumulative in that a line with multiple resistance will have in its name all of the analogues used in selection in the order in which the selective steps were carried out. (for example, see fig. 3.4) (Subak-Sharpe, 1965).

Derivation

The cell lines used in this current work are derived from the continuous line of hamster cells, BHK21/C13, described by McPherson and Stoker (1962). This line of cells is not useful for the isolation of spontaneous mutants of the type of interest here, because it only very rarely gives rise to colonies in the presence of the selective agents. More usually, the cells survive for long periods but do not divide, so that clones of resistant cells are extremely rare and difficult to isolate.

BHK21/C13 cells can be transformed by polyoma virus and one of these transformed lines, designated PyY (Stoker and McPherson, 1964) has proved useful in one production of resistant cell lines (Subak-Sharpe, 1965). All the cells used in this current work were derived from PyY by Professor Subak-Sharpe and his coworkers. The resistant cell lines were not in the first instance derived from single cells. The selection method employed was to seed 10^5 cells into Pyrex baby-feeding bottles, without prior mutagenesis. They were incubated in 10 ml. of Eagles medium supplemented with 10% foetal calf serum and containing the appropriate selective agent. Variable numbers of colonies appeared, but usually the number did not exceed 8 clones per bottle. These were allowed to grow for 10 days, before being passed for further growth and selection or frozen at $-70^{\circ}C$ for storage.

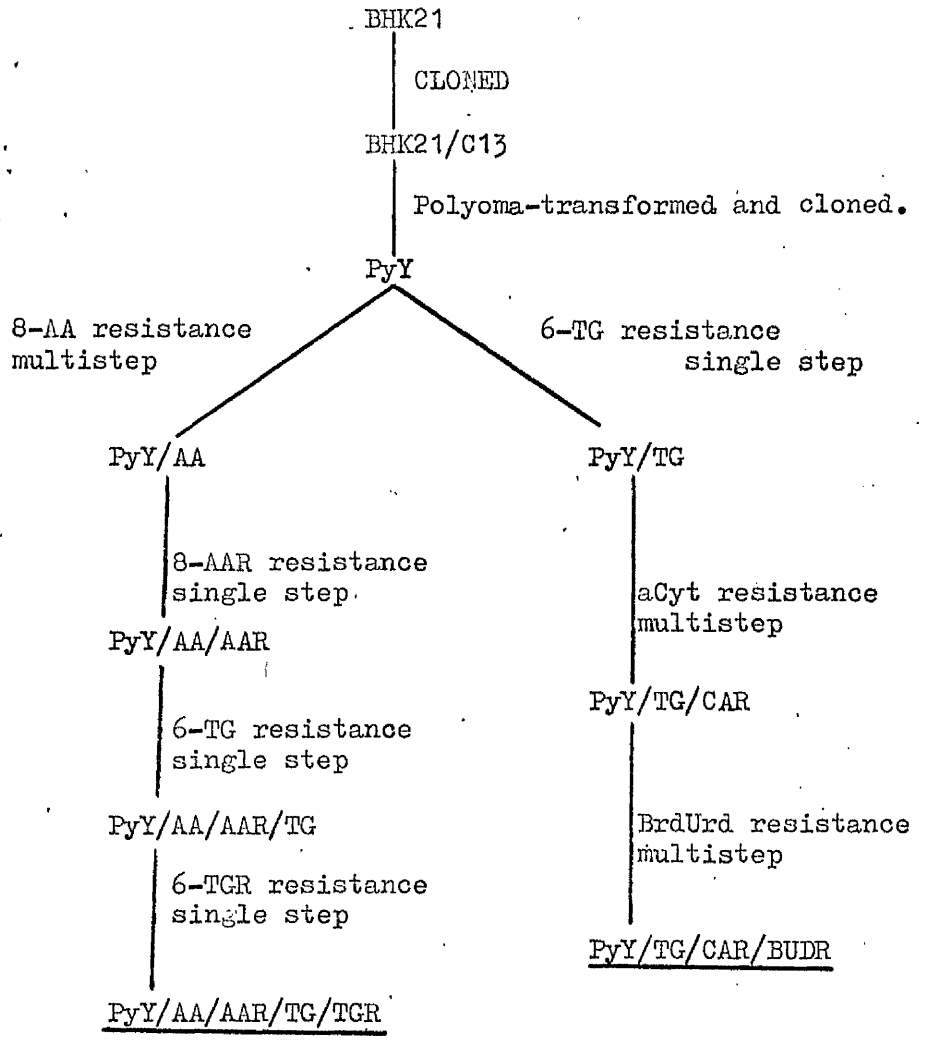
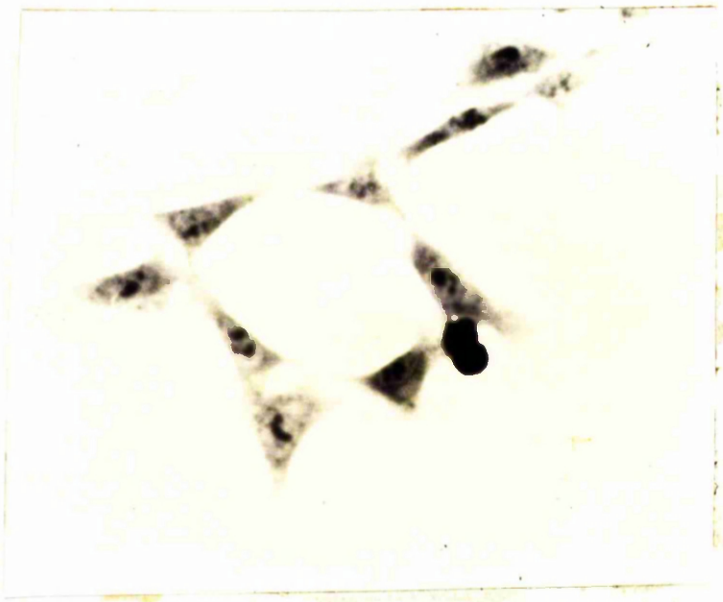
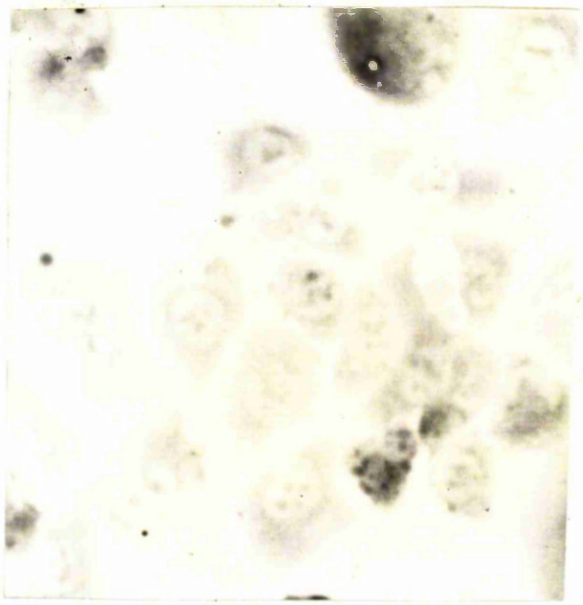


FIGURE 3.4. The derivation of the biochemically-marked mutant cell lines used in the present study. This diagram is partly taken from Subak-Sharpe (1969).



PyY/AA/AAR/TG/TGR



PyY/TG/CAR/BUDR

Fig. 3.5: Morphology of the mutant cell lines.
Giemsa stain.

Thus, the original mutant populations are derived from several clones. These cells have been cloned using the agar suspension-culture technique and all the work here described was carried out on lines derived from single mutant cells.

In some cases, resistance to an analogue was acquired in a single step (fig. 3.4) while in others the development of high resistance was a process involving many selective stages and a gradual increase in analogue-resistance. There is some correlation between this and the revertants which have been found.

Morphology

The resistant cell lines differ markedly in morphology from the wild-type, parental line, PyY. Detailed investigation of the changes in cell character in the course of selection procedure was not undertaken and no description is offered here, but the cells shown in fig. 3.5 are representative of the stable morphology of some cell types.

Section 3.3: PROPERTIES OF THE CELLS

3.3a: Analogue Resistance

In the mutant cell lines, there is no sharp upper limit to the analogue concentrations that are tolerated, so resistance curves were constructed by analysing cell growth in different analogue concentrations.

This was done by growing the cells from low cell numbers to a stage where the controls, i.e. those bottles of cells which received no analogues, were confluent. This usually took about a week, using an inoculum of 10^5 cells in "Pyrex" baby-feeding bottles or 2.10^4 cells in 5 cm. "Nunclon" plastic petri-dishes. When the controls reached confluence, each culture was brought to a single cell suspension and the total number of cells determined. Representative curves are shown in figs. 3.6 and 3.7. These figures also show the analogue concentrations required to completely inhibit the growth of the non-resistant cell line, PyY.

The 8-azaadenine resistance curve is of interest here, because it shows the highest degree of resistance found for any of the genetic markers involving purine pathways. It was also the result of multistep selection (fig. 3.4) and no revertants were ever found in autoradiographs of cells which had been exposed to ^{3}H-adenine, the substance of which 8-azaadenine is an analogue. The 8-azaadenosine resistance marker was one which proved difficult to develop. The cells whose resistance curve is shown in fig. 3.6 showed a perceptible background of incorporation in autoradiography after exposure to ^{3}H-adenosine, and showed an unacceptable frequency of reversion (see section 3.3e). This marker was not used in most of the transformation studies, although cell lines resistant to 50-60 µg/ml. of 8-azaadenosine have subsequently been developed.

The cell lines show a full range of cross-resistance to other analogues (see fig. 3.8). Lines resistant to one hypoxanthine analogue were resistant to others to which they had never been exposed. This, coupled with the en-

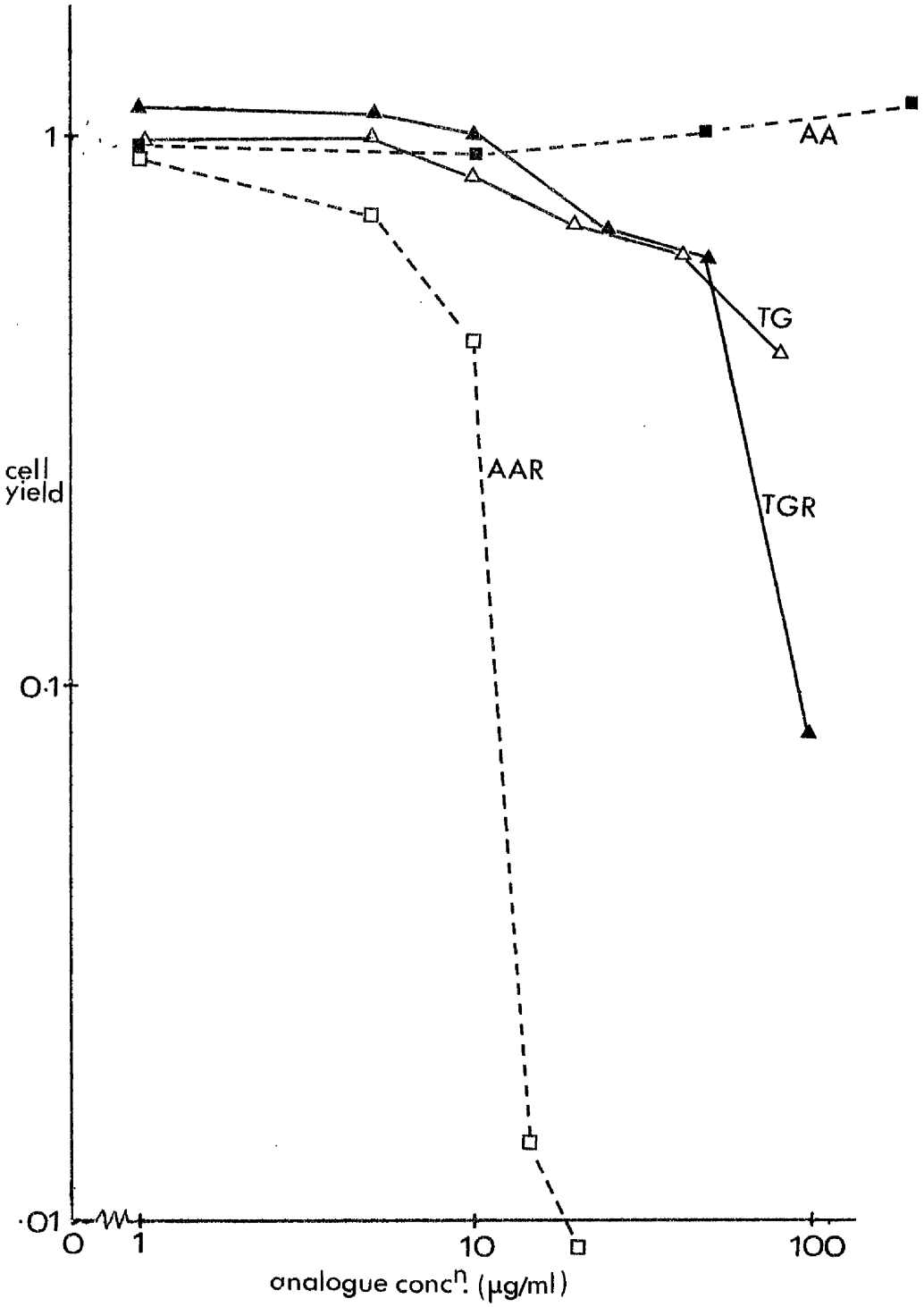


Fig. 3.6 Analogue resistance of PyY/AA/AAR/TG/TGR.Cells were grown in the presence of the single analogues for one week, and cell growth determined by counting the total number of cells in each dish. These were normalised to control (no analogues) = 1·00.

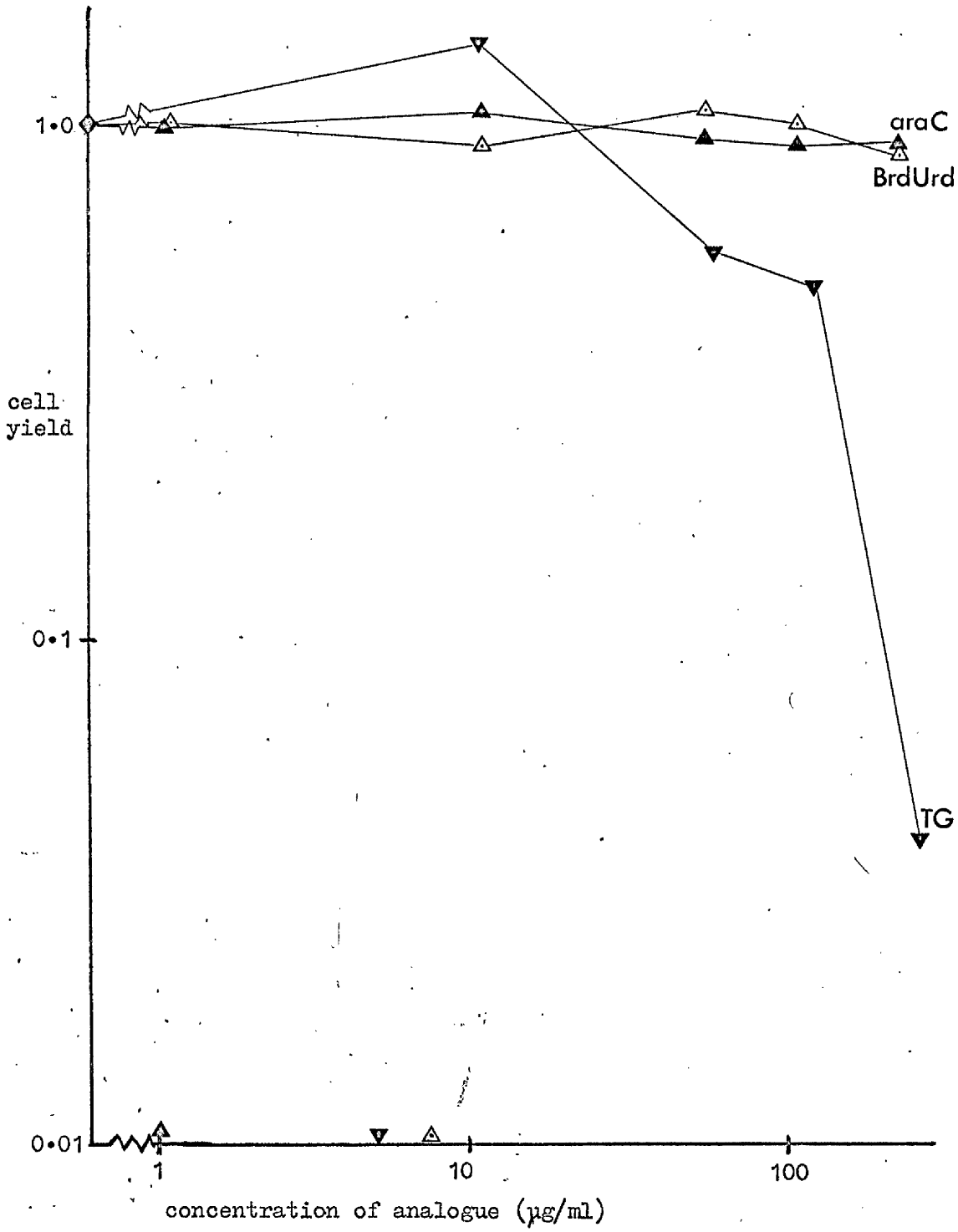


fig. 3.7. Analogue-resistance of PyY/TG/CAR/BUDR. Details are as for figure 3.6.

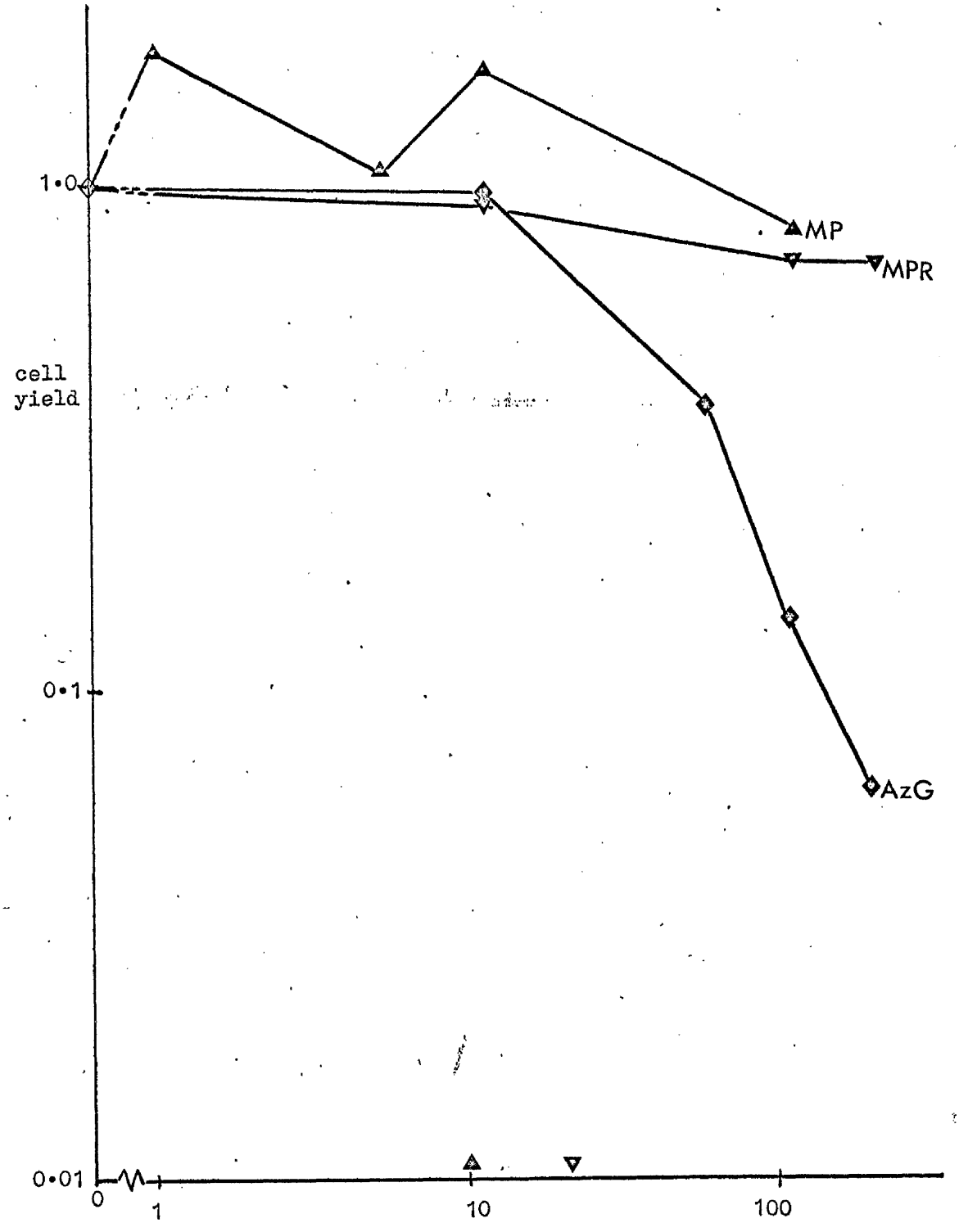


FIG.3.8. Resistance of PyY/AA/AAR/TG/TGR cells to mercaptopurine, azaguanine and mercaptopurine riboside, analogues to which the cell line had never previously been exposed. Details were as for fig. 3.6

zyme assays (Mrs. Llonwyn Edwards, personal communication) described in section 3.3c. below, confirms the mechanism of resistance.

3.3b: Autoradiography

The incorporation of radioactively-labelled bases and nucleosides into cellular acid-insoluble material was checked using autoradiography (section 2.5). It is found in all cases that the cells are unable to incorporate the compounds to whose analogue they had become resistant (see fig. 3.9).

3.3c: Enzyme Assays

Many of the mutant cell lines have been tested for enzyme activities by use of in vitro assays. This work was all carried out by Mrs. L. Edwards of this department (unpublished data). All the lines resistant to 8-azaadenine were totally deficient in adenylate pyrophosphosylase, and all those resistant to 6-thioguanine in inosinate-guanylate pyrophosphosylase. A similar situation was found in bromodeoxyuridine- and cytosine arabinoside-resistant cells, which were deficient in thymidine kinase and deoxycytidine kinase activity respectively (see figs. 3.1 and 3.2). These latter two enzymes are unstable, during extraction, and while the enzyme assays were carried out under the most gentle conditions, the enzyme assays do not exclude the possibility that there is a low level of activity remaining. The very high resistance of the cells to the two analogues in vivo and the absolute lack of incorporation of thymidine or deoxycytidine in autoradiographs are both consistent with total absence of enzyme activity.

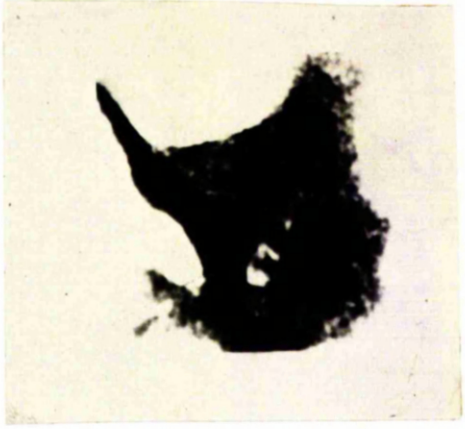
The situation in the purine nucleoside analogue resistance markers is not so clear. The enzyme assays show that there are normal adenosine kinase and guanosine kinase activities even in 8-azaadenosine and 6-thioguanosine-resistant cells. Examination of the incorporation of ^{14}C-adenosine into AAR-resistant cells shows that it reaches a level of 5% of the incorporation into PyY, and that the ^{14}C activity is found in nucleic

Table 3.2 Analogue resistance of PyY and the biochemically-marked cell lines. See figs. 3.6, 3.7, 3.8.

