Studies on biochemical variants of BHK 21/C13 cells

by

G. B. Clements

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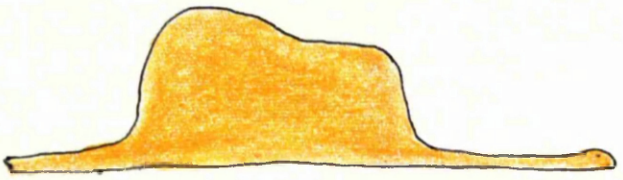
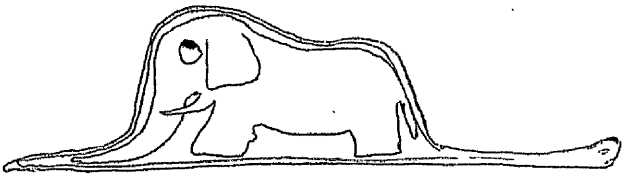
ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

J'ai alors beaucoup réfléchi sur les aventures de la jungle et, à mon tour, j'ai réussi, avec un crayon de couleur, à tracer mon premier dessin.
Mon dessin numéro 1. Il etait comme ça:



J'ai montré mon chef-d'oeuvre aux grandes personnes et je leur ai demandé si mon dessin leur faisait peur.
Elles m'ont répondu: "Pourquoi un chapeau ferait-il peur?"

Mon dessin ne représentait pas un chapeau. Il représentait un serpant boa qui digérait un éléphant. J'ai alors dessiné l'intérieur du serpant boa, afin que les grandes personnes puissent comprendre. Elles ont toujours besoin d'explications. Mon dessin numero 2 etait comme ça:



Antoine de Saint-Exupery

The Little Prince.

I wish to thank Professor J. H. Subak-Sharpe for his supervision and encouragement.

I also wish to thank everybody else working in the Institute of Virology, without whose help this work could not have been carried out.

I thank Dr. E. A. C. Follett for performing the electron microscopy of Sendai virus and Dr. R. Elton for statistical advice. Thanks are due also to Mrs. I. Ferguson for typing the thesis.

Finally, I thank my wife both for her patience and help with the diagrams.

SUMMARY

Polyoma transformed BHK 21/C13 cells and some of their variants were used during these investigations.

The variants had previously been derived from BHK 21/C13 cells by selection for resistance to several purine and pyrimidine analogues in tissue culture. The behaviour of these cells was compared in several ways. The levels of resistance of the cells to some purine and pyrimidine analogues were determined. The variants when exposed to an analogue to which they were resistant continued to grow at more than one hundred times the concentration at which the majority of wild type cells were killed. The variants generally survived no better than the wild type in the presence of those purine and pyrimidine analogues for which resistance had not been selected. The growth curves of the wild type and the variants under non-restrictive conditions were identical. The incorporation of radioactively labelled purines and pyrimidines from the growth medium was determined using both scintillation counting and autoradiography. Wild type cells incorporated each of the nucleic acid precursors used in large amounts. In most cases the variants were no longer capable of incorporating significant quantities of radioactive bases or nucleosides to whose analogues they were resistant.

The phenomenon of metabolic co-operation is well established. It occurs between cells in contact at light microscopic level and causes an alteration in the phenotype of a co-operating variant cell to that of the cell with which it is in contact. Metabolic co-operation requires a donor-recipient relationship between at least two cells, with the cell exhibiting the enzyme activity being the donor. The technique of double label autoradiography was used to distinguish between two types of cell in a mixture after one had been prelabelled with 14-C thymidine. Metabolic

co-operation could be studied at the level of the individual cell.

The effects of metabolic co-operation were investigated using cells growing under selective conditions. Under conditions in which the cells were auxotrophic for a purine source metabolic co-operation influenced the selection. Variants were capable of growing when present as a mixture under conditions in which they would not be able to survive alone. This effect of metabolic co-operation has been termed "the kiss of life". Metabolic co-operation has implications both for the selection of variant cells in culture and also for the diagnosis and possible treatment of inborn errors of metabolism.

The biochemical basis of metabolic co-operation was investigated. The phenotypic alteration was found to have a short half life which is compatible with there being an exchange of labelled material, possibly nucleotides, between cells. Metabolic co-operation may be suitable as an in vitro model of differentiating systems based on the setting up of concentration gradients of low molecular weight diffusible substances between cells.

The reactivation of chicken erythrocyte nuclei was followed after fusion with the BHK 21/C13/PyY cell variants. Sendai virus was grown up in embryonated eggs, inactivated with β-propriolactone and used to fuse the cells in suspension. The reactivation process followed the pattern described in other cells, the reactivating nucleus undergoing enlargement, synthesising DNA, RNA, forming a nucleolus and finally directing the synthesis of chick specified proteins. The process of reactivation progressed more rapidly within the hamster cells than the mouse A9 cells used by other workers. A variant selected for resistance to four purine analogues offered the possibility of following the reactivation of four enzyme activities simultaneously within a single type of cell. The time course of the appearance of all four was the same and correlated well with the appearance of a functional nucleolus within the reactivated erythrocyte nucleus.