CELL LINE	6-TG	6-TGR	8-AA	8-AAR	aCyt	BrdUrd
PyY	5	1	5	2		7
PyY/AA/AAR/TG/TGR	100	80	500	10	-	-
PyY/TG/CAR/BUDR	100	-	5	-	10^3	10^3

The figures given are the concentrations in µg/ml at which the cells are inhibited to 50% of their growth in the control cultures.

a. clump of cells. ^{3}H-hypoxanthine label. See also fig.3.5, in which the cells are labelled with ^{3}H-adenine (upper) or ^{3}H-thymidine (lower).



b. clump of cells. ^{3}H-uridine label.

Fig. 3.9: Autoradiographs of cells which had been grown in the presence of tritiated purines, showing the low level of incorporation into PyY/AA/AAR/TG/TGR cells.
Deatils of technique are on pages 54-56.

acid as well as in the intracellular nucleosides and nucleotides. The results suggest that the resistance to AAR in these cells is attributable to a deficiency in a specific permease.

These findings are summarised in table 3.1.

3.3d: Metabolic cooperation

The cell lines used in this work are closely related to those in which metabolic cooperation was first observed (Subak-Sharpe, Burk and Pitts, 1966, 1969; Subak-Sharpe, 1969). This phenomenon was found in mixed cultures of IPP^+ and $HGPRT^-$ cells which had been exposed to labelled hypoxanthine, and examined by autoradiography. The $HGPRT^+$ cells were heavily labelled, and the $HGPRT^-$ cells were not labelled at all, but there was a third class of cells which were very lightly labelled. These were always found to be in contact with labelled $HGPRT^+$ cells. $HGPRT^-$ cells close to $HGPRT^+$ but not linked to them by cell-to-cell contacts showed no labelling. The ultrastructural basis of this intercellular communication has been only partly investigated (Subak-Sharpe, 1969; Gilula et al., 1972), but the evidence is that it is connected with the appearance of "gap" junctions which form the low-resistance intercellular pathways that facilitate the passage of ions from cell to cell (Potter et al., 1966; Furshpan and Potter, 1968; Reese et al., 1971; Loewenstein, 1966; Sheridan, 1970).

There are several possible molecular mechanisms for metabolic cooperation (Subak-Sharpe, 1969; Pitts, 1971), and these are divided on the basis of the type of molecule that is transferred from one cell to the next.

Firstly, there could be a transfer of radioactive nucleotide formed by Phophorylation in the $HGPRT^+$ cell. It has been found (Subak-Sharpe, 1969 and unpublished data; Liebman and Heidelberger, 1955) that nucleotides cannot enter these cells without the loss of the phosphate group. This means that nucleotide transfer would require very close cell to cell contact, possibly with cytoplasmic continuity.

A second possibility is the transfer of nucleic acid. This may either be labelled nucleic acid from the $HGPRT^+$ cells, or informational nucleic acid which is biologically active and hence can direct the synthesis of HGPRT in the $HGPRT^-$ cell. In this latter case it would not matter whether the nucleic acid was itself labelled or not.

As it has still not been demonstrated that the lack of HGPRT activity in the mutant cells arises from a structural mutation, it is possible that metabolic cooperation is the result of the passage of regulatory substances.

Finally, the molecule that is passed to the $HGPRT^-$ cells could be a polypeptide. That is, molecules of active HGPRT could be passed.

There is now some evidence that it is the first of these mechanisms that is operating in cooperating cells. That is, that there is a transfer of nucleotide, which could be the immediate HGPRT product or one of the substances derived from it (fig. 3.1). Pitts (1971) approached this question by growing $HGPRT^+$ and $HGPRT^-$ cells in a confluent mixed culture. This was dispersed and divided into two in the ratio 99:1, and the two parts seeded into separate culture vessels. Each culture was exposed to ^3H-hypoxanthine for eight hours and immediately fixed for autoradiographic examination. Grain counts showed that the sparse culture contained two distinct cell populations, one showing very little or no labelling and the other showing the grain distribution characteristic of $HGPRT^+$ cells. The confluent culture formed from the 99% portion of the original unlabelled mixed culture contained only incorporating cells. From this, Pitts concludes that the ability to incorporate hypoxanthine that is passed to $HGPRT^-$ cells by metabolic cooperation is very unstable. Further experiments showed that the ability of $HGPRT^-$ cells to incorporate hypoxanthine was lost within 10 minutes of their separation from the $HGPRT^+$ population. Cox, Kraus, Balis and Dancis (in press) report similar experiments, carried out on cooperation between human Lesch-Nyhan cells ($HGPRT^-$; Seegmiller, Rosenbloom and Kelley, 1967)

and hamster cells (BHK21), which are $HGPRT^+$. They find results very similar to those of Pitts (1971), and conclude that the cells are transferring nucleotides. This is also consistent with their own earlier work (Cox et al., 1970) on cooperation between human cells in culture.

The PyY/AA/AAR/TG/TGR cells used in the present work showed metabolic cooperation in all four of the markers. The AA- and TG-resistance markers were those in which the phenomenon was first observed and the generality of the process has not been confirmed. For example, Cox et al (in press) find that G-6-PD deficiency is not corrected by contact with normal cells. The photographs in fig. 3.10, however, do show that the AAR- and TGR- resistance markers can also display metabolic cooperation.

3.3e: Revertants

In the course of the experiments aimed at the detection of transformants, it became obvious that the populations of mutant cell lines contained a small proportion of cells that could incorporate nucleic acid precursors that the bulk of the cell population could not by virtue of the enzyme deficiencies imposed on them in the course of the selective procedure. These incorporating cells were present at a frequency of about 5.10^{-4} in the TG marker ($HGPRT^-$) and about 10^{-3} in the AAR marker (see section 3.3c). There were no cells which incorporated ^3H-adenine, so that the corresponding frequency for the AA ($APRT^-$) marker was zero. The cells which displayed these frequencies had been grown for about 200 generations without prolonged exposure to selective conditions. The incorporating cells could have arisen from the infection of the cells with an organism, possibly a mycoplasma, which had the capacity to produce the missing enzyme activity, or from an inadvertant mixing of different cell lines or they could be revertants.

The possibility of the mixing of cell populations can be excluded because of the total lack of adenine incorporating cells. This would mean that the contaminating cells would have to be PyY/AA and no such cell line was handled. In addition, the usual precautions were taken to

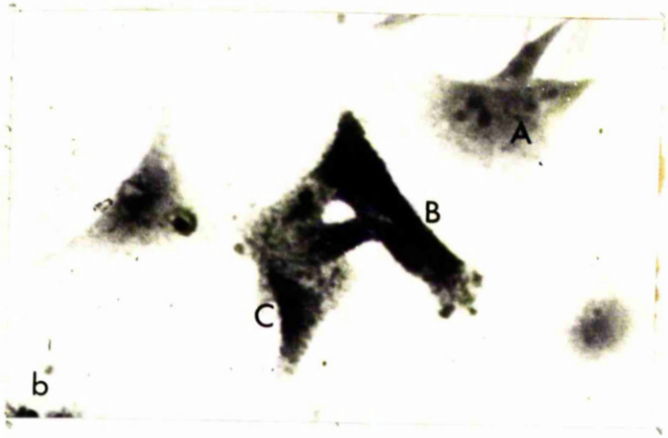


Fig. 3.10: Metabolic cooperation in the purine metabolism markers. Nonincorporating cells are PyY/AA/AAR/TG/TGR. Giemsa stain.

a. ^{3}H-adenosine incorporation. Note the relatively high background in the cell **A.**

b. ^{3}H-**guanosine** incorporation.

A. nonincorporating.

B. incorporating.

C. cooperating.

avoid accidental contamination by media, glassware and so on. The only other cell lines handled were PyY and BHK21/C13, both of which incorporate adenine.

Infection of the cells with Mycoplasma can also be ruled out because incorporating cells were present in cultures which were Mycoplasma-free by the orcein staining method (section 2.1). More importantly, some cultures which were known to be Mycoplasma contaminated were free of incorporating cells when they were labelled with ^{3}H-hypoxanthine. Labelling with ^{3}H-thymidine revealed considerable levels of cytoplasmic label in Mycoplasma-infected cells.

In an effort both to isolate clones that were free of any incorporating cells and to confirm that the incorporating cells that had already been observed were in fact revertants and not contaminants, the PyY/AA/AAR/TG/TGR cells were cloned under stringently selective conditions using the agar suspension culture technique described in section 2.1.

The population of cells that was showing incorporating cells at the frequencies given above were suspended in agar and poured onto 5 cm. petri-dishes at 10^{3} and 10^{4} cells per dish. The basal agar had been made up to contain the four types of analogue to which the cells were resistant, at the following final concentrations after diffusion into the top agar.

8-aza-adenine	50 µg/ml
8-aza-adenosine	10 µg/ml
6-thioguanine	50 µg/ml
6-thioguanosine	40 µg/ml

Antibiotics were also added to suppress mycoplasma (section 2.1e). After 10 days growth, the plates had developed many colonies. Those which had received 10^{4} cells contained so many colonies that it was not possible to pick them without risk of taking cells from more than one colony at a time, so all the colonies picked for further growth were selected from the plates which had been seeded with 10^{3} cells. The

large numbers of colonies that were formed confirms that the bulk of the inoculum was resistant to all four analogues. Thirty of the larger and more isolated colonies were picked and transferred to 3 cm. petri-dishes each containing 3 ml. of freshly made Eagle's/10% foetal calf serum (EFC10). The clones were subsequently identified by numbers ranging from 1 to 30. They were grown at 37°C in an atmosphere containing 5% carbon dioxide, and were inspected for growth daily. The media were changed every two days, with precautions to prevent inadvertant mixing of the clones. Three plates did not have any cells at all. Either the attempt to pick the colony failed or the cells were not viable in those dishes. Of the remaining clones, one was accidentally lost and one was discarded because of its slow growth and abnormal morphology. The others were passed to 8 oz. medical flats as they became confluent. The 8 oz. bottles were incubated until the cells were near confluence (about 4-5 x 10^6 cells per bottle). At this point the cells were suspended in EFC10 and some were placed in 5 cm. petri-dishes at 1-2 x 10^5 cells per dish. Each dish held five 13 mm. diameter coverslips. These cells were labelled overnight with tritiated hypoxanthine, guanosine, adenine and adenosine in separate dishes. The remainder of the cell suspension was passed into a 20 oz. bottle, in which they were grown to confluence before being suspended in eagle's-serum-glycerol for freezing and storage until they were needed. The labelled cells were examined by autoradiography for the presence of incorporating cells. These cell stocks had been grown through approximately 22-23 generations from single cells in the stock used to inoculate the agar plates.

All the cell lines incorporated ^{3}H-uridine normally. Of the clones examined by inspection of individual cells under medium power magnification, only three showed no incorporating cells in all markers. This is the result of very exhaustive examination of about 10^5 cells by systematic scanning of every cell on the coverslips. Eight clones showed the presence

of hypoxanthine-labelled cells, and three showed guanosine incorporation. This result for guanosine is probably a minimum estimate because the stock of labelled guanosine gave unusually light labelling in the control (PyY) cells. The reason for this was not apparent. The 8-AAR resistance marker was tested by looking for ^{3}H-adenosine incorporating cells, and these were found in 10 clones, one of which (clone 19) was almost exclusively composed of incorporating cells. Of course, this does not mean that all the cells were genotypically able to utilise adenosine, because in all the labelled cultures the incorporating cells were surrounded by a group of cells which, from their numbers and their relatively light labelling, were most probably involved in metabolic cooperation.

The actual numbers of incorporating cells found in individual coverslips was usually very low, varying between one and five in most cases. Although the cells had only been growing on the coverslips overnight, the groups of incorporating cells contained up to 20 cells each. Within these groups, one or two cells that were more heavily labelled than the rest were often discernible, and this was interpreted as indicating active metabolic cooperation between the revertant cells and the nonrevertants. Such metabolic cooperation was found in the three markers which showed reversion. The 8-aza-adenine resistance of the cells appeared to be sufficiently stable to make detection of revertants impossible. No adenine incorporating cells were found in any of the clones, and this is consistent with the observations made in the course of the biological experiments involving DNA or polyoma virus. In none of these experiments were any adenine-incorporating cells found. This is a multi-step mutation, and is so stable that it may well represent a deletion of the structural gene for APRT.

An estimate of the reversion rates in the various mutations was made. This is not precise, but it is based on the total number of cells examined in all clones, the number of generations that the clones had

gone through and the total number of incorproating cells that were found.

TABLE 3.3; Number of revertant cells found in clones of PyY/AA/AAR/TG/TGR.

CLONE NO.	UR	Hx	GR	A	AR
1	+	1	0	0	20
3	+	4	3	0	5
4	+	0	0	0	4
7	+	0	0	0	0
8	+	0	0	0	2
9	+	4	0	0	5
10	+	0	0	0	1
13	+	0	0	0	0
14	+	1	0	0	0
11	+	0	0	0	1
16	+	6	0	0	0
17	+	4	0	0	2
19	+	12	2	0	*
21	+	4	1	0	2
23	+	0	0	0	0
		36	6	0	42*

* clone 19 contained too many incorporating cells for counting (at least 200 per coverslip).

The figures in table 3.3 are based on the systematic scanning of about 2×10^6 cells, and do not include lightly-labelled cells that seemed to be in metabolic cooeration with each scored revertant. Calculations of the reversion rates were made by the methods of Luria and Delbruck (1943) and Breeze and Subak-Sharpe (1967).

1. $\mu = \frac{m.\ln 2}{N}$ $\quad m = -\ln(C_o/C_t)$ $\qquad$ 2. $\mu = \frac{2h.\ln 2}{N.g}$

μ=mutation rate per cell per division; N= average no. of cells per clone; C_o= number of clones with no revertants; C_t=total number of clones; h= average number of mutants per clone; g= number of cell generations since isolation of the single cell.

RESISTANCE MARKER	REVERSION RATES. METHOD 1	METHOD 2
AA	nil	nil (<0.02)
AAR	5.19	1.42*
TG	3.97	1.03
TGR	1.17	0.19

*excluding clone 19 for which there is no quantitative data.
All figures are $\times10^{-6}$. Approx. 1.33×10^5 cells/clone.

REFERENCES: Breeze and Subak-Sharpe; J.gen.Virol., 1, 81 (1967)

Luria and Delbruck; Genetics, 28, 491,(1943).

Fig. 3.11: Autoradiographs of some of the purine marker revertants in clones of PyY/AA/AAR/TG/TGR cells. See section 3.3e, p.71.
a. ^{3}H-hypoxanthine label; b. ^{3}H-adenosine label.

- FOUR -

BIOCHEMICAL INVESTIGATION OF DNA UPTAKE

Biochemical studies on the uptake of radioisotope-labelled DNA were undertaken to obtain information that would be both complementary to the genetic transformation work and helpful in designing the genetic experiments.

The experiments were carried out using several different cell lines, including analogue-resistant PyY lines and the untransformed BHK21/C13 line. The DNA used was labelled with thymidine-methyl-^{3}H (table 2.3). The specific activities of DNA preparations were determined using the channels-ratio method (section 2.2c) and varied between 2.5 and 8.0 x 10^{4} dpm/µg (11-36 µCi/mg) from preparation to preparation. An estimate of the sensitivity of the system, assuming a DNA of specific activity of 5 x 10^{4} dpm/µg and a tritium counting efficiency of 22% (section 2.2d) indicates that 10^{-2} µµg of absorbed DNA per cell would give about 40 cpm above background. BHK21/C13 cells contain about 7 x 10^{-6} µg of DNA per nucleus, so that the detection of DNA uptake to the extent of 0.2% of the cell DNA complement is possible.

A number of published papers have claimed that the uptake of DNA by mammalian cells in culture is a very rapid process, being complete in a few minutes (Glick, 1967a; Ayad and Fox, 1968). Accordingly, the early experiments were carried out with time scales of up to one hour. In these experiments, cells were grown in monolayer culture in petri-dishes and were exposed to solutions of labelled DNA in various buffers at 37°C. The temperature was maintained by carrying out the entire experiment in the hot room, so that there would be none of the cooling effects caused by frequent opening of the incubator door. The dishes were kept in a humid atmosphere and, in the case of those cells in Eagles medium, in the presence of 5% carbon dioxide to prevent fluctuations of pH. DNA-^{3}H concentrations of up to 100 µg/ml were used and the medium was tris-saline, PBS (table 2.1) or Eagles medium. At the appropriate time, the DNA-^{3}H solution was withdrawn and the cells thoroughly washed in PBS before being incubated in saline/

0.005M. magnesium chloride containing 50 µg/ml of DNase for 5 minutes at 37°C. The cell sheets were washed three times with saline and brought to a single-cell suspension by scraping them off the substrate and by vigorous pipetting. The cell suspension was lysed by adjusting it to a final SDS concentration of 0.5%, and the nucleic acids were precipitated by adding TCA to a final concentration of 5% and storing at 4°C for several hours, in the presence of 100 µg of unlabelled DNA. The precipitates were collected by centrifugation, washed in cold 5% TCA, and hydrolysed in a small volume of concentrated formic acid at 100°C. After dilution, an aliquot of the hydrolysate was added to Bray's scintillant for assay of the radioactivity. In the samples taken up to 1 hour after addition of ^{3}H-DNA to the cells, no radioactivity could be detected. There has been a report (Schimizu et al., 1962) that DNase can penetrate into the cytoplasm of cells in a functional form, so further experiments were carried out in which the incubation of the cells with DNase was omitted, and cells simply thoroughly washed and lysed, with the DNA precipitated as described above. Even in these conditions, there was no detectable radioactivity in the cells after one hour of incubation with ^{3}H-DNA. To exclude the possibility that DNA was being lost at some stage of the extraction procedure, a much simpler procedure was adopted. In this, the cell suspension was applied directly to Whatman 2.4 cm filter paper discs and dried. The discs were washed in two changes of 5% TCA and finally in ether, before being dried and immersed in toluene-PPO-POPOP fluid for radioactivity assay (section 2.2e). In the course of this simplified sample preparation, the only DNA that should be lost is that which is washed off the surface of the cells before they are dried onto the disc, and that which is of a sufficiently small molecular weight to be soluble in the TCA washes. This means that only very loosely adsorbed molecules and those which are less than about ten nucleotides in length should be lost. Despite the use of this procedure and the extension

of the sampling period to two hours, no radioactivity could be detected in the cells. The main point to emerge from these experiments was that macromolecular DNA does not appear intracellularly as rapidly in BHK21 or PyY cells as has been reported for other cell types. This does not exclude the possibility that DNA is absorbed and immediately degraded to small oligonucleotides, but obviously DNA absorbed in such a way can have no genetic significance.

The absence of early DNA uptake was also evident in experiments similar to the above but in which DEAE-dextran was present along with the ^{3}H-DNA during uptake. It has been reported (McCutchan and Pagano, 1968; Warden, 1968) that DEAE-dextran will increase the infectivity of a polyoma DNA preparation by a factor of up to 10^{4}, so that this is one obvious material to use in attempts to detect genetical activity in mammalian DNA preparations. One drawback of DEAE-dextran is that it forms insoluble complexes with DNA so that there is a level of concentration above which it cannot be used. With the mammalian DNA preparations used in the present work, visible precipitates were formed when DEAE-dextran and DNA were mixed at a final concentration of 10 µg/ml. each. All the experiments on DNA uptake in the presence of DEAE-dextran, the concentrations of both were 5 µg/ml or less. High concentrations of DNA alone also showed some cytotoxic effect, with the cells rounding up and floating loose after 2-3 hours incubation with the DNA solution.

The consequence of these difficulties and the need to extend the time of uptake to periods of many hours was that lower concentrations of DNA-^{3}H were used, and most of the subsequent experimental work was between 3 and 10 µg of DNA-^{3}H/ml. Keeping the cells in a healthy condition over the longer periods of time also placed restrictions on the composition of the incubation medium, and the first choice was obviously the growth medium, EC10 or EFC10, and this was checked for the presence of DNase activity, which

would damage the labelled DNA before it could be absorbed by the cells. Freshly-made Eagles medium plus calf serum had very little DNase activity (fig. 4.1), but medium in which cells had been grown for 4 days had a considerable amount. This release of DNase by the cells means that it is inevitable that some of the labelled DNA will be damaged or degraded in the medium, but the low level of DNase in fresh medium makes it as suitable, from this point of view, as any of the buffers, and it is obviously better for the maintainence of the cells.

DNA uptake was investigated under a number of different conditions. Firstly, uptake by cells in suspension culture is shown in fig. 4.2. This illustrates the lack of uptake in the first two hours after addition of DNA, but shows a very rapid rise in bound DNA-^{3}H from 4 hours up to 24 hours. This rapid rise and the subsequent plateau region are reproducible in this and other systems, and are observed in both PyY and BHK21/C13 cell lines. The plateau value of $\sim 2.5 \times 10^{-3}$ cpm per cell may be compared with the value of 2.1×10^{-2} cpm/cell calculated from the total amount and specific activity of the DNA-^{3}H and the total number of cells in the culture. As the first observed value is tenfold lower than the calculated value, it seems unlikely that the reduction of DNA uptake is a result of exhaustion of DNA-^{3}H in the medium. The peak value is equivalent to 0.3 μμg DNA per cell, which is about 5% of the total DNA content of the recipient cell.

A second culture method was to grow the cells in monolayers and examine uptake by scintillation counting and autoradiography. The cells could be prepared for liquid scintillation counting either by one of the filter disc methods (section 2.2d) or simply by fixing the cells, after growth on coverslips, according to the procedure used for autoradiography (section 2.5). This has the disadvantage that estimation of cell numbers and counting efficiency is difficult and very unreliable, but also has the advantage that the coverslips can be recovered from the scintillant and subjected to

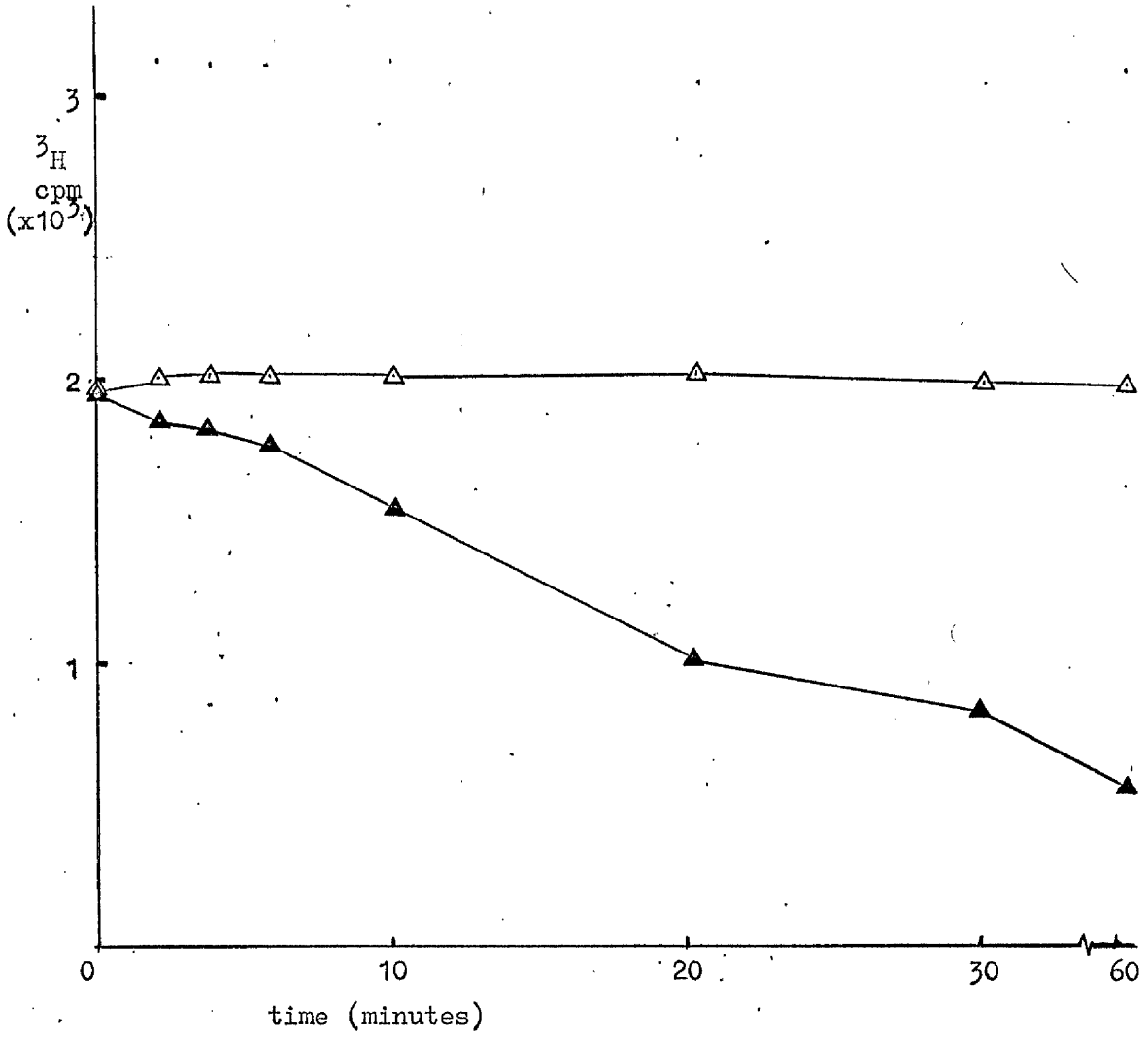


FIGURE 4.1. The nuclease activity of fresh (△——△) and conditioned (▲——▲) Eagle's- 5% foetal calf serum. The conditioned medium was spun at 1,500rpm twice to remove any contaminating cells. To aliquots of the media at 37°C was added DNA-^{3}H at 37°C. The reaction was stopped at appropriate times by the addition of cold TCA to a final concentration of 10%. The precipitates were collected on filters, washed in cold 5% TCA and ether, and dried. Radioactivity was assayed by using toluene-PPO-POPOP fluid.