PREFACE

The introduction describes the way in which the principles of genetic variation and selection were first appreciated. These may be used in the analysis of mammalian cells growing in tissue culture, which occasionally undergo changes to produce cells termed "variants". Variants differ in their behaviour from the wild type cells and may in some cases be separated from them when grown under selective conditions. Chapter 1 outlines the biochemical basis of the selective systems involving the purine salvage pathways, which have been much used to select mammalian cell variants. In Chapter 2 the selection of variant mammalian cells is described and discussed. Some approaches to the investigation of mammalian cells involving the use of variants and the transfer of genetic material between cells are described in Chapter 3.

The remainder of the thesis describes my own work using BHK 21/C13 cell variants resistant to several purine and pyrimidine analogues.

INTRODUCTION

Biological material is plastic and adapts to suit the prevailing environmental conditions.

Charles Darwin (1859), after studying variation in living organisms, deduced the principle of natural selection. He realized it was a mechanism whereby advantageous changes could be encouraged to remain within the population once they had appeared.

Darwin founded the principle of natural selection on three observations:

1. Offspring are initially present in greater numbers than their parents.
2. Under normal circumstances the numbers of a given species remain constant.
3. Variation continually appears within members of a species.

From the first two observations he drew the conclusion that there was competition for survival, which with the third observation led to the principle of natural selection. If there is a continual struggle for existence and not all individuals are alike, those exhibiting favourable characteristics will have a greater chance of survival than those with unfavourable characteristics. If variation is transmissible to the offspring, differential survival will gradually tend to accumulate advantageous characters within the population.

Despite the fact that the details of the transmission of characters to the next generation were not understood in Darwin's time, his theories remain intact. During the fifty years after the publication of "The Origin of Species", enough information accumulated to explain the mechanics of inheritance and to establish genetics as an experimental science. Using

the pea, Mendel (1865) showed the way in which simple characters could be distributed to the progeny during sexual reproduction by segregation and recombination. Later, chromosomes were recognised, together with the facts that they usually remain constant in number in the cells of any one species and reproduce by longitudinal splitting (Flemming, 1879 and van Beneden, 1884). Weismann (1885) first made the clear distinction between the somatic cells of the body which are not involved in reproduction, and the germplasm which alone contains the information transmitted to the offspring. The inheritance of acquired characteristics, as postulated by Lamarck (1815), fell from favour because of the difficulty in understanding how the genetic information for the adaptation could be added to the germ cells. Boveri (1890) and others observed the reduction in chromosome number during meiosis.

Chromosomes behave as if they carry the characters studied by Mendel. Segregation occurs during meiosis, the homologous pairs of chromosomes passing to different cells. Recombination takes place at fertilization, both paternal and maternal sets of chromosomes being present in the fertilized ovum. de Vries (1900), while studying the evening primrose, coined the term mutation to describe new types arising at a single step from their parents. The term mutation is now reserved to describe changes that occasionally take place in part of the genetic material of an individual which produce an alteration in the characteristic potentially specified. In fact, de Vries was not observing mutation, but aberrant segregation from translocation polyploids.

These studies, when viewed as a whole, provide an explanation of the mechanisms of inheritance and the production of variation, but it is incomplete. Mutation is a rare event and the new character may, far from being of survival value, be deleterious to the individual possessing it. Much of the observed variation is produced by the association of existing characters in novel ways. Characters behaving in a true Mendelian fashion are unusual, what is apparently a single character may be the result of a

complex interaction between a particular genetic composition and the environmental conditions.

In all species there appear to be a large number of metaholic disorders caused by the presence of abnormal genes. Garrod (1909) first appreciated the nature of these diseases and termed them inborn errors of metabolism. His ideas were based on the study of alkaptonuria, a disease of man in which large quantities of homogentisic acid are produced in the urine. Alkaptonurics are usually asymptomatic, but their urine turns black on standing, providing an easily noted characteristic. Garrod noted that homogentisic acid was readily metabolised by normal subjects but when fed to alkaptonurics was quantitatively excreted in their urine. He also showed that its excretion in alkaptonurics was augmented by increasing the dietary protein due to the presence of the aromatic amino acids phenylalanine and tyrosine. Garrod made two deductions from this evidence. Firstly that homogentisic acid is a normal intermediate in the catabolism of the aromatic amino acids, though he could not detect its presence in normal individuals. Secondly that in alkaptonurics the lack of an enzyme leads to a break in the degradative path and the accumulation of large quantities of metabolic intermediates. It is now possible to assay the relevant enzymes and show that there is an inability to convert homogentisic acid to maleylacetoacetic acid in alkaptonurics (La Du, Zannoni, Laster and Seegmiller, 1958).

Garrod also noted a characteristic familial distribution of alkaptonuria and concluded that the disease was due to a hereditary defect caused by a rare recessive factor. A corollary to this was that the presence of the normal factor was needed for the formation of enzyme activity, indicating that some genetic factors exert their effects on the organism by directing the synthesis of enzymes. The term gene is now used to describe these factors. Thus at the dawn of genetics, long before biochemistry had developed any understanding of the mechanisms involved, the study of a disorder of man provided evidence of a mechanism of gene expression.