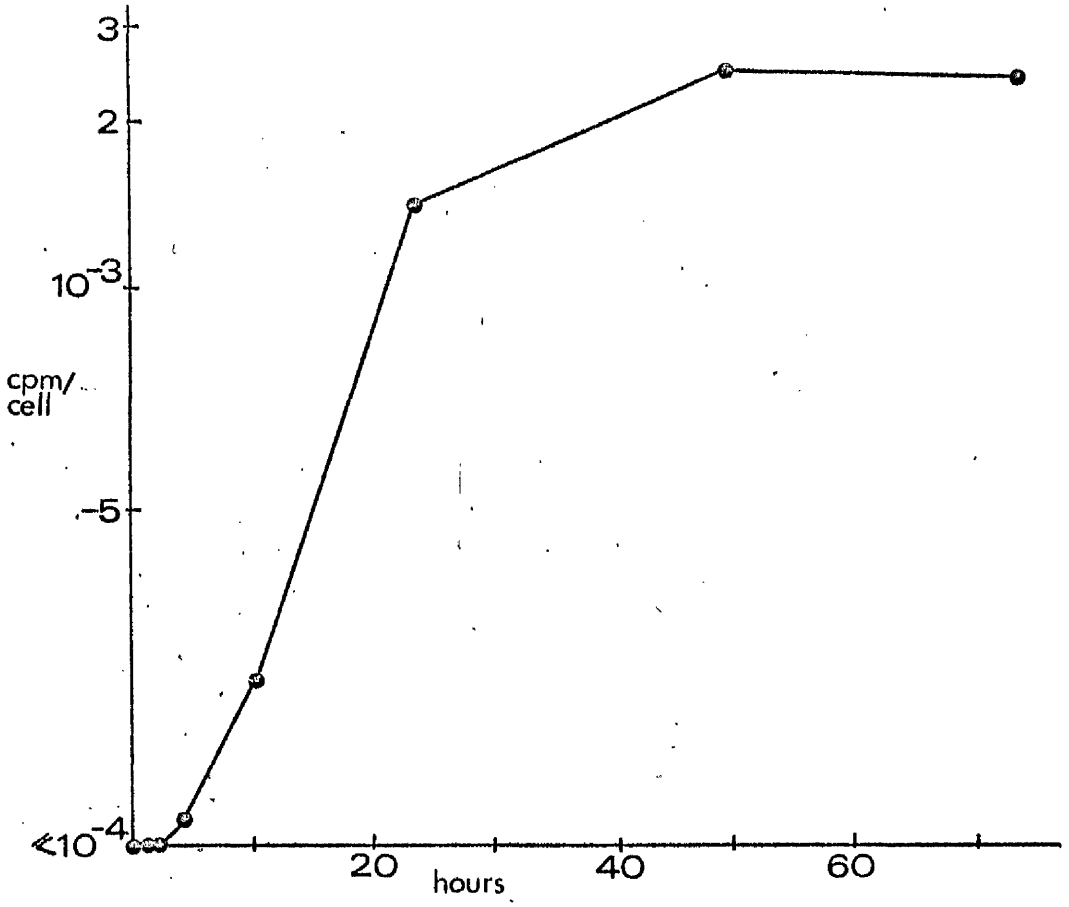


Fig. 4.2 Uptake of DNA by cells in suspension culture.

PyY/AA/AAR/TG/TGR/ cells were suspended in Eagles-10% foetal calf serum at a concentration of 2.10^5 cells/ml. The suspension was placed in a sterile culture flask (fig. 2.1) the atmosphere in the flask being adjusted to 5% CO_2 and the flask placed at 37°C. At zero time, ^{3}H-DNA (sp. act. = 17·7 µCi/mg.) was added to a final concentration of 3·3 µg/ml. At intervals 5ml samples were withdrawn, and the flask regassed. All handling of the cells was at 37°C. The sample was spun down and the cells resuspended in saline with ·005M Mg^{++} and 40µg/ml DNase. This was incubated at 37°C for 15mins. The cells were washed twice in SSC and lysed in 1% SDS. The lysate was sheared mechanically and 50µl samples were dried onto filter discs, which were acid- and ether-washed for radioactivity assay.The cells went through 2-3 doublings during incubation.

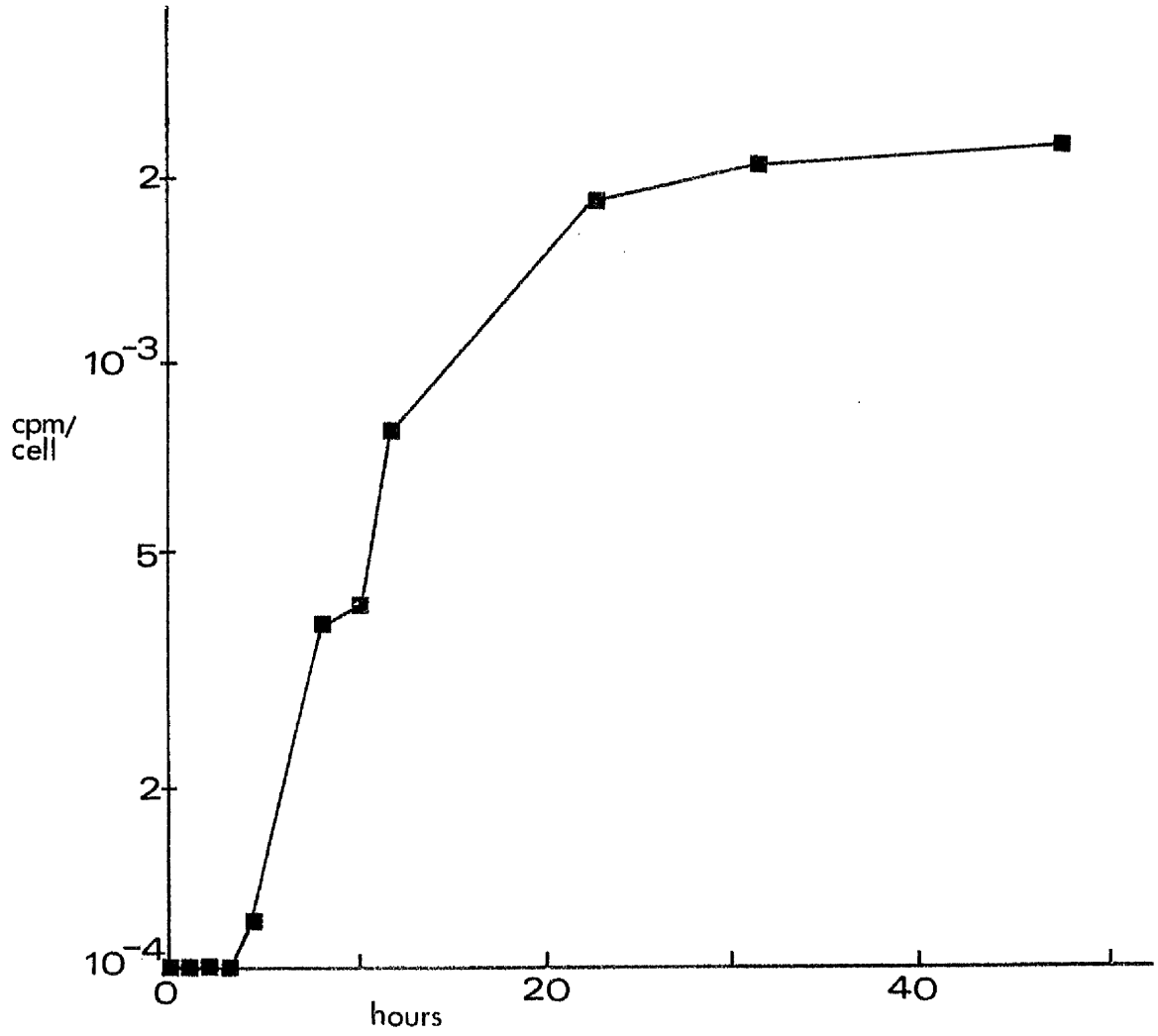


Fig. 4.3 Uptake of ^{3}H-DNA by BHK21-C13 cells in monolayer culture. 3cm petri dishes were seeded with 2.10^{5} cells in Eagles-10% calf serum over washed coverslips. The cells were incubated overnight at 37°C. The cells were washed in PBS and the medium replaced with Eagles-10% calf serum containing 2µg/ml of ^{3}H-DNA (11µCi/mg). At appropriate times, the cells were washed twice in PBS and fixed in formal saline at 4°C. The coverslips were then subjected to the standard autoradiographic washes (section 2.5). Coverslips with uniform cell cover were selected for counting under toluene-PPO-POPOP. The cell counts for cpm/cell calculations were performed on duplicate petri dishes, and cells/coverslip determined using the ratio of areas.

autoradiography after washing in ethanol and drying. Fig. 4.3 shows the results of experiments involving uptake of DNA-^{3}H by cells in monolayers. Once again the lag in DNA uptake is apparent. Fig. 4.4 shows the results of autoradiography carried out on the same coverslips. The interesting feature is the almost total lack of cytoplasmic label, even at the earliest times at which labelling of the cells is apparent. The grain count histograms show a wide variation in labelling from cell to cell, but do show that the vast majority of the cells are perceptibly labelled.

As pointed out in the introduction, autoradiography does not provide any information on the question of whether the DNA survives as intact molecules, or is degraded and reutilised. The lack of cytoplasmic label (fig. 4.5) suggests either that the DNA passes rapidly into the nucleus or that it is broken down to molecules too small to be retained in situ during the acid-washing procedure. When autoradiographs of cells exposed to DNA-^{3}H in the presence of DEAE-D are examined, a striking difference is evident (fig. 4.5). The DEAE-D-treated cells display clumps of labelled material in the cytoplasm and on the cell periphery. Very little label makes its way into the nucleus. This difference between DNA uptake in the presence and in the absence of DEAE-D will be mentioned again in the section on density-labelling experiments.

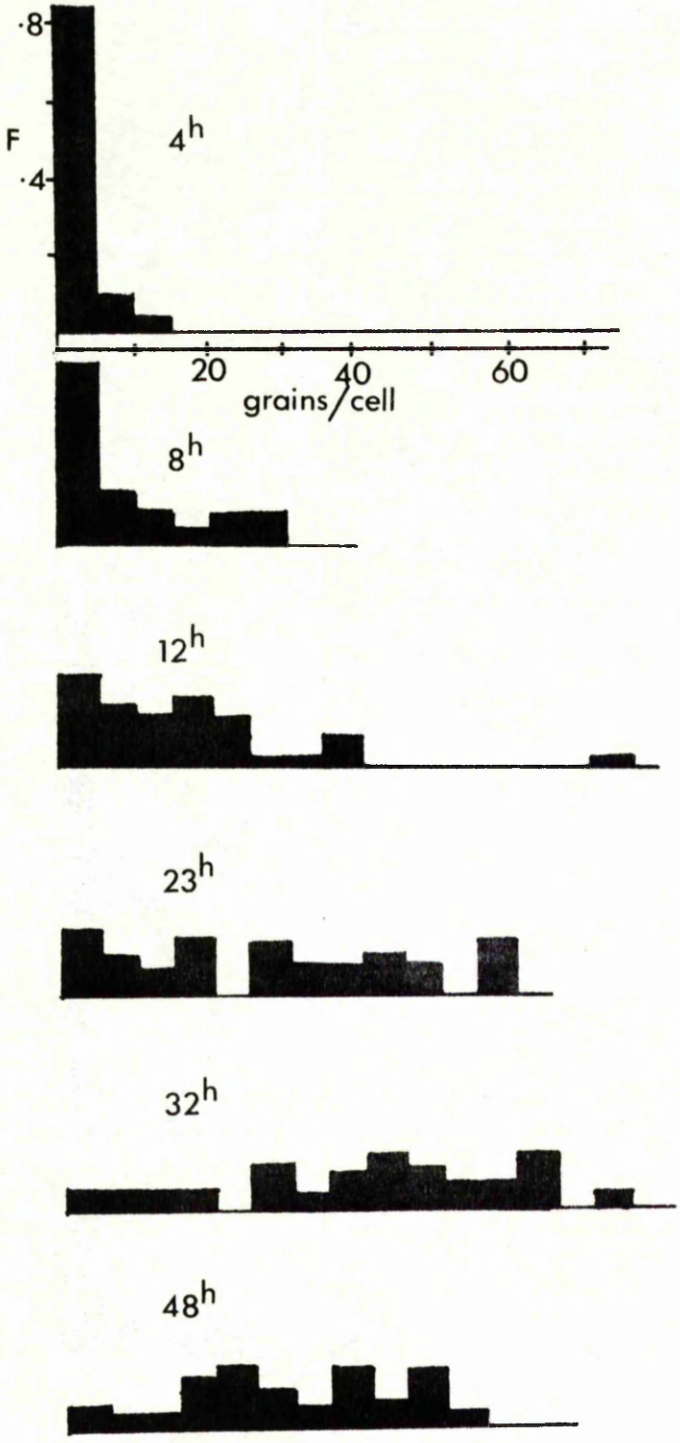


Fig. 4.4 Grain counts from autoradiographs of the coverslips represented in fig. 4.3. F = relative frequency.
Each histogram represents grain counts over 100 cells.

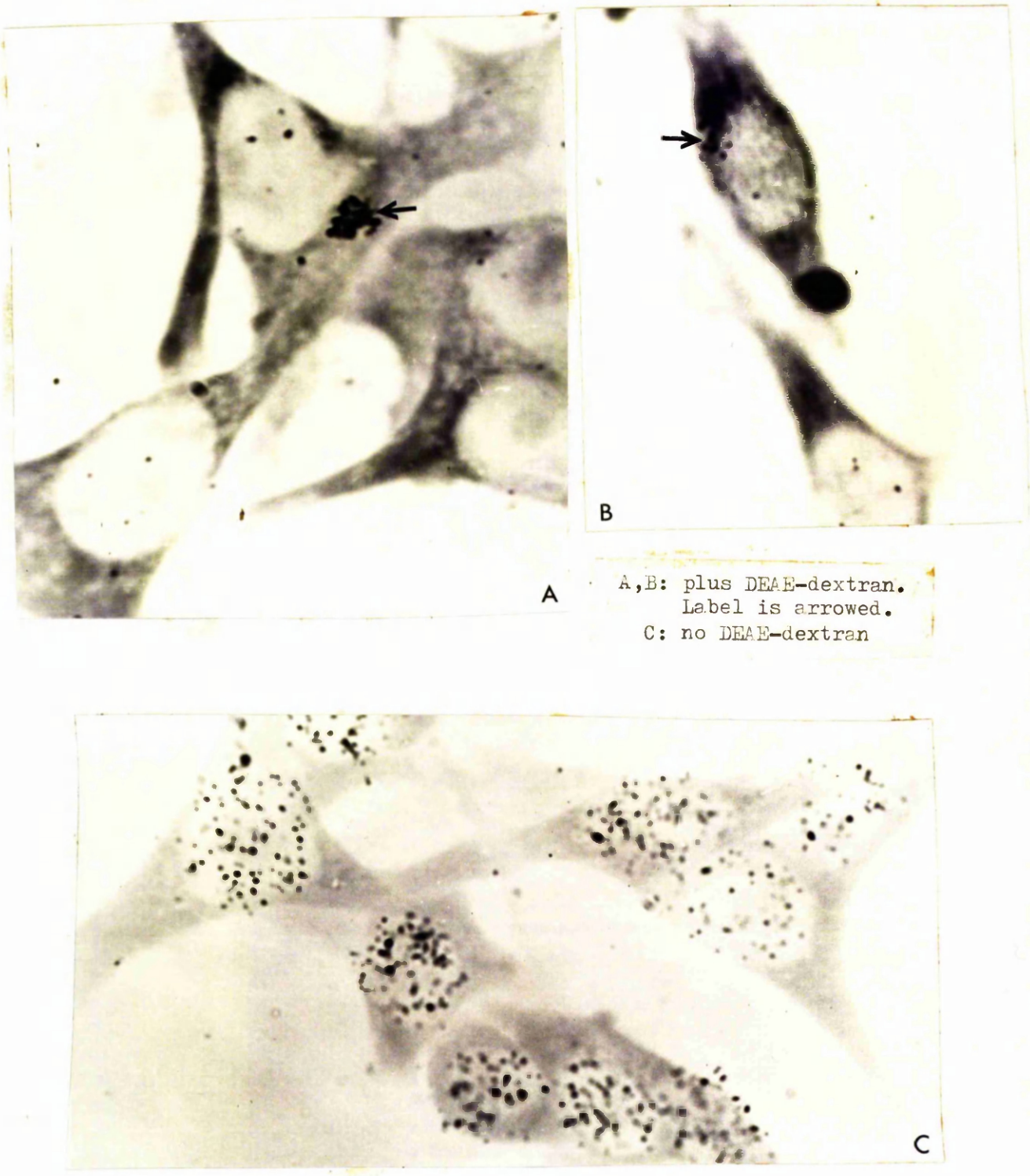


Fig. 4.5:Autoradiographs of cells which have absorbed ^{3}H-DNA in the presence or absence of DEAE-dextran.

Density-Labelling Experiments

Bromodeoxyuridine (BrdUrd) was used as a DNA density-label in experiments designed to provide information on the question of whether the absorbed DNA entered the cells and survived as macromolecules. The method involved labelling the cells with BrdUrd and following the uptake of ^{3}H-DNA which was not density-labelled by means of caesium chloride equilibrium density gradients. The converse approach, the treatment of the cells with BrdUrd-labelled DNA, was not used because of the problems described in section 2.4. The labelling and gradient conditions were such that three DNA peaks could be resolved, representing the unlabelled, the fully density-labelled and the hybrid DNA duplexes. An example of the separation that was possible is shown in fig. 4.6a, which also helps to confirm the interpretation of these peaks. The cells from which this DNA was extracted were grown in the presence of BrdUrd for 2 days, during which time the DNA replicated, giving rise to half- and fully-labelled duplexes (fig. 4.6b). On removal of the BrdUrd, and further incubation, the fully-labelled duplexes replicated to form half-labelled molecules, and the half-labelled, on replication, generated half-labelled and unlabelled duplexes. This is summarised in fig. 4.6b. In fig. 4.6a, the non-BrdUrd-labelled peak (L-L) is labelled only with the ^{3}H supplied in the final 24 hours. The hybrid (H-L) peak has ^{3}H activity in the light chains synthesised in the final 24 hours, and ^{14}C activity in the BrdUrd-labelled chains that were synthesised in the first 72 hours. At the bottom of the gradient can be seen a small peak of ^{14}C activity which represents DNA labelled with BrdUrd in both strands which has not replicated in the final 24-hour period.

In the experiments on the uptake of DNA, the density label was used as a means of distinguishing the donor from the recipient DNA. The buoyant density profile represented in fig. 4.7 was obtained when cells were labelled for 48 hours with ^{14}C-TdR and BrdUrd, and then exposed to ^{3}H-DNA for 24 hours.

Fig. 4.6

An 8 oz. medical flat bottle was inoculated with 2.10^6 PyY/AA/AAR/TG/TGR cells in 15 ml. Eagles-10% foetal calf serum containing 5 µg/ml BrdUrd and 0.05 µCi/ml ^{14}C-TdR (57 µCi/mmole). The bottle was incubated for 2 days after which the cells were washed and incubated for 24 hours at 37°C in 15 ml. Eagles-10% foetal calf serum containing 0.3 µCi/ml ^{3}H-TdR (0.1 Ci/mmole). The cells were washed in tris-saline pH 7.4 and lysed in tris-saline/1% SDS/pH7.4. The lysate was heated to 60°C for 15 mins. and sheared mechanically, after which it was adjusted to refractive index 1.401 with solid caesium chloride. The solution was spun for 3 days at 40,000 rpm and 15°C in a titanium-50 fixed-angle rotor, and the gradient relaxed at 35,000 rpm for 15 hours. Fractions were collected from the bottom of the tube and 50 µl aliquots dried onto filter discs for radioactivity assay.

H-L indicates DNA with one strand BrdUrd-labelled.

L-L indicates DNA with both strands unlabelled.

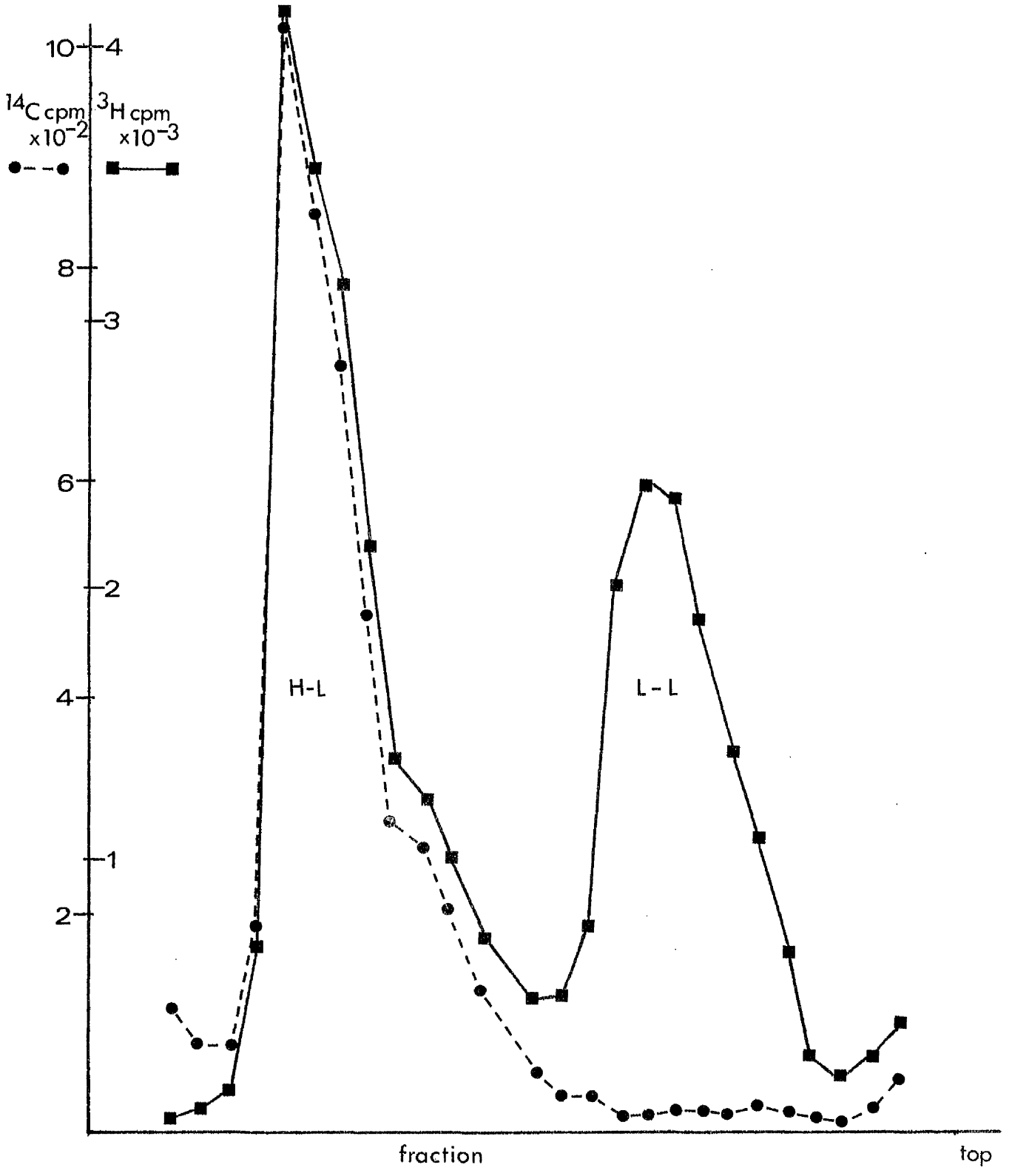


Fig. 4.6 Caesium chloride equilibrium-density gradient of DNA from cells labelled with ^{14}C/BUdR and then with ^{3}H-TdR. Details are given opposite.

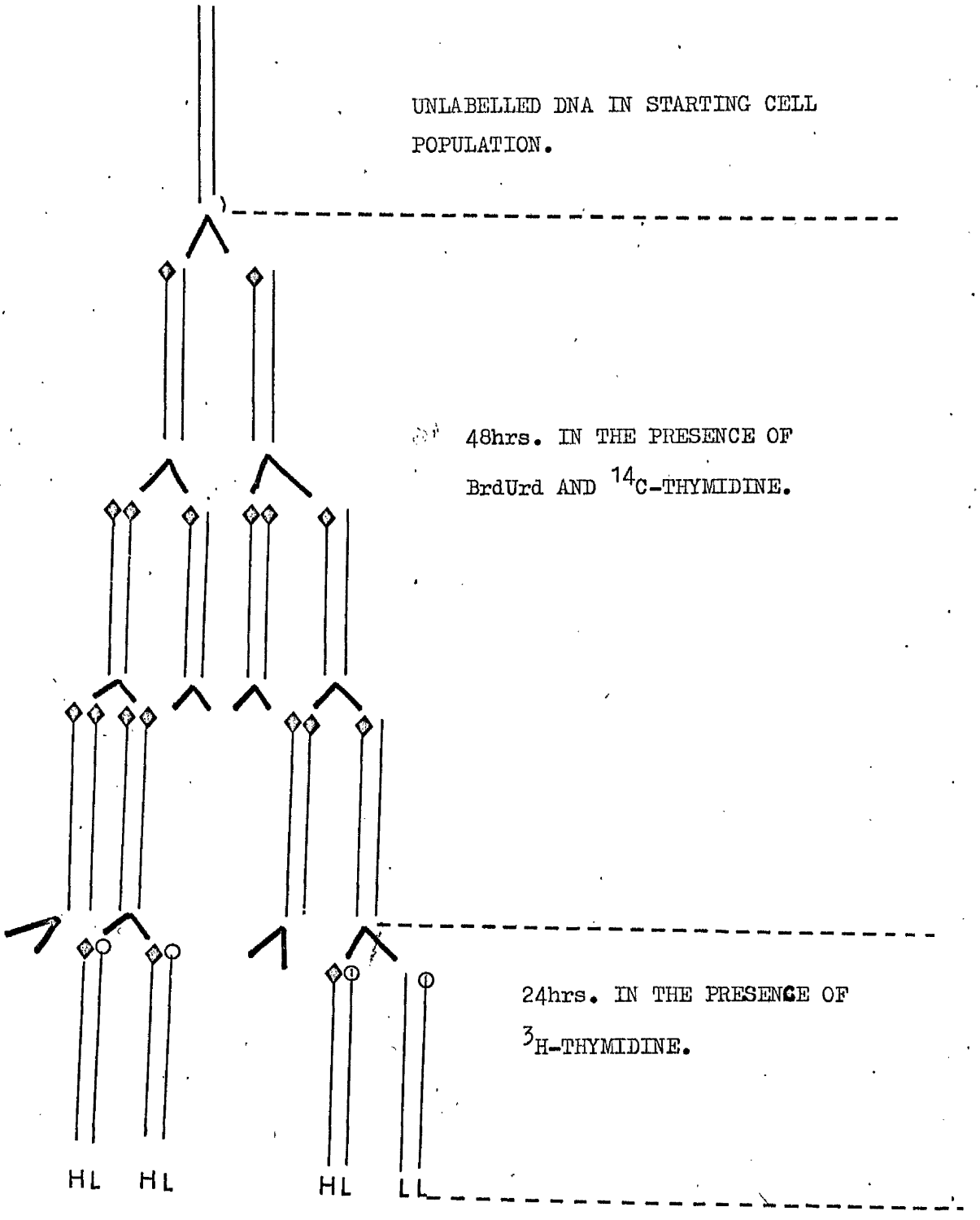


FIG 4.6b; Outline of the labelling of cells with BrdUrd. Conditions and timing is the same as for fig. 4.6a.

———— ; unlabelled polynucleotide strand.

———◆ ; strand labelled with BrdUrd and ^{14}C-thymidine

———○ ; strand labelled with ^{3}H-thymidine.

The fully-labelled and hybrid recipient molecules are evident as peaks in the ^{14}C profile, while the position of DNA which is not density-labelled is shown by the inclusion of a BHK21/C13 DNA marker. Figure 4.8 shows a gradient in which light ^{3}H-labelled DNA was added after lysis of BrdUrd-labelled cells. Once again, the separation of the unlabelled DNA is quite clear. What is evident from the ^{3}H distribution in figure 4.7 is that very little of the donor DNA label remains at the density position it would have occupied had it been added to the cells after they had been lysed. The result of absorption of DNA by BrdUrd-labelled cells in the presence of free BrdUrd is that it becomes associated with density label during its passage to the intracellular acid-insoluble nucleic acid fraction. Parallel autoradiographic experiments described in the previous section indicate that in cells at the same stage of DNA uptake as those used in fig. 4.7, all the donor DNA label is in the nucleus.

Uptake of DNA-^{3}H in the presence of DEAE-dextran presents a different picture, and two parallel caesium chloride gradients which illustrate this point are shown in figures 4.9 and 4.10. Cells were incubated in the presence of BrdUrd and ^{3}H-DNA under conditions which meant that the majority of the recipient DNA was in the hybrid position in the gradients - that is, having undergone one cycle of replication in the presence of BrdUrd. What is striking about the ^{3}H profile after uptake in the presence of DEAE-dextran is that the vast majority of the donor DNA remains in the light band (fig. 4.9). This contrasts sharply with the situation shown in fig. 4.10, in which only a little of the total donor label that is absorbed remains in the light band. Another difference between figs. 4.9 and 4.10 is that the total ^{3}H activity which has been absorbed is very much higher in the presence of DEAE-dextran. While the ^{14}C profiles in the two figures are closely similar, the peak of ^{3}H activity is around 70 cpm in fig. 4.10 and around 700 cpm in fig. 4.9. This increase in the amount of DNA absorbed in the

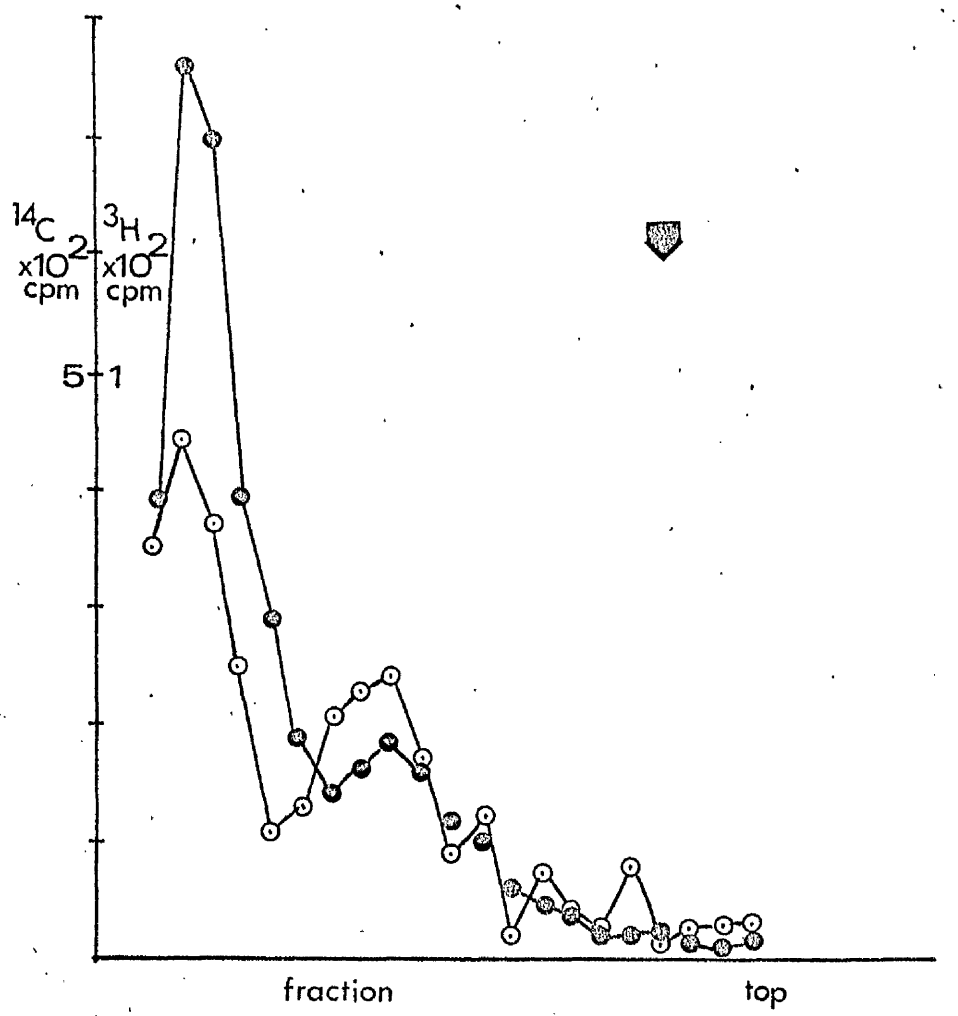


Fig. 4.7; caesium chloride equilibrium-density gradient profile of DNA extracted from BHK21 cells after uptake of ^{3}H-DNA. The cells were grown in 5µg BrdUrd/ml and 0·05 µCi ^{14}C-thymidine/ml for 48 hours, washed, and incubated for 24 hours in EC10 containing 5µg BrdUrd/ml and 2µg ^{3}H-DNA/ml. The cells were thoroughly washed and lysed in tris-saline/1% SDS/pH7·4. The lysate was adjusted to refractive index 1·402 after addition of 200µg of unlabelled BHK21 DNA. The mixtura was spun at 40,000rpm and 15°C for 3 days in a titanium-50 fixed-angle rotor. Fractions were assayed for radioactivity on filter paper discs.

^{3}H ○—○ : ^{14}C ●—●

Unlabelled DNA marker ⛉

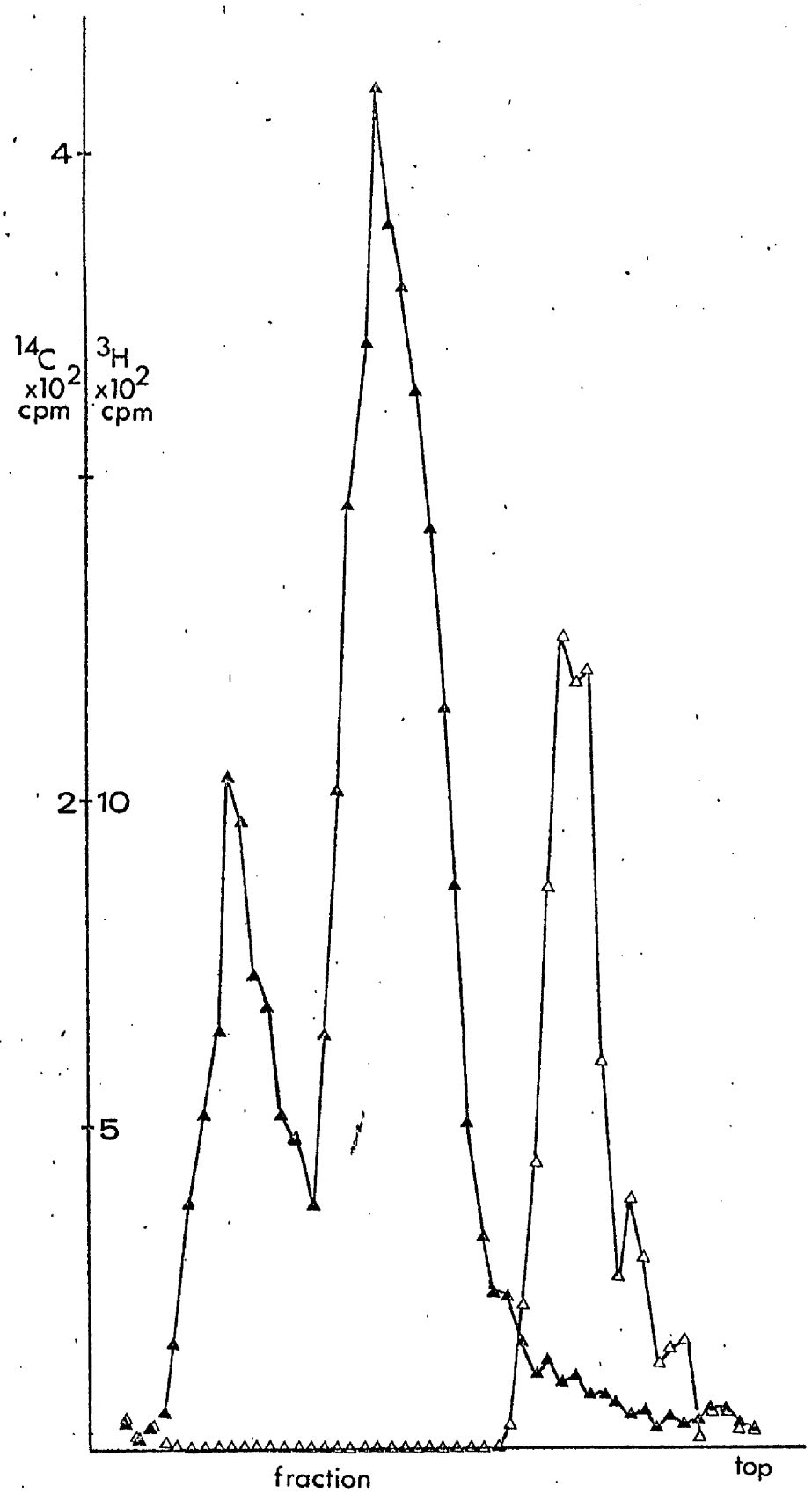


Fig 4.8; Caesium chloride equilibrium-density profile of DNA extracted from cells labelled with BrdUrd (5µg/ml) and ^{14}C-thymidine (0·05µCi/ml). Details as for fig. 4.7. ^{3}H-DNA was added to the cell lysate.

^{3}H △ ; ^{14}C ▲

presence of DEAE-dextran has been observed in other systems (Warden, 1968).

A further point emerges from a comparison of the ^{3}H profiles in figs. 4.7 and 4.10. Although the ^{3}H-DNA has been absorbed under almost precisely the same conditions in the two cases, the final density distributions differ in a way that clearly depends on the way in which the recipient cells have been labelled. In particular, the distribution in fig. 4.7 shows considerably more ^{3}H in the fully-labelled band than would be expected if the donor DNA was all gaining density label independently of the recipient DNA, because the ^{3}H-labelled donor strands are not BrdUrd-labelled and thus would never pass into the fully-labelled band in the course of normal replication. What is indicated here is that the donor DNA is associating with the recipient DNA in such a way that its presence in the duplex does not significantly alter the buoyant density. This could arise either by the total degradation of the donor DNA and the reutilisation of the nucleotides released, or by the insertion of small pieces of donor DNA into the recipient chromosomes.

The uptake of ^{3}H-DNA is affected by other chemical treatments in the course of incubation with the cells. For example, the addition of 50 μM thymidine will diminish the acquisition of donor DNA label by the recipient cells. This concentration of thymidine is well below the level at which the cells are inhibited, but represents a 20-fold excess over the total amount of labelled thymidine that would be released by the total degradation of 2 μg/ml of ^{3}H-TdR-labelled DNA. This again suggests that at least a part of the input DNA is degraded and reutilised as mononucleotides. Cytosine arabinoside (aCyt), a potent inhibitor of DNA synthesis (see section 3.1b), will also considerably diminish the uptake of donor DNA label. Dr. T. Friedmann (personal communication) has found that aCyt will prevent the passage of donor DNA label into the nucleus, but has no effect on its movement into the cytoplasm. In the BHK21 and PyY cell system, however, no label is detectable by autoradiography in either the nucleus or the cytoplasm of cells which have been simultaneously treated with aCyt and

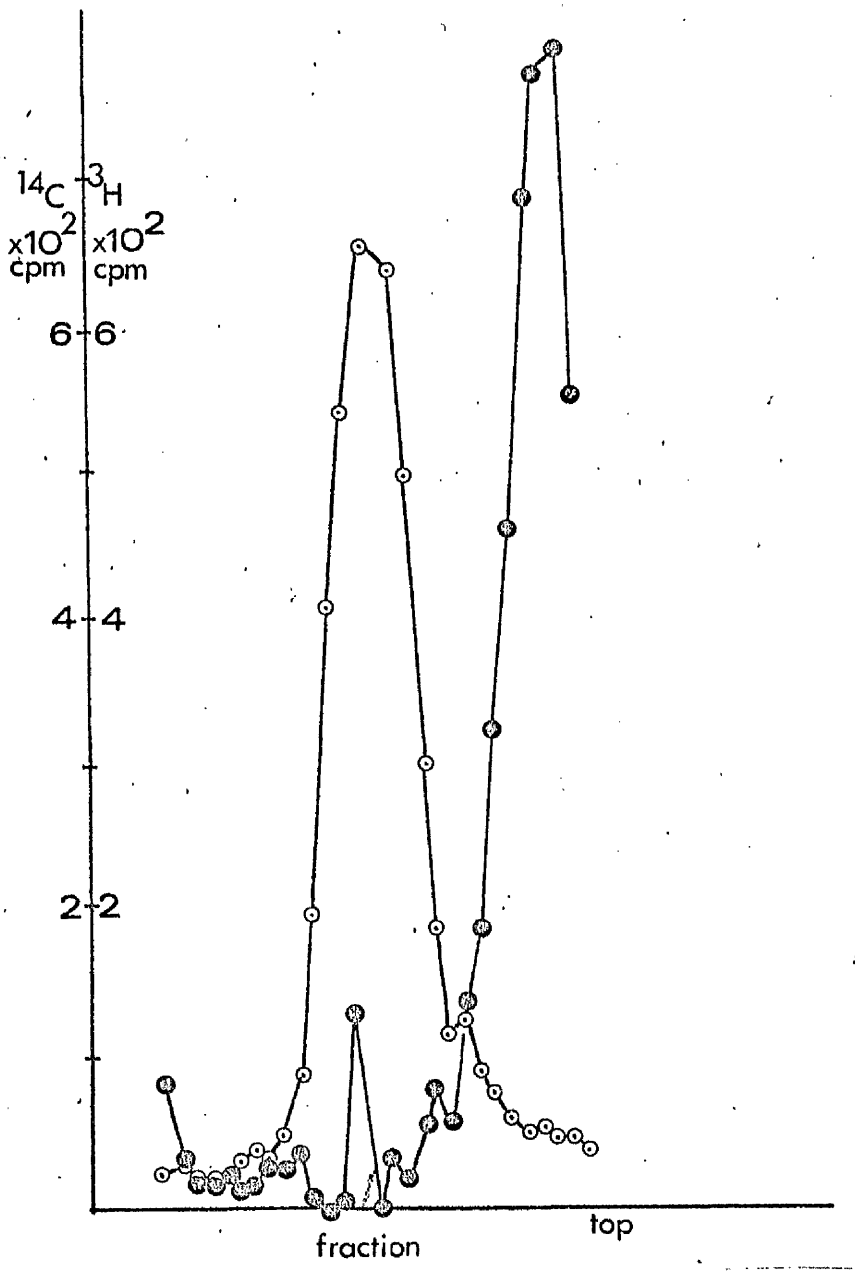


Fig. 4.9; Caesium chloride equilibrium-density profile of DNA extracted from cells labelled with BrdUrd (5µg/ml), ^{14}C-thymidine (0·05µCi/ml), ^{3}H-DNA (2µg/ml) and DEAE-dextran (2µg/ml) in EFC10 for 24 hours. The cells were lysed at that time in the manner described in fig. 4.7. ^{3}H ● ; ^{14}C ⊙

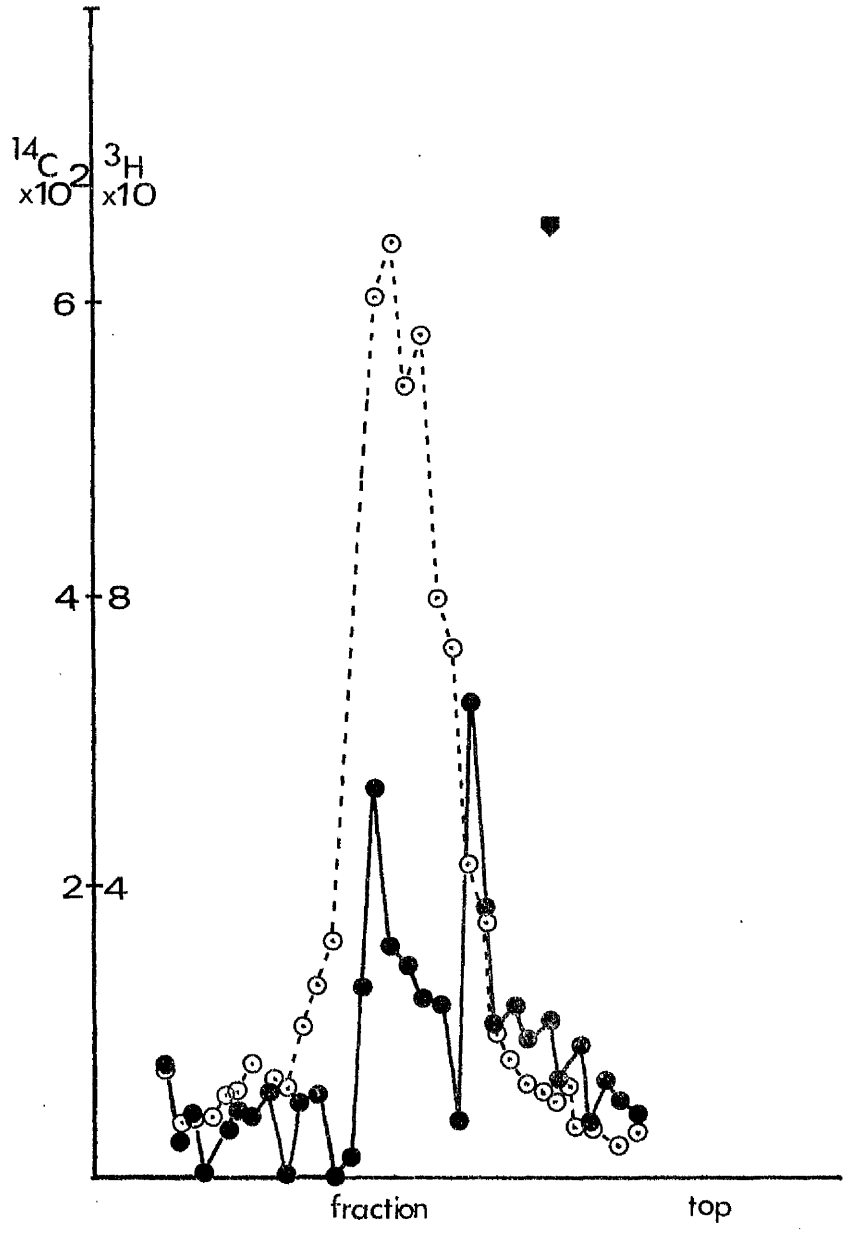


Fig. 4.10 Caesium chloride equilibrium- density gradient of DNA from cells labelled as in fig. 4.9, except that DEAE-dextran was omitted during the incubation.

^{3}H ● ^{14}C ○

^{3}H-DNA.

The presence of DEAE-dextran alters the effect of aCyt on DNA uptake. In fig. 4.11, two equilibrium-density gradients are represented, showing the effect of aCyt on the incorporation of ^{14}C-TdR into recipient cell DNA and its effect on DNA uptake in the same cells. The ^{14}C profiles show the incorporation of thymidine in the presence (open squares) and absence (black squares) of 20 µg/ml of aCyt. The dramatic drop in the count rate is evident. In contrast to this is the small effect on the uptake of ^{3}H-DNA (black triangles) when aCyt is added to the incubation medium (open triangles). There is a considerable reduction in the amount of ^{3}H label which shifts to the density-labelled bands, but the donor DNA which maintains its starting density is, if anything, increased in quantity.

The interpretation of these experiments is not entirely unambiguous, because they are consistent with a DNA synthesis-dependent integration of the donor DNA as well as with the more obvious possibility of breakdown and reutilisation.

If, after incubation of density-labelled cells with ^{3}H-DNA and DEAE-dextran for 24 hours, the cells are washed and further incubated in the absence of DNA for 24 or 48 hours, results such as that shown in fig. 4.12 are obtained. Fig. 4.12a is the density distribution of the radioactivity after 24 hours of ^{3}H-DNA uptake and it is typical in that most of the donor label remains in the light band. After a further 48 hours incubation, the profile shown in fig. 4.12b was found. It shows that most of the label in the light band has been lost and that very little of it has passed into the two density-labelled positions. The profile at 24 hours (not shown) after removal of ^{3}H-DNA showed a similar effect. This seems to indicate that the majority of the DNA label absorbed by cells in the presence of DEAE-dextran is never used by the cells at all, but is eliminated without participating in synthetic processes within the cell. It is possible that

FIG. 4.11. Caesium chloride equilibrium-density gradient analysis of the incorporation of ^{3}H-DNA into cells labelled with BrdUrd and ^{14}C-TdR. Two gradients are shown.

1. Cells prelabelled with BrdUrd (5µg/ml) for 24 hours and subsequently incubated with 2µg/ml ^{3}H-DNA (14uCi/mg), 2µg/ml DEAE-dextran, 0·05µCi/ml ^{14}C-TdR (57 mCi/mmole) and 5µg/ml BrdUrd for 24 hours.

2. Labelled under the same conditions as in 1., but the addition of 20µg/ml aCyt during the second 24 hour period.

The cells were processed as in fig. 4.6, and spun for 3 days at 40,000rpm in a titanium-50 rotor.

1. ^{14}C ■—— ^{3}H ▲- - -
2. ^{14}C □ ^{3}H △——

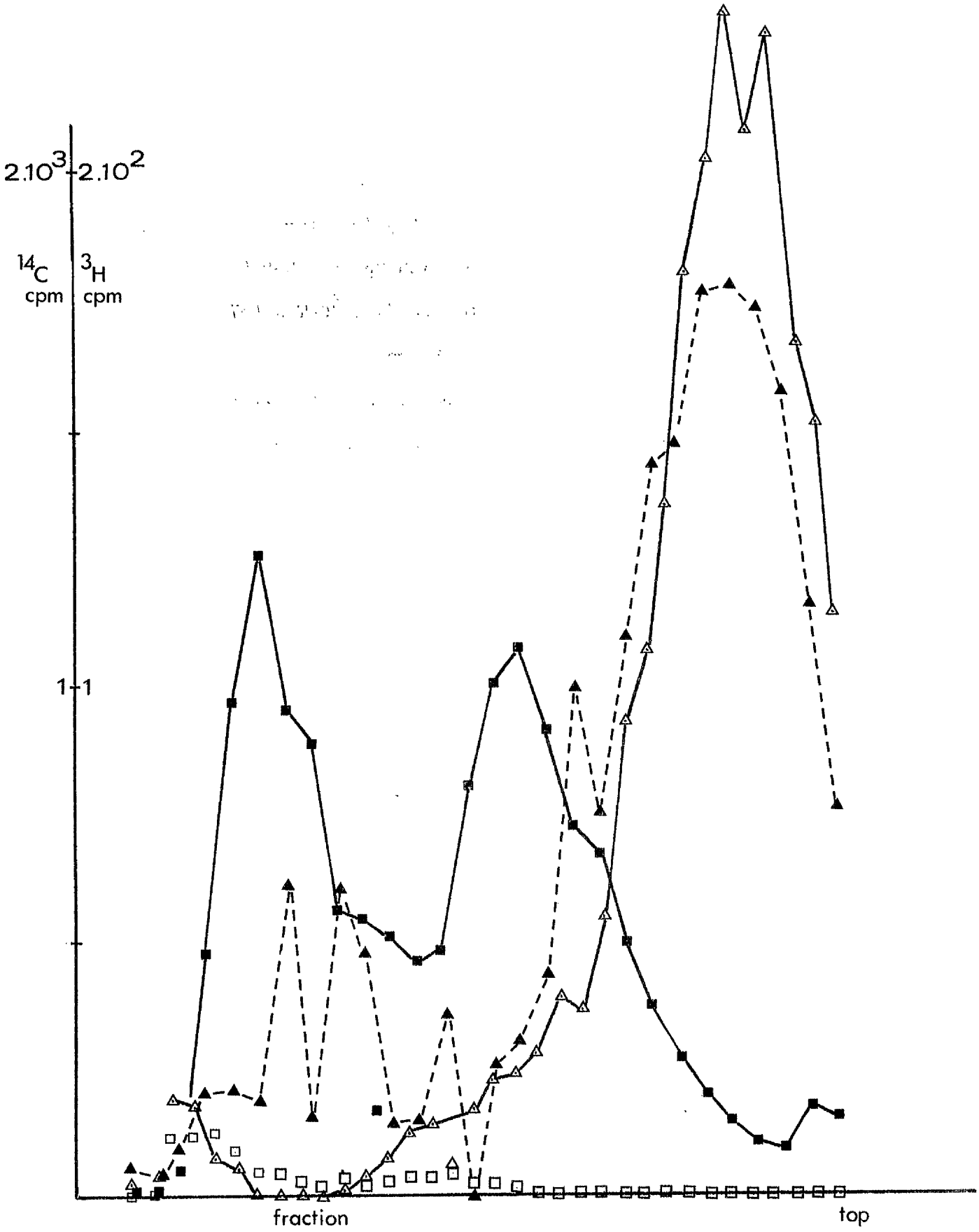


Fig. 4.11

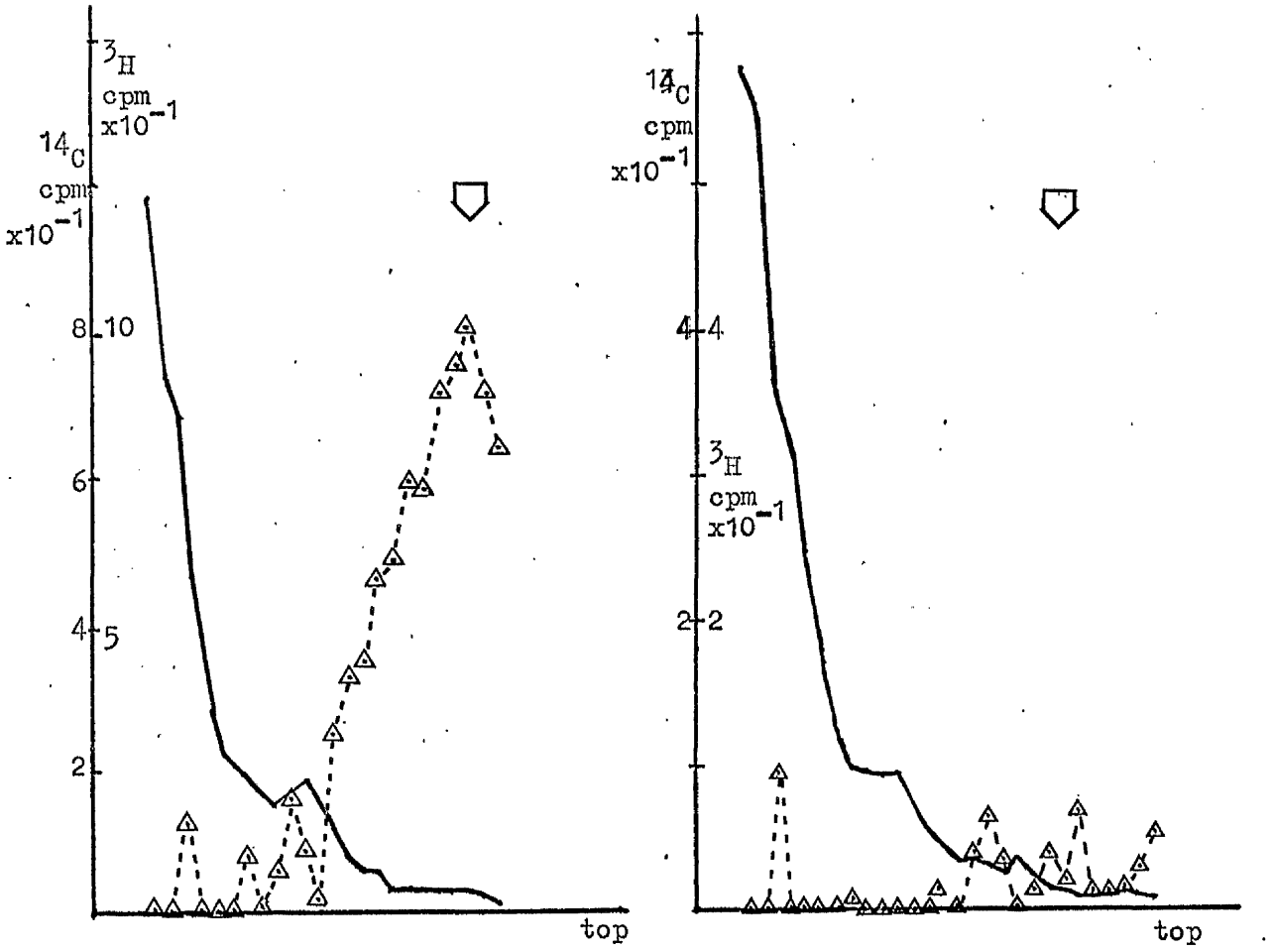


FIG. 4.12. Caesium chloride equilibrium-density gradients.

Cells were grown in 5µg/ml BrdUrd and ^{14}C-TdR (0·05uCi/ml) for 48 hours. They were then incubated for a further 24 hours in the presence of 2µg/ml ^{3}H-DNA and 2µg/ml DEAE-dextran together with 5µg/ml BrdUrd. At this time the cells were thoroughly washed in tris-saline pH 7·4.

A. lysed in 4ml tris-saline pH 7·4 containing 1% SDS.

B. incubated for a further 48 hours in EC10 containing 5µg/ml BrdUrd before lysis.

Gradient conditions were as for fig. 4.7.

△- - △ ^{3}H: ——— ^{14}C: ▽ position of BHK DNA marker

the DNA-DEAE-dextran complexes are largely inert and are egested by the cells. The autoradiographic evidence quoted in the previous section is consistent with this type of mechanism, for the donor label is almost exclusively found in clumps of material in the cytoplasm after uptake in the presence of DEAE-dextran and the metabolic inertness of the polycation could easily prevent the breakdown of these complexes by cellular enzymes.

It is also possible by the use of double labelling to analyse the uptake of DNA on sucrose gradients and thus to determine the size of the molecules with which the labels are associated after uptake. The details of the sucrose gradient technique are given in section 2.7. It involves lysing the cells on the top of a gradient so as to avoid any shearing of the recipient cell DNA and thus to ensure that recipient DNA is separable from the donor DNA which is extensively sheared by the procedure used for its purification. The radioactivity profiles found when ^{14}C-TdR labelled cells were lysed on top of an alkaline sucrose gradient with the subsequent addition of ^{3}H-DNA to the top layer before centrifugation is shown in fig. 4.13. The ^{14}C-profile shows a large peak of activity at the bottom of the gradient, while the ^{3}H-DNA is evident as a peak of activity close to the top. The ^{3}H activity at the bottom of the gradients was found each time this experiment was performed and probably represents aggregation of the single-stranded DNA into clumps which sediment to the bottom of the tube as rapidly as the almost unsheared cellular material. The ^{14}C-labelled material at the top of the gradient was also a constant feature and represents either membrane-bound DNA from imperfectly lysed cells, or residual low molecular-weight material. If DNA-^{3}H was added to cell lysate together with DEAE-dextran, a similar profile was obtained. It would appear that in the alkaline conditions prevailing in these gradients not only is the DNA itself denatured, but the DEAE-dextran-DNA complexes are also broken down.

Fig. 4.13 PyY cells were grown for 3 days in Eagles-10% calf serum containing 0·05μCi/ml ^{14}C-TdR (57mCi/mmole) . They were brought to a single cell suspension using trypsin-versene and washed twice in saline.About 4.10^6 cells in 0·2ml saline were introduced into 0·6ml of 0·3N.NaOH/M.NaCl/0·01M.EDTA/5% decon layered on top of a 10 - 30%(w/v) sucrose gradient in 0·3N. NaOH/M.NaCl/0·01M.EDTA. The gradient had been formed with a total volume of 32ml in a cellulose nitrate tube for an SW.25 swing-out rotor using an MSE gradient-maker at a flow rate of 120-140ml/hr. Into the top layer was added 4·5μg of ^{3}H-DNA in 30μl. The gradient was left in the cold room for 1 hour and spun at +5 C for 5½ hours at 22,000rpm. 25 drop fractions were collected by air diplacement in the apparatus shown in fig.2.15. Each fraction was brought to a final TCA concentration of 5% by addition of 50% TCA, and left overnight at +4 C. The precipitates were collected by suction filtration on 0·22μ pore size millipore filters and washed with cold 5% TCA, before drying and washing in ether. The dried discs were assayed for radioactivity under toluene-PPO-POPOP.

^{3}H ▲——▲ : ^{14}C .-----.

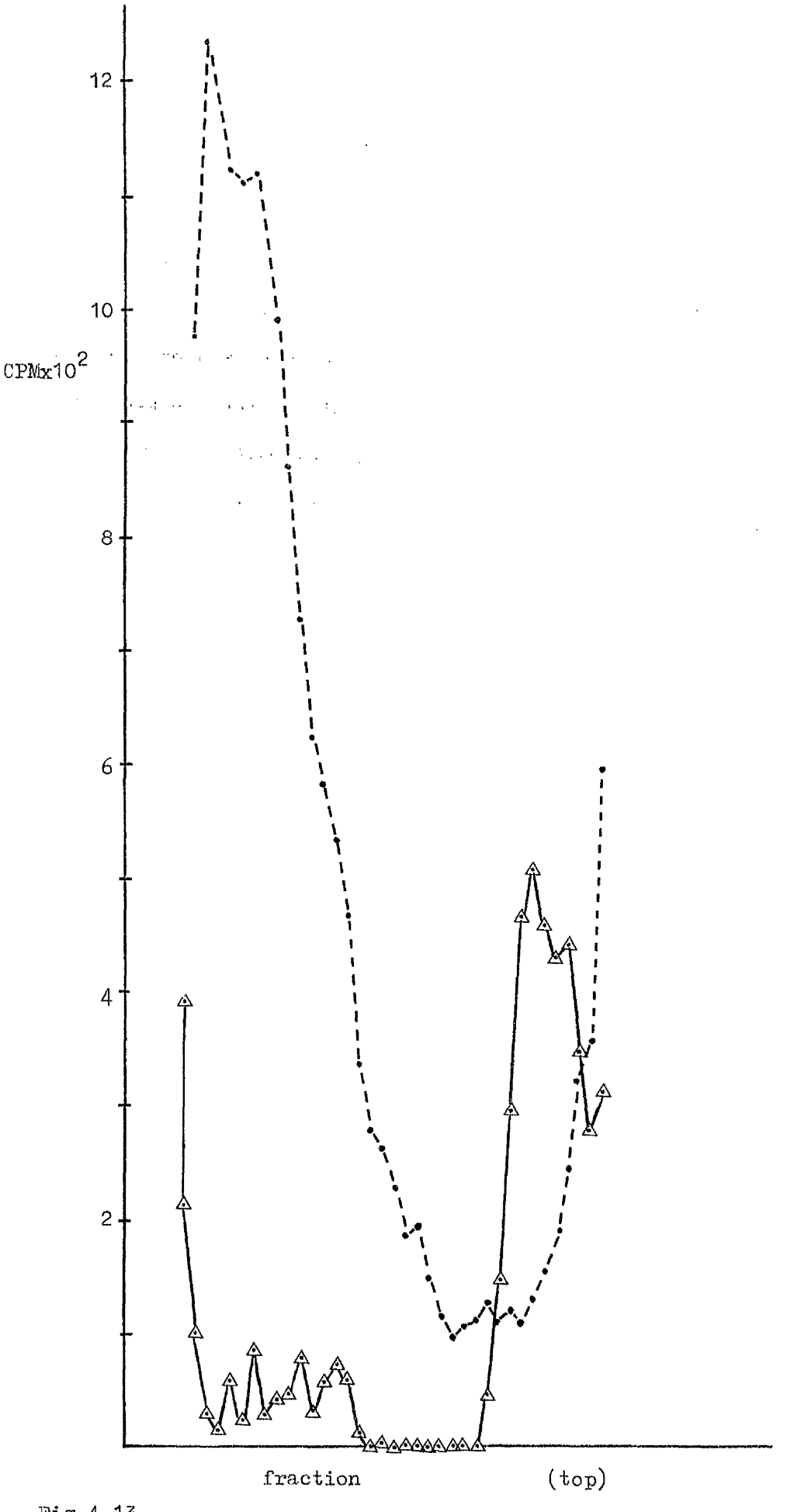


Fig.4.13

Incorporation of labelled thymidine follows the expected pattern in the sucrose gradient profiles (fig. 4.14), with the later ^{3}H-label following the ^{14}C-TdR prelabel very closely. Uptake of ^{3}H-thymidine-labelled DNA, however, shows a different pattern (fig. 4.15). In the experiment illustrated, the cells were labelled and exposed to ^{3}H-DNA under the same conditions as were used for the equilibrium-density gradients which have been described, except for the omission of BrdUrd. The two types of gradient should be directly comparable. Fig. 4.15 shows that the ^{3}H-label from donor DNA is bimodally distributed, with a peak corresponding to the size of the recipient DNA and another close to the position characteristic of purified donor DNA (fig. 4.13). No such peak normally is found in the ^{3}H-thymidine incorporation profiles, so that it is not likely that the second peak in fig. 4.15 represents a small molecular species involved in the synthesis of cellular DNA. It is difficult to estimate what proportion of the total donor DNA label that falls under the second peak because the recipient cell material is very heterogeneous.

Figs. 4.7 and 4.10 show buoyant-density analyses of DNA from cells labelled under conditions comparable to those used to obtain the profiles in fig. 4.15. The two types of analysis, taken together, show that, at a time when the vast majority of donor DNA has become associated with density-labelled molecules, there is still a significant proportion of the donor DNA which maintains approximately the same molecular size as it had before uptake. The sucrose gradients are alkaline, so that it is single-stranded DNA which is being analysed.

Alkaline gradients were used because they would more clearly show the survival of donor macromolecules not covalently bound to the recipient DNA. If neutral gradients were used, it would not be possible to distinguish surviving donor DNA associated with the recipient DNA from recipient DNA labelled by reutilisation of donor material.

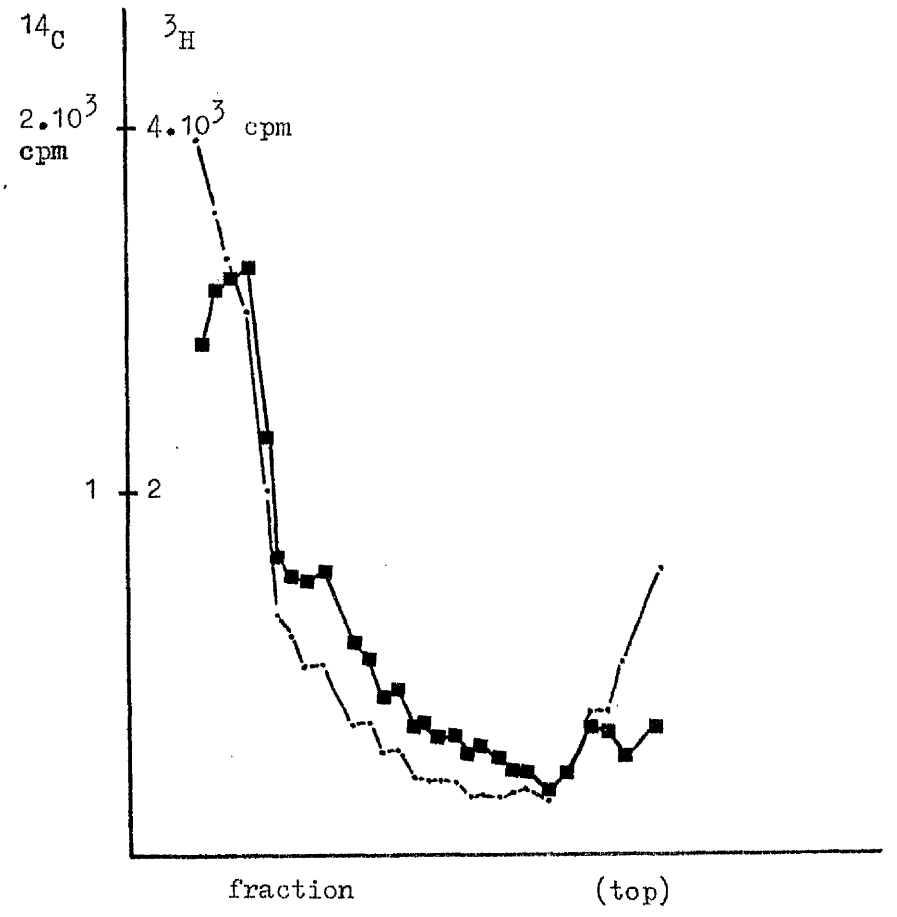


Fig.4.14 Sucrose gradient analysis of the incorporation of ^{3}H-TdR into cells prelabelled with ^{14}C-TdR. The cells were labelled with ^{3}H-TdR for 24 hours. Other conditions were as described in fig. 4.13.

^{3}H ■——■ : ^{14}C •—•

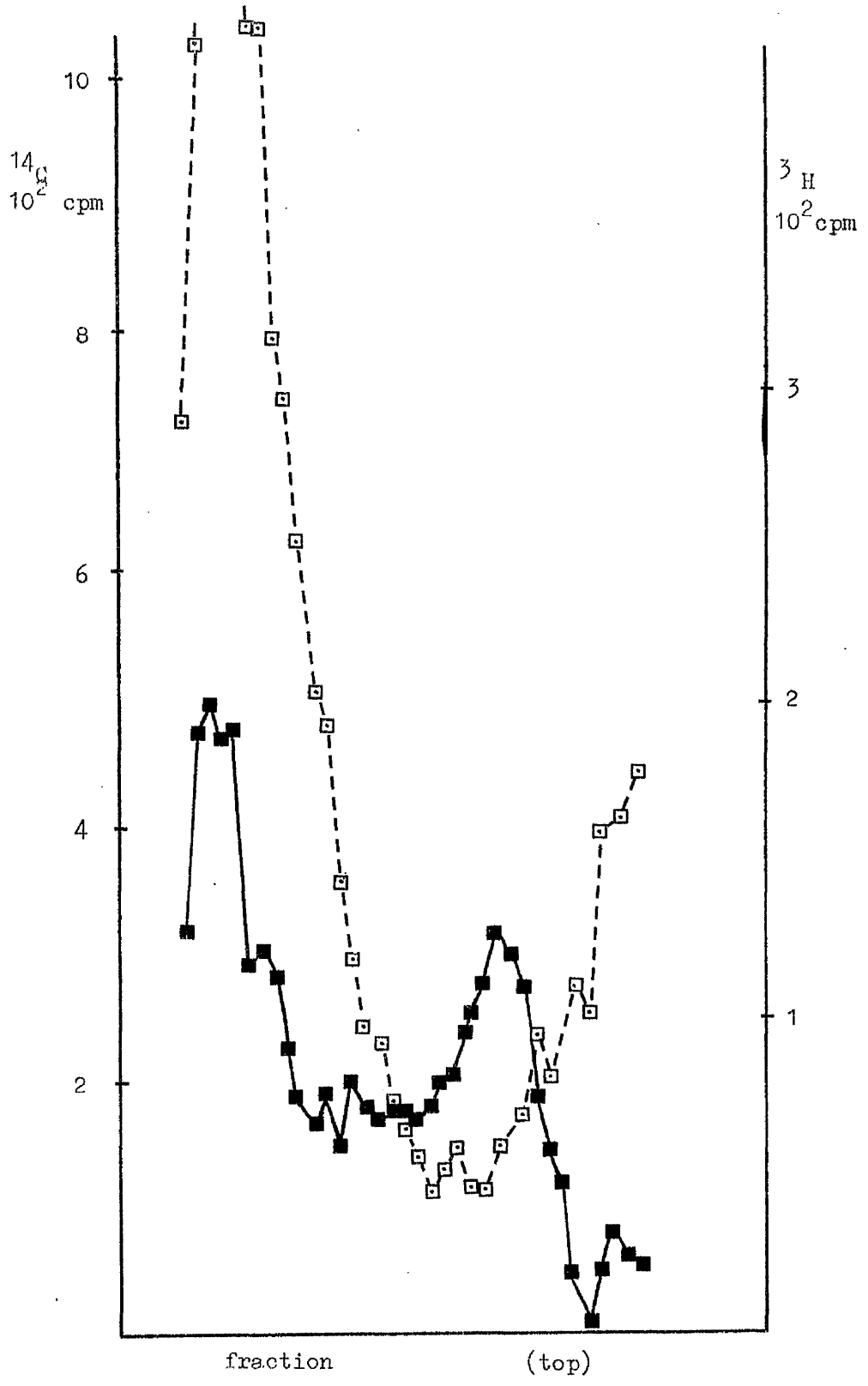


Fig. 4.15 Sucrose gradient analysis of the uptake of ^{3}H-DNA by PyY cells prelabelled with ^{14}C-TdR. The cells were treated with ^{3}H-DNA at a concentration of 2µg/ml in Eagles-10% calf serum for 24 hours. Other details were as described for fig.4.13.

^3H ■——■ : ^{14}C ⊡---⊡

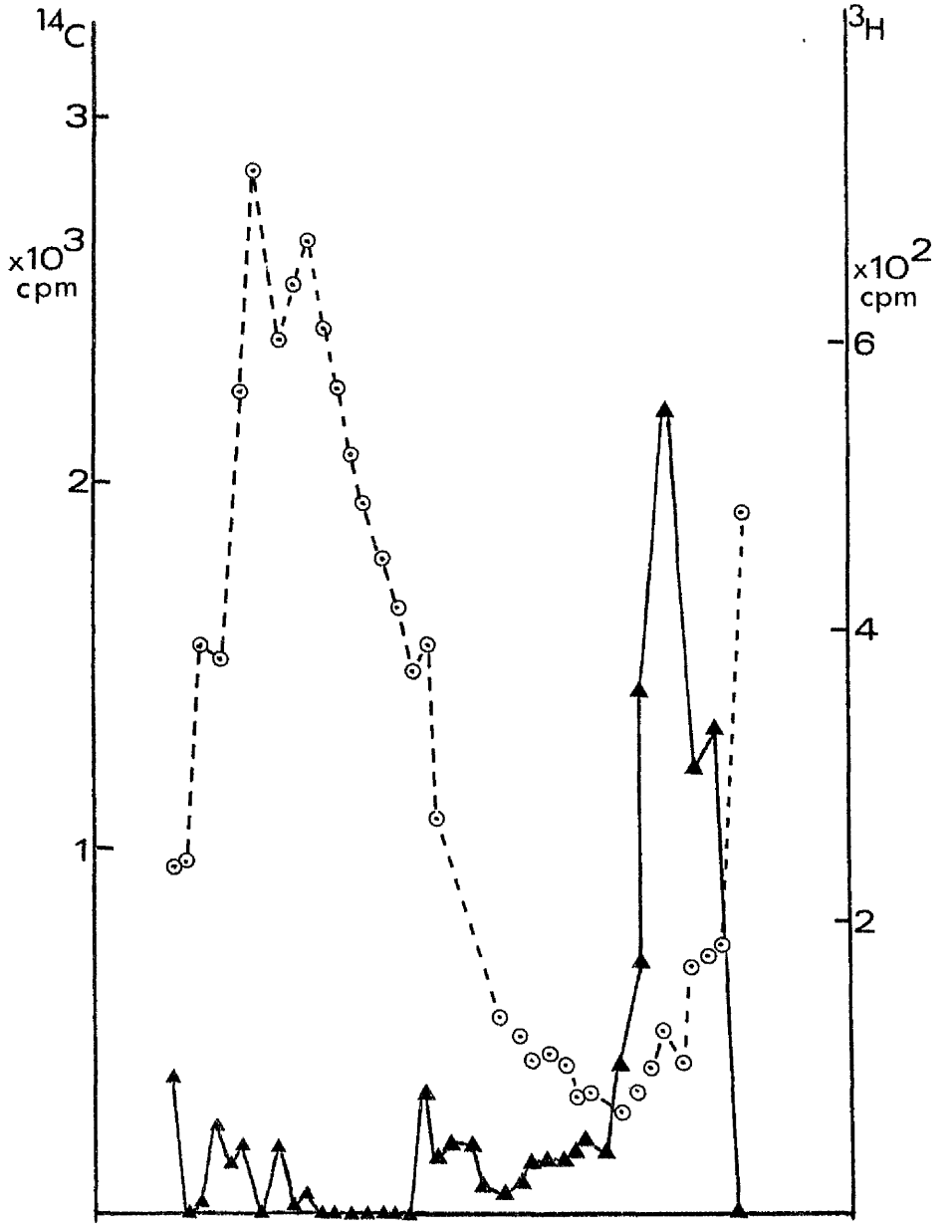


Fig. 4.16 Sucrose gradient analysis of ^{3}H-DNA uptake by cells prelabelled with ^{14}C-TdR. The cells were treated with ^{3}H-DNA for 24 hours in the presence of 2µg/ml of DEAE-dextran. The other conditions were as described for fig. 4.13.

The uptake of DNA in the presence of DEAE-dextran shows an effect consistent with its effect as shown by autoradiography and buoyant density analysis. The DNA-^{3}H molecules do not change in size in the course of absorption (fig. 4.16; compare with fig. 4.13). The small proportion of the donor counts which appear in the region of the recipient cell material does not exceed in quantity that which is found in that position in reconstruction experiments (fig. 4.13), and which is probably the result of a small degree of aggregation in the gradient.

- FIVE -

GENETIC EFFECTS OF EXOGENOUS DNA

BIOLOGICAL ACTIVITY OF ABSORBED DNA

One of the great advantages for genetic studies of the PyY-derived mutant cell lines which are used in the present work is the ease with which small numbers of wild-type cells can be identified among a population of the mutants. This is possible because the genetically-marked cells are unable to incorporate certain nucleic acid precursors, and thus appear as unlabelled cells in autoradiographs after they have been exposed to these labelled substances. In such conditions, the wild-type cells are heavily labelled. That this method is capable of detecting genetically-altered cells is shown by its use in demonstrating that vaccinia and herpes simplex induce certain enzyme activities, such as thymidine kinase, in the course of lytic infection (Subak-Sharpe, Gentry and Jamieson, in preparation), and by the fact that it was the means by which metabolic cooperation was first detected (see section 3.3d). In the genetical experiments to be described, the cell lines carried multiple genetic markers, so that they were each unable to utilise several classes of nucleic acid precursors. The two cell types were PyY/AA/AAR/TG/TGR and PyY/TG/CAR/BUDR. The general experimental plan involved treatment of these cells with DNA extracted from wild-type cells, and subsequent incubation in media containing, singly or in combinations, the various classes of labelled precursors that the cells were unable to utilise prior to the DNA treatment. This procedure has an advantage over experiments involving selection of genetically-altered cells in that it permits the detection of cells in which the absorbed DNA is only temporarily genetically active. The characteristics of the experimental system are more fully discussed in section 3.

In order to identify with certainty any transformant cells, a number of controls are required. Firstly, it is necessary to know at what frequency incorporating cells occur in the mutant population before it is treated with DNA. Secondly, the genetic specificity of the event must be

established by treating the cells with isologous DNA, i.e. DNA extracted from the mutant cell line itself, and showing that the genetic change does not occur. The conditions and kinetics of DNA uptake must also be taken into account, although it should be borne in mind that the genetic effect of absorbed DNA is a self-amplifying phenomenon and may therefore be detectable at levels of DNA uptake and survival which are too low to be detected by techniques involving the use of radioactively-labelled donor DNA.

One of the main problems encountered in the use of these cells was the presence of revertant cells (see section 3.3e) in the standard experimental line, PyY/AA/AAR/TG/TGR. While this would not necessarily prevent the detection of transformant cells, it would certainly make the result less clear-cut, particularly if the revertants appeared at a frequency that was large in relation to that of transformants. One of the genetic markers, that of resistance to adenine analogues (AA or $APRT^-$), showed no revertants at any time throughout the work, but the others gave rise to incorporating cells at such a rate that the genetic experiments had to be carried out using cells that had recently been grown in selective conditions. The standard way of doing this was to inoculate a 20 oz. medical flat with $2\text{-}3 \times 10^6$ cells in medium containing the following analogues.

6-thioguanine	50 µg/ml.
6-thioguanosine	40 µg/ml.
8-azaadenine	50 µg/ml.
(8-azaadenosine	8 µg/ml.)

The effect of these purine analogues on sensitive cells differs from that of aCyt or BrdUrd in that the cells are rapidly damaged and detach from the glass within a few hours of the addition of the analogues. When the pyrimidine analogues are added, even sensitive cells can survive for a matter of days, although usually with grossly changed morphology and with a greatly diminished growth rate. This rapid cell death caused by the

purine analogues means that any revertant cells that are present in the inoculum of the 20 oz. bottles described above will almost certainly be dead by the time the culture reaches confluence. Autoradiographic checks of cells from the confluent bottles confirmed that no incorporating cells were present after the selection procedure. Table 6.2 shows the levels of revertant cells before and after selection in one typical instance.

The background of silver grains over individual mutant cells was a problem only with the AAR-resistance marker. In the others, the background grain-count was effectively zero, so that even partial restoration of enzyme activity should have been detectable. In view of the high grain-count and reversion frequency found in it, the AAR marker was not used in many of the genetic experiments. The PyY/AA/AAR/TG/TGR cells were thus treated as triply-marked cells. In order to more fully exploit the potential of the genetic system, some DNA-treated cells were always labelled with tritiated hypoxanthine, guanosine and adenine in the same culture vessel. In this way, the cells could be simultaneously screened for genetic changes in three markers - a procedure which will treble the sensitivity, making the reasonable assumption that the three characters are equally likely to be transformed.

In none of the experiments was there detected any genetic change which could be ascribed to the DNA with which the cells had been treated, but the nature of the system was such that even these negative findings are of interest. In the absence of any unknown property of these cells rendering them unsuitable for the detection of transformation, the system is certainly sufficiently sensitive and versatile to detect any genetic changes that might take place.

The autoradiographic experiments always involved the growth of cells on coverslips of 13 mm. diameter in 5 cm. plastic petri-dishes. Each combination of DNA treatment and subsequent labelling was used for a set of

6 - 10 coverslips, which were, after development of the autoradiographs, scanned systematically at 100x magnification so that every cell could be scored as labelled or not labelled. In some cases, dark-field illumination was used. Under these conditions, silver grains appeared as bright dots on a dark background of cells. By means of this systematic and exhaustive examination of the labelled cells, the sensitivity of the experiments was optimised. In a single experiment, such as those described below, up to 5×10^6 cells were examined for incorporation of each type of precursor.

Two typical experiments will be described, and the variations to these basic plans will be discussed.

In the first type, the cells were exposed to relatively low concentrations of DNA for long periods. The PyY/AA/AAR/TG/TGR cells were grown for 3-4 generations under conditions which selected against revertants. The cell monolayers were washed thoroughly, and the cells incubated for an hour in analogue-free medium, before being trypsinised, plated out and incubated overnight in analogue-free medium. The marginally selective conditions and the thorough washes were designed to ensure that no analogues were carried over into the transformation procedure. The likelihood of a low analogue concentration interfering with the experiment is not large, but in dealing with so elusive a phenomenon as transformation any reasonable precaution is valuable. The cells were washed again, and replicate plates treated in the following ways:-

1. incubated in Eagles-10% foetal calf serum.
2. as in 1., but containing 5 µg/ml BHK21 DNA.
3. as in 1., but containing 5 µg/ml PyY/AA/AAR/TG/TGR DNA.

Five petri-dishes from each of these treatments were labelled with tritiated uridine, hypoxanthine, guanosine, adenine and a mixture of the last three, respectively. The plates were labelled from 0 - 24 hours after the addition of DNA, after which period they were fixed and washed by the standard autoradiographic method as described in section 2.5. Five plates

were similarly labelled for 24 hour periods from 24, 48, 72 and 96 hours. DNA was present in the incubation medium throughout this time.

This type of experiment covers the period of maximum uptake of DNA shown by the labelling experiments, and screens the cells for delayed expression of the DNA for three days afterwards. One possibility that it may not cover is a very early, transient expression, but it seems unlikely that molecules phosphorylated as a consequence of the addition of the DNA would have passed out of the acid-precipitable material by 24 hours. This was tested anyway, and no incorporation was found even with 12 hour pulses. The effect of variations in the incubation conditions was also tested. One of the most interesting variations was the addition of polycations to the incubation medium. Warden (1968) and others have found that diethylaminoethyl-dextran (DEAE-dextran) will greatly increase the infectivity of isolated viral DNA (Pagano, 1970), and this seemed to be worth investigating in the mammalian DNA system. The problems of precipitation of DNA with DEAE-dextran have been mentioned in the section on uptake of labelled DNA (section 4.1), and the DEAE-dextran was usually added at the same concentration as the DNA, that is, about 4 µg/ml. Other polycations used were poly-lysine and poly-ornithine, neither of which have been found to enhance infectivity of viral DNA. Cells were exposed to DNA under the conditions described above, and in the presence of one or other of the polycations. Although the labelling periods totally covered the period from 0 to 108 hours, no transformants were found in any of the treatments, either in the presence or absence of polycations. The total number of cells screened in these experiments sets an upper limit of about 2×10^{-8} on the frequency of expression of exogenous DNA in the presence of these polycations. The genetic markers tested in this type of experiment were AA, TG, TGR, CAR, BUDR and, less thoroughly, AAR (see table 3.1).

The second form of experiment was to expose the mutant cells to DNA

at higher concentrations and for shorter periods. The labelled DNA experiments did not show any significant absorption of DNA during these periods, but, as indicated above, the genetic effects could be detected at levels of DNA uptake not easily detected by labelling of the donor DNA. In these experiments, the cells were treated with DNA at concentrations of up to 100 µg/ml, whether in monolayers or in suspension culture. A typical experiment will be described.

PyY/AA/AAR/TG/TGR cells were grown for 8 days in selective conditions as described above. They were then grown overnight in Eagles-10% foetal calf serum, at 37°C. The medium was removed and the cells washed twice in fresh medium. To replicate plates were added the following:-

1. 0.2 ml of Eagles-5% foetal calf serum.
2. as in 1., plus 20 µg of PyY DNA.
3. as in 1., plus 2 µg of PyY DNA.
4. as in 1., plus 20 µg of PyY/AA/AAR/TG/TGR DNA.

The plates were incubated in a humid atmosphere containing 5% CO_2 for 1 or 2 hours, with occasional rocking, in the same way as virus is adsorbed to cell monolayers for plaque assays. At the end of adsorption, the DNA solution was sucked off and the cells washed with fresh medium. The cells were subsequently incubated in Eagles-10% foetal calf serum, with individual plates being labelled for 24 hour periods at 0, 24, 48 and 72 hours post adsorption with tritiated uridine, hypoxanthine, guanosine, adenine or a mixture of the latter three.

Only the PyY/AA/AAR/TG/TGR cells were extensively used in this type of experiment, and there were some difficulties arising from the marked cytotoxic effect of concentrated DNA solutions, which had the effect of removing large numbers of cells from the coverslips so that there was an appreciable loss of sensitivity, particularly if those cells which were lost were also those that had absorbed the most DNA.

In a total of five large-scale experiments involving DNA absorption under these conditions, 2×10^7 cells were examined, after wild-type DNA treatment, for each marker. Sporadic incorporating cells were found in both DNA-treated and control coverslips (at a frequency of less that 10^{-7}), but there was no evidence at all that any specific genetic changes were being induced by the DNA. In these, as in other experiments, the controls included the same number of cells as did the experimental cultures, and the two types were coded for mounting on microscope slides, the code not being broken until the slips had been examined. These experiments fix the transformation frequency at less than 5×10^{-8} for the AA, TG and TGR markers and one of not more than ten times this value for CAR and BUDR.

Three factors were varied from experiment to experiment in this system. These were the composition of the incubation medium, the presence or absence of cationic substances and the growth habit of the cells. In the last heading, both monolayer and suspension culture was tried.

The concentration of DNA used in these experiments was varied from 20 µg/ml to 100 µg/ml. At 100 µg/ml, the cells were damaged after about one hour of incubation, but the effect was not as marked at lower concentrations of DNA. The figures given below are the approximate number of cells screened after treatment with wild-type DNA, for each marker and under the conditions indicated. In each case, an equivalent number of cells that had not been treated with DNA of any type, plus a similar number that had been treated with the isologous (mutant) DNA under similar conditions, were included in the experiment.

Medium	Max. DNA concn.	Cations.	Cells screened.
EFC10	100 µg/ml	-	10^6
tris-saline	100	-	10^6
PBS	100	-	10^6
PBS	100	spermine 50µg/ml	10^7

All of these experiments made use of cells that had recently been grown in selective conditions, so that the number of incorporating cells was low in all coverslips. Most often, there were no positive cells at all, but in each experiment a few did appear. There was no obvious difference between test and control coverslips and the accumulated number of positives was too low to be used in a statistical analysis. It seemed very likely that the positives represented the low level of reversion that occurred after removal of the selective medium.

Szybalski and Szybalska (1962a) reported that the DNA-mediated transformation of their human fibroblast cell line occurred only when the DNA was absorbed in the presence of spermine, but their work has never been confirmed. In the above table, the PBS/spermine combination was an effort to mimic the conditions used by Szybalski and Szybalska, the only difference being in the cell line and in the means used to detect transformants. Szybalski and Szybalska used selection in HAT medium for this purpose. Although the genetic marker used here was the same as in that paper, no genetic transformation could be detected. This is in spite of the fact that the transformation frequency reported by Szybalski and Szybalska should have been easily detectable.

To summarise the results so far; cells were treated with DNA at various concentrations between 2 and 100 µg/ml for periods of between 1 and 96 hours and under different conditions of pH, ionic strength and cationic additives - including spermine. After these treatments, the cells were exposed to radioactively-labelled nucleic acid precursors and examined by autoradiography. The labelling periods covered the time from 0 to 108 hours after the start of DNA treatment. There was no difference in the number of incorporating cells found in controls and in DNA-treated cultures.

The nature of these negative results needs clarification. In some of the earlier experiments, there was a high frequency of incorporating

cells - up to 10^{-3} - which had arisen from the revertants that had accumulated since the cells were last exposed to selective conditions. The negative results in these experiments took the form of large numbers of labelled cells in treated coverslips and controls, except in the AA marker. No adenine-incorporating cells were found in any experiment either among the treated or control cells. This purine marker was consistent throughout. In the experiments in which newly-selected cells were used, at most one or two labelled cells were found on each coverslip, including the controls. Most often, no labelled cells at all could be seen. The most significant part of these results, in view of the apparently complete stability of the AA-resistance marker, is the total lack of adenine-incorporating cells in all the experiments. It might have been expected that transformation would become evident in this marker at least, as a result of the accumulated number of treated and tested cells.

One further variation was tried in the experiments on PyY/AA/AAR/TG/TGR. This came from an observation of Dr. R. R. Bürk in this laboratory that BHK21/C13 cells are held in G_1 after incubation for 4 days in low-serum medium. Bürk found that the levels of various cellular enzymes were considerably reduced after such treatment, and it seemed worth trying to reduce the level of intracellular DNase activity by this means, despite the fact that PyY cells are not as effectively synchronised by low-serum treatment. The PyY/AA/AAR/TG/TGR cells were incubated for 4 days in Eagles-¼% foetal calf serum and in the presence of 2 µg/ml of PyY DNA. Labelling with the non-incorporated purines during this period showed no incorporating cells. After the 4 day incubation, the medium was replaced with EFC10, and sample petri-dishes were labelled over 12-16 hour periods from the addition of the EFC10 to 110 hours afterwards. Incorporating cells were found in the TG and TGR tests, particularly in the later coverslips, in which the few heavily-labelled cells were clearly in a state of metabolic

cooperation with their neighbours. The number of incorporating cells was less than one per coverslip on average, and no difference was detectable between test and control cells.

The experiments with the PyY/TG/CAR/BUDR cells took the same form as those described above, but were not as extensive. There was not the same tendency to revert as was found in the PyY/AA/AAR/TG/TGR line, although a few incorporating cells were found in the course of the experimental work. These were always in the TG system. The CAR and BUDR markers were stable. Calculating the total number of cells from the number of coverslips examined and the number of cells per coverslip (obtained from cell inoculum per dish and the area ratio of coverslip to petri-dish) gives the following limits of sensitivity for the transformation experiments with PyY/TG/CAR/BUDR, and, as the results were negative, these figures represent the maximum transformation rate that would be undetected.

culture conditions	time (hrs)*	DNA (µg/ml)	label**	sensitivity
EFC10	0-100	4	0-100 hrs	7×10^{-7}
EFC10	1-2	100	0-100	5×10^{-7}
PBS	1	50	0-96	10^{-6}
PBS + spermine (50µg/ml)	1	50	0-96	10^{-6}

*period of DNA treatment

**period screened by labelling.

These results deal with 6 genetic markers, if the TG resistance of the two cell lines are considered as separate markers, although they are deficiencies in the same enzyme. They deal with many conditions of DNA uptake, including some which have been reported to give transformation in other systems. The need to scan individual cells on the coverslips imposes a timing limitation on the sensitivity of the method, but the repeated negative results put an upper limit on the frequency of transformation of $10^{-7} - 10^{-8}$. On the assumption that each cell absorbs, in the course of

an experiment, 1% of one cell-complement of DNA - and the labelling experiments indicate that this is a low estimate - these transformation experiments would have detected expression of the absorbed genetic material had only 10^{-5} of the genes under investigation been functioning.

- SIX -

GENETIC EFFECTS OF POLYOMA VIRUS

Section 6: THE GENETIC EFFECTS OF POLYOMA VIRUS

In section 1.4, the evidence for the encapsidation of cellular DNA in otherwise normal polyoma virus particles was reviewed and the possibility of the expression of the genes derived from the original host cell was raised. With this in mind, a number of experiments were undertaken, involving the infection of mutant cells with purified polyoma virus at high multiplicity, and subsequent testing of the infected cells for any genetic change in the various markers. Polyoma is a small virus with a genome consisting of a single molecule of double-stranded DNA of molecular weight 3×10^6 daltons (Crawford, 1964), and the encapsidated mouse DNA has been shown to be of a similar size (see section 1.4). This is sufficient to specify 6-8 polypeptides of average size. Of the various enzyme activities in which the mutant cell lines are deficient, only one is considered to be possibly specified by the normal polyoma virus genome. This is thymidine kinase (TK), the kinetic properties of which have been shown to be altered by infection with polyoma virus. On the other hand, it has been shown that infection of TK^- cells with polyoma virus does not result in the appearance of TK activity. The most probable explanation of these findings is that the virus modifies what is largely a host-specified structure. There is no evidence for the occurrence of such a phenomenon in the case of the other enzyme activities missing from the mutant cells. It is clear that the induction of these enzyme activities following infection of the mutant cells with polyoma virus would most probably arise from the functioning of the mouse cell genetic material contained in the pseudovirion fraction of the polyoma population. This is not the only possible mechanism, for others could be constructed on the basis of enzyme modification. The experiments did not detect induction of enzyme induction in the cells, although one or two observations suggest that is might be occurring at an extremely low frequency.

The plan of the experiments was basically similar to the DNA-mediated transformation experiments described in the previous section. In outline, they involved the infection of the mutant cells with polyoma virus at very high multiplicities, exposure of the cells to tritiated nucleosides, and autoradiographic examination of the cells with the aim of identifying any cells which have gained the ability to incorporate the labelled precursors into their nucleic acids.

The virus was purified as described in section 1.8 and was examined under the electron microscope as a negatively-stained preparation. The preparations were composed of around 98% "full" particles, and contained up to 10^{13} virus particles per ml. The suspensions were stored at $4^{o}C$ in the dark.

A typical experiment will be described and then the results of it and other experiments will be discussed.

A confluent culture of PyY/AA/AAR/TG/TGR was suspended in EFC10 and seeded into 5 cm. petri-dishes at 10^{6} cells per dish. Each dish also held 4 or 5 sterile coverslips of diameter 13 mm., so that each coverslip would have held 7×10^{4} cells. The cultures were incubated overnight at $37^{o}C$, and the cells were then washed once in tris-buffered saline. They were infected with 0.2 ml. of a dilution of stock virus in tris-buffered saline during an adsorption period of 1 hour at 37^{o}. Duplicate dishes were mock-infected with the same volume of tris-buffered saline without the virus. The adsorption period was one hour at $37^{o}C$, after which time 5 ml. of EFC10 was added to each plate. Three sets of ten plates each were used, each of the following compounds being added to one plate of each set.

i. ^{3}H-hypoxanthine
ii. ^{3}H-guanosine
iii. ^{3}H-adenine

at 2 µCi/ml.

iv. ^{3}H-uridine

y. Mixture of i, ii and iii at 2 μCi/ml each.

Of each set of ten, five were infected and five mock-infected, with one plate of each of these types receiving one or other of the above compounds. The labelling periods were 24 hours in length, beginning at 0, 24 and 48 hours after infection. Cells were fixed and subjected to the standard autoradiographic process immediately after removal of the labelled compounds.

In this particular experiment, the multiplicity of infection was 4×10^4 particles per cell. If 10% of these virions contain mouse DNA - and this is probably a minimum estimate - each cell will potentially be infected with 4×10^3 molecules of mouse DNA of molecular weight 3×10^6 daltons, which amount to a total of about 10^{10} daltons per cell. This is the equivalent of the DNA content of 3×10^{-3} cells, so that, if a total of 10^6 cells are infected and examined, this experimental system provides for the handling of a population of cells which has received a total of 3×10^3 cell complements of DNA. If this DNA is drawn at random from the genome of the original mouse cells, about 3×10^3 copies of each of the mouse genes should be present in the virus DNA. Not all of this DNA will enter the cells, and not all of that which does can be expected to express genetic activity, but this rough calculation does show that detection of transduction should be possible in this system unless the efficiency of expression is very low indeed.

The scanning of autoradiographs was thorough and was carried out under 100x magnification and bright field optics. Starting at the end of a diameter of the coverslip, the slide was moved so as to scan from end to end of a line perpendicular to the diameter. The slide was then moved by the width of the field of view and the adjacent part of the slip was scanned. This was repeated until the whole of the coverslip had been covered and

every cell on it examined. Both infected and control cells were subjected to this same process.

In the experiment being described, five coverslips of each type were examined in this way, and the results are summarised in table 6.1.

Table 6.1

LABEL	SET	Py-INFECTED	CONTROL
^{3}H-hypoxanthine	1	0:	0
	2	7	5
	3	0: all cells (+)	6: all cells (+)
^{3}H-guanosine	1	4	7
	2	0	0
	3	?	0: all cells (+)
^{3}H-adenine	1	2	0
	2	0	0
	3	0	0
mixture	1	6: all cells (+)	all cells (+)
	2	all cells (+)	all cells (+)
	3	all cells (+)	all cells (+)

^{3}H-uridine incorporation was not affected by polyoma infection.

? = preparation lost (bacteria)
(+) = visibly labelled, but not as heavily as the wild type PyY.
Figures indicate the total number of heavily-labelled cells on the 5 coverslips.
(but see comments below)
set 1 = 0-24 hours; set 2 = 24-48 hours; set 3 = 48-72 hours.

Several features emerge from these results and they will be discussed in parallel with the results of similar experiments.

Firstly, it is evident that incorporating cells are found in both infected and control cultures. This is a manifestation of the reversion described in section 3.3e, and was a complicating factor in many experiments. In one particular group of experiments carried out before the reversion of these cells was first observed, the numbers of incorporating cells reached as high as 50 per coverslip, which is a frequency of 10^{-3}. This

figure represents the frequency of heavily-labelled cells and excludes those cells which were relatively lightly labelled and were obviously in a state of metabolic cooperation with heavily-labelled cells. The number of revertants was reduced to zero by growing the cells for use in the experiment in the presence of the three analogues, TG, TGR and AA immediately before they were transferred to the petri-dishes for infection. The procedure adopted was to seed a 20 oz. medical flat with 2×10^6 cells in 50 ml. of EFC10 containing the following concentrations of the analogues.

8-azaadenine	50 µg/ml
6-thioguanine	50 µg/ml
6-thioguanosine	40 µg/ml

The table of results shown above represents the infection of cells which had been grown in selective conditions a few generations before the experiment. In table 6.2, two experiments are presented. In one of these, the frequency of revertants is at the highest level observed.

Table 6.2

		69/67 nonselective		70/6 selective	
LABEL	TIME	INFECTED	CONTROL	INFECTED	CONTROL
^{3}H-hypoxanthine	0-24	41	45	0	0
	24-48	38	54	0	0
	48-72	all +	all +	0	0
^{3}H-guanosine	0-24	2	11	0	1
^{3}H-adenine	0-24	0	0	0	0
	24-48	1?	0	0	0
	48-72	0	0	0	0
mixture*	0-24	33	37	0	0
	24-48	35	35	0	0
	48-72	all +	all +	0	0

* = ^{3}H-labelled adenine, hypoxanthine and guanosine at 1 µCi/ml each
? = this was heavily-labelled, and not of typical cell morphology. It could not be certainly identified as a cell.

The absence of genetic effect of polyoma infection is most clear in the cells which had been selected prior to use, and was consistently observed

through a series of twelve experiments involving a total of about 108 cells, along similar lines to that described above. There is one feature of the results which could indicate that enzyme is induced by polyoma infection at an extremely low efficiency, but it is not consistently observed. The adenine incorporation marker was the only one which never showed revertants in any of the preparations, whether preselected or not, but the tables above show two cases in which adenine incorporation was seen in polyoma-infected cells. It is difficult to estimate what weight should be placed on this observation. On one hand, the only incorporating cells were in infected cells. On the other hand, only three incorporating cells were found altogether, so that their significance is doubtful, and there is no consistency in their distribution, two being found in the 0-24 hour labelled samples and one in a 24-48 hour sample. In the majority of the experiments, no adenine-incorporating cells were found. It should also be mentioned that the single incorporating cell in table 6.2 was not of typical morphology, being rounded and not even certainly identifiable as a cell.

It has been found by previous workers that pseudovirions are slightly less dense on caesium chloride density gradients than are the normal virions. This has been used by Osterman et al (1970) as a method of purifying the pseudovirions. The high titre polyoma suspension used in the present study was also centrifuged in caesium chloride gradients at a starting density of 1.310 for 17 hours at 40,000 rpm. The virus band was identified by monitoring the absorbance at 260μ and the less dense side of the peaks from the gradients were pooled and dialysed exhaustively against tris-buffered saline. The resulting virus suspension was used to infect PyY/AA/AAR/TG/TGR for the same type of experiment as is described above, but no genetical activity could be detected in a total of 10^8 infected cells.

The apparent lack of genetical activity of the mouse DNA found in polyoma pseudovirions could be attributed to one of several reasons, which will be discussed in section 7.

- SEVEN -

DISCUSSION

Section 7: DISCUSSION

The uptake of DNA by mammalian cells is a topic which has given rise to considerable confusion in the past, partly because of the lack of suitable experimental systems. Even the uptake of DNA macromolecules has only recently been satisfactorily demonstrated. The most convincing proof of this would be to show that the genetic functions encoded in the absorbed DNA are expressed within the recipient cell. The reports that indicate that this actually occurs are open to question for a variety of reasons, and no such expression was detectable in the cell lines used in the present work. This is more fully discussed below.

The use of radioactively-labelled DNA to demonstrate uptake of macromolecules presents problems in experimental design and in interpretation, principally because of the difficulty in distinguishing between label in intact donor molecules and that which has been reutilised by the recipient cells after breakdown of the donor DNA. This distinction has not always been made (Kraus, 1961). Among the means which have been used to avoid this difficulty is the labelling of the donor DNA with ^{14}C-formate. This precursor is used in the de novo syntheses of DNA bases. The ratios of the specific activities of the bases can be determined by chromatography after hydrolysis of the donor DNA. If the labelling pattern of DNA extracted from the recipient cells after they have been exposed to the ^{14}C-DNA closely resembles that of the untreated donor, it is taken as an indication that the donor DNA has been absorbed as macromolecules. The published work which has made use of this method (Kay, 1961; Rabotti, 1962; Meizel and Kay, 1965; Gibb and Kay, 1968; Robins and Taylor, 1968) has consistently shown that the ratios remain approximately constant throughout uptake, which implies that only very little of the absorbed DNA is degraded. Considering the high levels of nuclease activity in the cytoplasm of mammalian cells, this seems unlikely enough, and the observation

of the decrease in DNA-label uptake in the presence of thymidine or arabinosyl cytosine makes it even more so. The evidence in favour of at least partial breakdown of donor DNA (Ayad and Fox, 1968; Cocito et al., 1962; Robins and Taylor, 1968) is sufficiently strong to cast doubt on the validity of the interpretation of this type of experiment.

Some workers have used, as recipients, cells which were deficient in thymidine kinase (TK) activity. The rationale of this is that uptake and degradation of DNA labelled with ^{3}H-thymidine will release radioactive molecules which cannot be reutilised because of the deficiency in thymidine kinase. In fact, this reasoning is fallacious. If the donor DNA is broken down intracellularly, it will release tritiated TMP, which can be incorporated into nucleic acid without the intervention of thymidine kinase. Extracellular breakdown of DNA will also release TMP, but this can only cross the plasma membrane if it loses the phosphate group, so that lack of TK will prevent its reutilisation by the cells. Hence the use of TK^{-} cells, while it will distinguish between uptake of macromolecules and uptake of mononucleosides released by extracellular degradation of the donor DNA, gives no information as to whether those molecules remain intact for any length of time after uptake. The results will be the same whether the DNA-^{3}H reaches the nucleus or is degraded at the inner surface of the plasma membrane.

The present work uses density-labelling to investigate DNA uptake. The approach that was adopted was to label the recipient cell DNA with BrdUrd and ^{14}C-thymidine, and to look at the uptake of ^{3}H-DNA, which could thus be distinguished from the recipient material on equilibrium-density gradients. The survival of significant quantities of donor DNA of unchanged density could only be demonstrated if absorption of the DNA had taken place in the presence of DEAE-dextran. The analysis of DNA in the recipient cells after uptake in these conditions showed that the vast

majority of the absorbed donor label banded at the position in the gradient characteristic of the donor DNA. Only a very small proportion became associated with density-labelled material. Reconstruction experiments showed that ^{3}H-DNA added to cell lysate in the presence of DEAE-dextran banded at the same position as in its absence. Sucrose gradient analysis of recipient DNA after uptake of donor label showed that the donor material sedimented at a rate very similar to that which it displayed before uptake. The picture that emerges is one in which the donor DNA is not changed either in size or in density during absorption by cells in the presence of DEAE-dextran. Unless the DEAE-dextran forces the donor DNA into some unusual degradative pathway, the small amount of donor label in the density-labelled bands and the high molecular weight regions of the sucrose gradients indicates that very little of the donor DNA is broken down. In addition, the experiment illustrated in fig. 4.11 shows that the uptake of DNA in the presence of DEAE-dextran does not depend on the continuation of recipient DNA synthesis, because the addition of arabinosylcytosine will abolish the latter while not diminishing passage of donor label into acid-precipitable material in the "light" band. The results of autoradiographic experiments are consistent with this picture of inertness. In cells exposed to ^{3}H-DNA and DEAE-dextran, the absorbed radioactive label is found only in the cytoplasm of the recipient cells and is always seen as clumps of silver grains, rather than as single grains dispersed through the cell. It was found that DNA and DEAE-dextran would form precipitates when mixed even at concentrations as low as 5 μg of each per millilitre, and it is known that the two substances do form complexes with each other. It seems likely that the observed behaviour of the DNA after uptake is the result of the inability of the cells to break down these complexes, particularly as there is no known intracellular enzyme which will degrade DEAE-dextran. These findings may be correlated with those of Warden (1968),

who showed that DEAE-dextran will enhance the infectivity of isolated polyoma DNA, while polyaminoacids such as polyornithine, which also increases the amount of polyoma DNA that is absorbed, have no such effect. DEAE-dextran may in fact be acting as a physical protection against the action of nucleases. The limited release of absorbed DNA from the complexes is shown by the small amount of radioactive label that is found in the recipient bands of the gradient profiles (for example, ^{3}H profile no. 1 in fig. 4.11). The experiments in which the cells were incubated for various lengths of time after replacement of the medium containing the DNA and DEAE-dextran showed that the label was gradually lost from the culture, so that it never appeared in the density-labelled (recipient) DNA band. This indicates that the labelled nucleotides never become available for host syntheses. While there is a possibility that the DEAE-dextran enforces the use of an unusual breakdown pathway so that the products are not available for reutilisation, the most likely explanation for these observations is that the majority of DNA that is held in complexes with DEAE-dextran is not degraded at all by the cells. The complexes are in fact lost from the culture either by egestion from the cells or by the death of cells damaged by the absorbed complexes. The significance of these findings for genetic and infectivity studies is discussed below.

In the absence of DEAE-dextran, a different picture emerges. The donor DNA is all, or almost all, found in the density-labelled bands in caesium chloride gradients, although its distribution is not identical to that of ^{3}H label which has been given to the cells as thymidine-5-^{3}H. The association of donor with density label could come about in one or two or all of three ways. It could be the result of the reutilisation of labelled thymidine or the insertion of intact donor polynucleotide into the density-labelled recipient molecules or the synthesis of new polynucleotide on the donor template. If the first mechanism were solely

responsible, it would be expected that the donor label would be distributed with respect to density in a manner identical to that of ^{3}H-thymidine incorporation. The profiles in fact differ considerably, especially in that the ^{3}H-DNA profile is very heterogeneous. This heterogeneity does not arise from the uncertainty inherent in low count-rates, because it persists even when the samples are counted for periods sufficient to accumulate some thousands of counts. The diminished uptake of ^{3}H-DNA label in the presence of thymidine or BrdUrd suggests, however, that there is some considerable degree of breakdown and reutilisation taking place. There is also some evidence that one or both of the other two mechanisms is operating. Firstly, the caesium chloride profiles often show, as in fig. 4.7, a relatively large fraction of the donor label in the hybrid peak. This is consistent with some of the donor DNA acquiring some density label without being broken down. In the conditions illustrated in fig. 4.7, that is, in the continuous presence of BrdUrd, one would expect the vast majority of the donor label if it is degraded and reutilised, to pass directly into the fully-labelled band. Instead, about 50% of it appears as a fairly broad band around the hybrid position. Again, in fig. 4.10, in which the cells are not prelabelled before the combined density-labelling and DNA uptake step, a considerable quantity of donor material is found between the unlabelled peak and the hybrid (recipient) material. Total breakdown would have led to its appearance in the hybrid peak only. Secondly, alkaline sucrose gradient analysis of the DNA after uptake reveals that some of the donor material sediments in the same position as does the unaltered donor DNA preparation, while no such material is found in cells labelled for the same period with ^{3}H-thymidine. The recipient DNA sediments much faster. It was not possible to compare the sedimentation rate of the donor DNA before and after uptake with any precision, because this would require cosedimentation of the two types of DNA in the same

gradient, and the necessity to use only one DNA preparation precludes the use of double-labelling. The best that can be said is that the absorbed DNA in the smaller component seems to sediment slightly more rapidly than the pure DNA when the two were run in similar and parallel gradients. Taking into account the size heterogeneity in the sucrose gradients, the proportion of the donor DNA which maintains its original sedimentation behaviour is probably quite small. These alkaline gradients indicate that this donor DNA is not covalently linked to the recipient genome, but do not rule out the possibility that there is association by hydrogen-bonding. The distinction between this sort of mechanism and that in which the donor DNA is reutilised is a difficult one to make experimentally. Simple use of neutral sucrose gradients would of course not make the distinction. The only attempts to answer this question have been made by Hill and Hillova (1971) and by Ayad and Fox (1968). The latter group used DNA that was labelled with ^{3}H-IdUrd, and found that, after uptake, they could detect a band at an intermediate density in caesium chloride gradients. As has been pointed out in the introduction (section 1.3) the significance of this material is not clear in view of the lack of data about the labelling pattern of the original donor DNA preparation. The authors appear to interpret it as representing a duplex of which one strand is from the donor and one from the recipient, but the fact that the cells went through only one doubling in the DNA labelling step suggests that the ^{3}H-IdUrd-DNA was in fact labelled in only one strand. Their data do however suggest that a component in the DNA after absorption is relatively rich in density-label, which suggests that some of the recipient DNA is associated with intact donor molecules. Alkaline gradients showed that the recipient DNA in these associations was always that which had been synthesised before the addition of donor DNA. This rules out the insertion of donor DNA single-strands into the recipient strand which is synthesised during the uptake

period, and suggests that the donor-recipient interaction involves only that recipient DNA which has not replicated during uptake.

The third group who have recently produced data from density-labelling experiments are Hill and his colleagues (Hill and Huppert, 1970; Hill and Hillova, 1971). They investigated the uptake of tritiated DNA by cells that had been prelabelled with ^{14}C-BrdUrd and their most striking findings were that the ^{3}H-labelled material near the donor band in caesium chloride gradients contained a component of density greater than that of the donor DNA, and that the material around the recipient (BrdUrd-labelled) position displayed a lack of coincidence between the ^{14}C and ^{3}H activity peaks. This lack of coincidence was not found when the ^{3}H activity was supplied as thymidine under the same conditions as were used for the DNA uptake. It was found that there was a ^{3}H peak which was always rather lighter than the recipient DNA after uptake of ^{3}H-DNA, and that this difference became more marked when the reextracted DNA was sonicated. The authors (Hill and Hillova, 1971) interpret the data as an indication that there are regions of the reextracted DNA which are relatively rich in the donor label and which most probably arise from the insertion of donor macromolecules of unknown size into the recipient DNA. There is, however, the possibility that the splitting of the peak of donor label is an artefact arising from delayed breakdown of the donor DNA. If the ^{3}H-thymidine is not released immediately after uptake, there will be a period during which the newly-synthesised recipient DNA will incorporate only the ^{14}C-BrdUrd and will thus be density-labelled to the same extent as the prelabelled molecules. Only after the input ^{3}H-TMP becomes available for DNA synthesis will there be competition between donor label and density label, and during this time the newly-synthesised molecules will have a density less than that of the prelabelled recipient DNA. One experiment in the cells used in the present work and involving the addition of labelled ^{3}H-thymidine at

different times after mock-addition of DNA to the medium indicated that delayed release of thymidine gives rise to considerable spreading of the donor label profile on the lighter side of the recipient band, although this was not resolved into two separate peaks. One finding that was reported by Hill and Hillova was that DEAE-dextran prevented association between donor and recipient label. This is consistent with the data presented here.

It appears that the donor DNA is at least partly broken down and reutilised in the absence of DEAE-dextran, but that a detectable amount of it survives at about the same molecular weight as before uptake. Under the same conditions, all the donor DNA becomes associated with density label so that there must be some synthetic process connected even with those donor molecules which survive at roughly the same molecular weight as before uptake. In the presence of DEAE-dextran there is no detectable change in the majority of the absorbed donor molecules. In the former case, it remains to be proven whether the surviving donor molecules are or are not associated with the recipient genome, but in the latter case they certainly are not. Inhibition of recipient DNA synthesis prevents the passage of donor label into the acid-precipitable fraction of the cells, a finding which is perhaps unexpected in view of the survival of donor molecules. It is probable that the input DNA is after absorption either associated with a part of the synthetic apparatus or rapidly degraded. In such a case, interference with DNA synthesis will force donor DNA molecules into the degradative pathway, and all the absorbed label will become acid-soluble.

The numerous reports of successful DNA-mediated transformation which have been published are described in the introduction, and it is clear that they have very little in common with each other. The experiments with whole animals or with tumours suffer from the defect that they often work with poorly-defined genetic markers. One of the clearest of such studies

is that of Fox and Yoon (1966, 1968, 1970), who made use of the classical colour and morphological characters of Drosophila. The genetics of these traits have been well worked out and in some cases the biochemical mechanism has been elucidated. In addition to this, Fox and Yoon applied their donor DNA preparations to oöcytes, a procedure which removes the difficulties inherent in treating whole organisms by such means as intravenous injection (Podgajetskaya et al., 1964), or simple immersion of the organism in DNA solution (Ledoux and Huart, 1969). The results of Fox and Yoon are not quite clear-cut in that transformation was not detectable in all the markers that they tested, and that there were certain irregularities in expression in those cases where it was detectable. For example, the transformed individuals were invariably genetic mosaics, and the frequency of transformation in single genes reached as high as 3.10^{-2}. This latter finding suggests that, unless the genes under investigation fortuitously had high frequencies, each individual must be transformed in up to several hundred loci. If this is the case, genetic transformation should be much more easy to detect than it has historically proved to be. The mosaicism of the transformed flies is consistent with the idea that integration of a piece of DNA need not be complete and need not occur at the same chromosomal locus as it occupied in the donor. This integration of the donor gene without loss or inactivation of the recipient allele could be tested by using known dominance relations among Drosophila genes, but the authors give no information on this. They do however postulate a model involving an "exosome" or a piece of donor DNA which is not integrated into the chromosomes at all, but replicates independently, and with a finite chance of being lost at various stages of the life cycle. This accounts for the observed loss of donor genes, while the mosaicism is explained in terms of a copy-choice mechanism within individual nuclei. The transformation events observed were also found, by treating oöcytes with DNA from flies of

various genotypes, to be highly specific for the donor genotype. The body of evidence set out by Fox and Yoon is impressive and further work may well show that this is the first fully-authenticated case of DNA-mediated genetic transformation in a higher organism. The other reports which have appeared have not been developed, apparently because of a lack of reproducibility (Kraus, 1961; Szybalski and Szybalska, 1962a; Fox, Fox and Ayad, 1969; Majumdar and Bose, 1968). In addition to these, there is a long list of negative findings in systems mainly involving the use of whole animals (Benoit et al., 1957a, 1957b, 1957c, 1958, 1959, 1960a, 1960b; Perry and Walker, 1958; Bearn and Kirby, 1959; Svoboda and Haskova, 1959; Chepinoga et al., 1960; Beatty and Billet, 1961; Schoffinnen et al., 1961; Astauroff et al., 1960; Horstadius et al., 1954; Billett et al., 1964; Novikov et al., 1961).

The cells used in the present work are the best genetic system that is currently available for the study of genetic transformation. The defects are thoroughly defined and are in single enzyme activities. The reversion rates are low and in some cases are undetectable or zero, so that any transformants that arise do not have to be distinguished from a background of revertants or of cells which for one reason or another mimic revertant cells (Fox, Fox and Ayad, 1969). Finally, it is possible to detect "abortive" transformation in single cells, and the change that is sought is one towards the expression of a single dominant gene (see section 3.1). The advantages of the system are such that the lack of transformed cells in the experiments described in sections 5 and 6 is in itself significant.

Of course, the genetic inactivity of DNA in these cells may have a trivial explanation, or one specific for these particular cell lines. There is the possibility that there is some intracellular factor or condition which is peculiar to these cells and which precludes the

expression of exogenous DNA. To suggest a mechanism for this would be pure speculation, but it is not hard to imagine some process similar to the host-controlled restriction of bacteriophages. This would be a trivial reason in the sense that it would not necessarily mean that expression of absorbed DNA would not occur in other cell lines. Of course, the achievement of transformation is to some extent a question of finding the correct technical trick. This was certainly the case in the early work on the isolation of infectious viral nucleic acid, which could only be assayed at a usable efficiency in the presence of DEAE-dextran or if the cells were previously exposed to hypotonic conditions (Warden, 1968; McCutchan and Pagano, 1968; Pagano, 1970). It was the reported efficiency of DEAE-dextran in enhancing viral DNA infectivity which prompted its use in the present work. McCutchan and Pagano (1968) describe a number of methods of treating the cells with DEAE-dextran, including pretreatment with relatively high concentrations. The PyY cell lines used here, however, would not tolerate high concentrations so that the pretreatment method was not used at all. This should not effect the results, because Pagano (1970) reports that treatment with DNA and lower concentrations of DEAE-dextran at the same time is equally efficacious. Spermine has been reported as an essential factor for the expression of absorbed DNA (Szybalski and Szybalska, 1962a), and it was also tried in the experiments described in section 5. Neither of the cationic substances that were tried was effective in allowing the expression of the donor DNA, but this does not mean that no other substance would have done so. This sort of argument can be applied to the other factors defining the cellular environment, such as pH and concentrations of particular ions. If the detection of transformation has so far been prevented by this sort of technicality, the problem becomes less interesting, though not less rewarding scientifically.

There are however more fundamental barriers to the expression of exogenous DNA in eukaryotic cells. Some of the more obvious ones are

discussed in the introduction (section 1.1), but there are other possibilities. The regulation of gene expression in eukaryotic cells is only very imperfectly understood, and it is not known what regulatory sites, if any, would need to be present on a fragment of DNA before the genetic information encoded in it could be transcribed and translated. If, for example, the particular gene under investigation absolutely required the functioning of a regulatory gene which was, in molecular terms, distant from it, the probability of a single fragment of DNA carrying both the regulator and the structural gene would be vanishingly small, so that expression of the gene would depend on the unlikely event (see section 1.1) of the two essential fragments being present in potentially active form in the same cell. Other types of regulator function could be shared with the recipient cell, and thus would not prevent the translation of absorbed genetic material. Another possibility is that the expression of genetic functions depends on the structure of chromatin and on the presence and configuration of the proteins in it. This would mean that purified DNA would necessarily be genetically inactive. That the purification of viral DNA does not destroy its infectivity does not invalidate this argument, because the viral DNA is adapted to deliver intact, genetically-functional units into cells. Mammalian DNA is not so adapted. Its role is to function within the chromatin, the structure of which is destroyed by the necessarily rigourous procedures used in DNA purification. If this is indeed what is preventing genetic transformation, the work, discussed below, on the uptake of chromosome fragments would be a more promising approach.

The size of the genome of a mammalian cell is a considerable difficulty. If a DNA complement of 10^{12} daltons is sheared into pieces of 10^{7} daltons, each cell will give 10^{5} pieces of DNA of which probably only one will carry the gene which is being investigated. The probability of any particular gene being sheared in the course of the extraction

procedure is, assuming random breakage, of the order of 10^{-2} and can be neglected as a source of inactivation. Thus, each DNA molecule that is detectable in the genetic system being used must compete with 10^5 other molecules for uptake and for the sites within the cell at which transcription is possible. This factor if 10^5 could take the possible frequency of expression below the level that is at present detectable. As an example, if each cell absorbs DNA amounting to 5% of the total cell genome, then 5% of the cells will be "infected" with any particular, non-reiterated gene. The efficiency of expression cannot at present be even roughly estimated, but if it is only as low as 10^{-2}, the frequency of detectable transformation will be about 10^{-9}, which would be extremely difficult to handle experimentally. Once again, the use of isolated chromosomes could be helpful, and this is discussed below. The factors which determine whether or not an isolated viral DNA molecule is or is not infectious are not fully understood. Pagano (1970) points out that viral DNA extraction loses at least 99.9% of the original infectivity even in the best preparations achieved so far. The reasons that he suggests for these losses, apart from the shortcomings of the techniques, include the possibility that the conformation of the viral nucleic acid plays a vital role in determining infectivity. For example, polyoma DNA is separable into three components, of which only the supercoiled and the circular forms are infectious. This could indicate that only circular DNA can be infectious or that the opening of the circle during extraction is a random process so that the correct initiation sites on the DNA molecule are not exposed. Infectivity is of course a much more stringent test of biological activity than is expression of a single gene. It is also possible that the loss of circular conformation is associated with loss of an internal protein which is essential if transcription is to procede. Applying this sort of thinking to the uptake of cellular DNA, several points

emerge. Firstly, if circularity is essential, then cellular DNA is ruled out as a means of transferring genetic information. The second point, that concerning initiation sites, has already been discussed, as has the possible need for proteins associated with the DNA in its normal situation. The calculations described above and relating to the probable proportion of the absorbed DNA molecules which will be active in a given experimental system must also be seen in the context of the very low recovery of infectivity in extractions of viral nucleic acid. If a molecule, contained in a simple protein protective structure and adapted to the initiation of the transcription of exogenous genes in a eukaryotic cell, can be extracted in active form by the most gentle procedures available at a maximum efficiency of only 0.1%, it is most likely that the extraction of a molecule not so adapted from a situation requiring more vigourous procedures will recover biologically-active molecules at a very low efficiency indeed.

Up to relatively recently, the failure of repeated attempts to demonstrate DNA-mediated genetic transformation in mammalian cells could be attributed simply to the shortcomings of the experimental systems that were available. The widespread availability of well-characterised and stable mutants makes it at least likely that transformation has been attempted in a large number of laboratories. The lack of recent, proven positive findings, the doubts cast on the reproducibility of the published positive results and the negative results obtained using the advantageous genetic system of the present work suggest that DNA-mediated genetic transformation either requires highly specific conditions or that it cannot be done.

Polyoma Pseudovirions

Infection of cells with polyoma pseudovirions (see section 1.4) is another possible method of introducing genetic information into cell, and it has received some attention, although not all the work has been published in full. The advantages offered by this system are the known ability of polyoma virions to reach the cell nucleus in a form recognisable by electron microscopy, and to release the viral DNA in a form capable of initiating an infectious cycle. There exists, therefore, the possibility that the pseudovirions, which are composed of normal virion protein, may introduce the mouse DNA that they contain into the nucleus and may release it at the sites that are suitable for transcription. It is not a completely sure method, because the encapsidated mouse DNA may lack nucleotide sequences or bound proteins which are essential for the initiation or continuation of genetic activity.

What little work has been done has dealt mainly with the nature of pseudovirions and of the DNA that they contain. No reports of genetic activity have appeared, and there have been some negative findings (Hirt, personal communication, 1971).

Transfer of genes by such a mechanism is analogous to generalised transduction in the phage-bacterium system, and it is interesting to note that a report has appeared in which it is claimed that a bacterial genetic function into cultured mammalian cells by means of a transducing phage (Merril et al., 1971). This report awaits confirmation but, whether or not this is forthcoming, it is certain that a good deal of work will be stimulated. If the bacterial DNA can be transcribed in this way after being introduced into a mammalian cell, there seems to be little reason why mammalian DNA should not function under similar conditions, especially if it can be delivered to the nucleus by a virus particle.

The paper by Merrill and his colleagues does no more than demonstrate the acquisition of an enzyme function. It appears that the controls

necessary to exclude the possibility of spurious results arising from mutation or from infection by bacteria or mycoplasma have been included in the experiments, although no attempt was made to characterise the enzyme as bacterial or otherwise. The unexpectedness of these results makes it imperative that they be confirmed.

The work described in section 6 is obviously a preliminary study of the interaction of polyoma pseudovirions with the lines of PyY cells. The primary aim was to find if there was any genetic interaction and the experiments were designed with that in mind. The lack of detectable genetic change in the pseudovirion-infected cells could be due to a number of reasons. Firstly, the proportion of pseudovirions in this particular line of polyoma virus is not established. If it happens that this is a line producing few pseudovirions, the lack of genetic activity needs no other explanation. In fact, some attempts to produce radioactively-labelled pseudovirions by infecting mouse cells that had been prelabelled with ^{3}H-thymidine, yielded only very little labelling in the band on the less dense side of the main virus band in caesium chloride gradients. Previous reports have shown that cellular DNA which had been synthesised before infection is included in pseudovirions. The only studies on the intracellular fate of the pseudovirions are those by Osterman et al., (1970) and Qasba and Aposhian (1971). The former workers showed that 6% of the DNA absorbed by cells during infection with purified pseudovirions becomes DNase-sensitive, which they interpret as indicating release from the virus particles by uncoating. Qasba and Aposhian (1971) labelled mouse embryo cells with ^{3}H-thymidine and then infected them with polyoma virus. ^{3}H-labelled pseudovirions were purified from the progeny virus by means of repeated equilibrium-density gradients. The purified, labelled pseudovirions were used to infect further mouse embryo cells, from which DNA was subsequently extracted for hybridisation studies. Their

finding that labelled DNA extracted from these cells would hybridise with mouse embryo cell DNA was interpreted as indicating the survival of pseudovirion DNA, although the same result might be expected if the DNA-^{3}H had been totally degraded and reutilised. The reasons for infecting mouse embryo cells with pseudovirions derived from mouse embryo cells are not clear.

This work was all carried out in permissive cells, and its relevence to the nonpermissive PyY cells used in the present work is questionable. The mechanism by which virus replication is prevented in these cells is not known, and there is the possibility that it is of a nature which totally precludes the expression of any DNA contained in polyoma capsids. It is not even certain that the virus even enters the cells with reasonable efficiency. What is clear from the results reported is that there is, for whatever reason, no genetic expression of pseudovirion DNA in the mutant PyY cells. The possibility of expression in other cells is, of course, not excluded.

Uptake of Isolated Metaphase Chromosomes

Many different methods have been employed for the isolation of metaphase chromosomes, and there is considerable variation in the conditions used, particularly with respect to the pH and ionic concentration (Chorazy et al., 1963a and b; Cantor and Hearst, 1966; Huberman and Attardi, 1966; Schneider and Salzman, 1967; Maio and Schildkraut, 1967; Chorazy, 1970, personal communication). The advantages offered by the use of isolated chromosomes in attempting to introduce exogenous genetic information into a cell are twofold. Firstly, the DNA is supplied to the recipient cell in a state more nearly resembling its original condition than does isolated DNA. It is still in association with many of the components on chromatin, and thus has a better chance of arriving in the recipient cell as a functional genetic unit. The extent to which chromosome structure is destroyed by the isolation procedures is not known. In most cases, the criterion of intactness of the chromosomes is simple staining and microscopic examination. It has been shown, however, that isolated chromosomes can be treated with pronase or DNase under conditions in which a large proportion of the chromosomal proteins or DNA are demonstrably removed, without destroying their characteristic morphology (see Hearst and Botchan, 1970 This means that the purified chromosome preparations may well have lost more of their original components than is realised. Of course, the available chemical analyses have been carried out on purified material, and offer no guidance on this point.

The second advantage of the use of isolated chromosomes in genetic studies stems from the fact that it is possible to fractionate chromosomes into size classes (e.g. Mendelsohn and Satzman, 1968) - a procedure which implies the enrichment of different parts within the DNA of the various chromosome fractions. As is pointed out in the introduction (section 1.1), the complexity of the eukaryotic genome greatly reduces the chance of

detecting genetic transformation in a particular cellular character. As each gene is carried on a particular chromosome, size fractionation amounts to a concentration of each genetic marker into a smaller population of DNA molecules. The only other method at the present available for such fractionation of DNA is differential renaturation, which is less specific. Ideally, each chromosome type should be separated from all the others, for this would open the possibility of ascribing each gene to a particular chromosome simply by looking for genetic transformation mediated by different chromosome fractions. In actual fact, there is a considerable degree of overlapping in the sizes of metaphase chromosomes so that the best that can reasonably be expected is a separation into five or six classes. This in itself will allow an independent check of chromosomal locations deduced from cell hybridisation experiments, and will decrease by a factor of about five the amount of DNA competing for entry and transcription with that whose genetic activity is being sought in a transformation experiment.

The reactivation of heterochromatic nuclei of chick red blood cells after fusion with other cells suggests that there is at least the possibility that exogenous chromosomes or fragments of chromosom[illegible] become active after they enter the recipient cell. If the losses of material suffered by the chromosomes in the course of extraction are not too severe, then it might be expected that they will carry with them either the enzymes required for the initiation of transcription or the mechanisms necessary for the production of those enzymes, because the chromosomes must be, in their normal state, capable of decondensation at the end of mitosis. The control of chromosome function is not fully understood, and it is possible that some factors necessary for reactivation are supplied by the nucleoplasm. This would not prevent genetic functioning of the absorbed chromosomes if they reached the nucleus of the recipient cell.

It has already been demonstrated that chromosome fragments can enter

cells in culture, and that they will penetrate into the nucleus while still in a form identifiable by staining or by autoradiography (Chorazy et al., 1963b; Bootsma, personal communication). Chorazy and his colleagues (1963a and b) isolated chromosomes at pH5.6, by hypotonic lysis of the cells and differential centrifugation. Fragments taken up by cells were detected by autoradiography, and were found in the cytoplasm 6 hours after they were added to the medium. After 16 hours, they were visible in the nucleus. The pH at which the isolation was performed was important in the maintainence of the shape of the chromosomes, and subsequent work by Chorazy (personal communication) showed that those isolated at pH5.6, as used in the work quoted above, were extremely fragile. In fact, there was difficulty in repeating the extraction, and a new method was adopted involving extraction at pH 4.6. No uptake studies have been carried out on these chromosomes. The question of how closely the extracted chromosomes resemble their original state is unsettled, but the use in the extraction process of conditions so different from the intracellular environment must markedly reduce the chance of obtaining complete genetic units.

- EIGHT -

CONCLUSION

Section 8: CONCLUSION

Only in the past few years has it been possible to make a beginning on the genetic analysis of cultures eukaryotic cells. The advances have involved little that is new in conception, but have followed hard on the heels of improvements in technique. For example, the relative scarcity of suitable genetic markers has been circumvented by the introduction of analytical methods which are capable of making very subtle distinctions between different proteins. Cook (1970) exploited such a technique in order to show that the HGPRT activity which appeared in HGPRT$^-$ cells after fusion with chick erythrocytes was due to the appearance of an enzyme which had an electrophoretic mobility identical to that of the enzyme found in normal chick cells, and which was therefore specified by the reactivated chick nucleus. A similar method of distinguishing variants of HGPRT is described by Der Kaloustian et al (1969). This combination of electrophoresis with localised assay of enzyme activity is potentially very powerful because it permits the detection of an "alien" enzyme even in the presence of endogenous enzyme activity, and hence removes the necessity of selecting mutant cell lines for genetic analysis (see Miggiano et al., 1969).

In the determination of the chromosomal locations of particular genetic markers, two stages may be distinguished. The first has no parallel in bacterial genetics because it involves the assignation of the various genes to linkage groups and to the associated chromosome type. The second stage is the determination of the relative positions of the genes on their respective chromosomes, and it is this which may be compared to genetic mapping of bacteria because it involves the construction of a linear genetic map. It differs in that the eukaryotic cell has a physically fragmented genome. With the exception of the few linkage maps which have

been constructed on the basis of breeding experiments for organisms such as Drosophila, the genetics of eukaryotic cells has not passed beyond the first of these ~~three~~ two stages. Certainly the genetics of cultured cells is at a very early stage indeed.

The construction of linkage groups in cultured mammalian cells has been made possible by the development of cell fusion techniques and in particular by the fact that some combinations of cells will give rise to a hybrid from which one set of chromosomes is gradually eliminated. Incubation of these hybrids in selective conditions allows the isolation of clones in which one region of the karyotype that is usually eliminated has been retained. Subsequent examination of these clones will reveal which of the unselected markers are retained with high frequency and are therefore closely linked to the selected gene. This type of work has attracted a considerable amount of interest, and linkage information is accumulating, particularly with respect to human cells, the chromosomes of which are lost after fusion with Chinese hamster (Kao and Puck, 1970) or with mouse (Ruddle et al., 1971) cells. The identification of the chromosome types with which the linkage groups are associated has been more difficult because only recently have methods of unambiguously identifying chromosome types been developed. Thus, Kao and Puck (1970) were unable to identify the chromosomes associated with their linked genetic markers, and Ruddle et al (1971) were only able to assign to their chromosome those markers which were linked to HGPRT, which is known by other means to be X-linked (see Fujimoto et al., 1971). The most promising current methods for identification of chromosomes or of fragments which have been involved in rearrangements involve the use of staining procedures which reveal patterns of transverse bands. Firstly, there is the range of quinacrine dyes (Caspersson et al., 1970a, 1970b) which produce fluorescent bands in patterns which are reproducible and unique to each chromosome type.

The second type of staining is the use of modifications of giemsa, which also give patterns of transverse bands (George, 1970; Sumner et al., 1971). A start has been made on the exploitation of these techniques for the identification of chromosomes (for example, Rowley and Bodmer, 1971). A direct demonstration of the genetic composition of each class of chromosome, the introduction into cells of genetic changes by absorbed chromosomes, is not at the moment possible. The necessary degree of purification is well beyond the reach of current chromosome fractionation techniques, and, while uptake of isolated chromosomes has been demonstrated, there is no indication that they can display genetic activity (Chorazy et al., 1963b; Bootsma, personal communication).

The advance of eukaryotic cell genetics into the second stage - that of the determination of the linear sequence of genes - will almost certainly depend on the development of techniques similar to those used for the same purpose in bacterial genetics. It does not seem that the uncontrolled processes of hybrid karyotype reduction will ever provide data of sufficient precision to lend any degree of certainty to the mapping of closely-linked markers. It is difficult to assess the chances of such methods as transformation and transduction becoming available for use in eukaryotic cells. Despite repeated attempts in many laboratories and over many years, there are no confirmed cases of the genetic of exogenous DNA. The alternative possibilities - that such expression either is very rare or requires very special conditions or cannot occur - will probably only be finally distinguished by a better understanding of the processes of DNA transcription and replication in eukaryotic cells, but there remains the chance that there is on some lab shelf a substance which will have as powerful an effect as DEAE-dextran has on the infectivity of viral DNA. Genetic activity expressed by the host-cell DNA in pseudovirions has also proved elusive, but this could well be a reflection of the lack of a really systematic investigation. It is becoming clear that

viruses other than polyoma produce pseudovirions (see section 1 and Lavi and Winocour, 1972), and any one of these may prove capable of transducing cellular genes.

Considerable resources of conceptual and experimental tools are being built up, but the difficulty remains that there is no method for the controlled transfer of genetic information from one eukaryotic cell to another. Once this blockage is overcome, the way will be open for developments as exciting as and more fruitful than the revolution in bacterial genetics in the nineteen-fifties and sixties.

SUMMARY

The published evidence relating to the uptake and genetic effects of purified DNA is reviewed, and the nature of polyoma pseudovirions is discussed. The characteristics of the biochemically-marked cell lines used in this work are described, and conditions for the labelling of DNA with bromodeoxyuridine and tritium are investigated.

Experiments on the uptake of isolated DNA indicate that up to 5% of the DNA complement of the recipient cell is absorbed from the medium. There is a lag of 2-3 hours after addition of DNA to the medium before uptake becomes detectable, after which time uptake is much more rapid. Even at the earliest time at which donor DNA is detectable in recipient cells by autoradiography, all of it is found in the nuclei, unless DEAE-dextran is present in the medium, in which case the donor label appears in clumps in the cytoplasm. Density-labelling experiments are consistent with this, for in the presence of DEAE-dextran the donor DNA does not become associated with the density band characteristic of the recipient material. DEAE-dextran also prevents the association of donor DNA with high molecular-weight DNA in sucrose gradients, and renders the majority of DNA uptake insensitive to arabinosylcytosine. In addition to this, DNA absorbed in the presence of DEAE-dextran is lost from the cell over a period of about 48 hours after its removal from the medium. There is evidence that, even in the absence of DEAE-dextran, there is a fraction of the donor DNA which survives intact, although they are associated with the recipient density band.

Experiments are described which did not detect genetic changes in cells treated with polyoma pseudovirions or with pure DNA under a variety of conditions.

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