It is now known that one structural gene specifies one polypeptide chain. One protein, for example myoglobin, may be composed of a single polypeptide chain. Many proteins are made up of two or more chains. The chains may be identical, as in the case of peptidase A1 which is composed of two α^1 chains. They may however be different: haemaglobin A has four chains, two identical α and two identical β.

The genetic information of an individual consists of a large number of genes arranged in a linear fashion along the chromosomes. Each gene has a particular position termed a locus. At any one locus there may exist a whole series of alternatives termed alleles. Different alleles of a structural gene determine different versions of the polypeptide chain specified at that locus. Mammals are diploid organisms so each allele usually exists in duplicate, one on each of the homologous pairs of chromosomes. When the same allele is present at both loci, the organism is homozygous for that character. If different alleles are present, the organism is heterozygous. Alleles which are expressed in the heterozygous state are described as being dominant. Alleles that fail to be expressed in the heterozygous state are termed recessive, those carried on autosomes are only expressed when present in the homozygous state. Recessive alleles of X-linked genes are expressed under different conditions. Females heterozygous for an X-linked recessive allele may express the character in cells whose X-chromosome carrying the dominant allele is inactivated (Lyon 1961). A recessive allele on this region in a male, for example that resulting in haemophilia, is unpaired and expressed whenever present.

The total genetic information available to an individual, whether expressed or not, is described as the genotype. The actual appearance of the individual is termed the phenotype. If the organism is homozygous for an allele, the phenotype will reflect the genotype. If the organism is heterozygous and one of the alleles is dominant, this alone will be expressed and the phenotype will differ from the genotype. In some cases neither allele is dominant

and both continue to be expressed in the heterozygote, resulting in a phenotype intermediate to that of the two homozygotes.

Different alleles of the same gene result from mutational events which took place in bygone generations. A large number of mutant alleles may be present in different members of the species, though individually most of them are very rare. Two classes of mutations result from altering the sequence of purine and pyrimidine base pairs in the DNA of a gene. In the first class one base is exchanged for another, termed a single base change. Most single base changes are capable of reversal by back-mutation. In the second class, one or more bases are added to or removed from the gene. Mutations involving the loss of nucleotides are termed deletions, and those involving the gain of nucleotides insertions. Sometimes a long sequence of nucleotides or even whole genes may be lost. Back-mutation to the normal sequence is impossible for large deletions and highly unlikely even in the case of the deletion of a single base. The phenotypic alteration from mutant to wild type produced by back-mutations is termed a reversion.

There are a great number of variant forms of haemoglobin A that have been identified in man, (Lehmen and Carell, 1969). Over one hundred of these have been sequenced and the majority differ by only one amino acid from normal haemoglobin A. Each of these differences can be accounted for by a single base change in the DNA of the gene. There are also some haemoglobin variants in which there is absence of one or a sequence of amino acids. These are caused by deletions within the structural gene. Different spectra of amino acid substitutions will no doubt be shown to occur in every protein. Sometimes a mutation may affect a region of the protein which is not essential for its function leaving the phenotype unchanged. In many cases a mutation will result in the production of a malfunctioning protein. When the function of a protein vital for survival is seriously affected, the mutation may be lethal. If the protein functions poorly, or is non-functional but not essential for survival, the mutation may manifest

itself as an inborn error of metabolism.

Most of the inborn errors of metabolism are inherited as autosomal recessive characteristics. Though the diseases themselves may be rare, the abnormal alleles causing them must be very much more common, occurring in the majority of cases in heterozygous carriers which show no symptoms. A disease present in 1 in 100,000 births may be due to a gene present in the heterozygous form in 1 in 160 individuals. Fresh mutations are continually arising at random, but since the gene will on average only be transmitted to half the children of the individual who possesses it there is a distinct chance that it will not be transmitted to the next generation. Disregarding selection, the majority of new mutant genes are likely to be eliminated in a random manner during the course of 10 to 20 generations. By chance however some persist and spread, a phenomenon which has been called random genetic drift. One special example is termed the founder effect. A small number of individuals may form an isolated new community. If one of the founder members is heterozygous for a rare mutant gene, this might happen to have a high frequency among the descendants of the original colonists. For example hereditary tyrosinaemia is very common in an isolated French-Canadian community living in Quebec. Even though an individual carrying a mutation may not be at a selective disadvantage, the likelihood is that the mutant character will be eliminated within a few generations. Natural selection is superimposed on these random phenomena. If individuals carrying a mutation are at a selective disadvantage with respect to the wild type, producing fewer offspring, the elimination will be hastened. If the mutation produces individuals which are better adapted to the environment and produce more offspring, it will be more likely to be conserved.

The variation that is the essential prerequisite for selection is produced in two ways. Firstly there is a continuous process of mutation producing fresh alleles. Secondly there is reassortment of the alleles carried by members of a species during sexual reproduction. Both processes

tend to produce a population containing individuals differing in their fitness to survive and reproduce. Natural selection encourages the persistence of characteristics in combinations leading to the development of individuals most suited to the environmental conditions prevailing.

The principles of genetic variation and selection have been described in the context in which they were first appreciated. They are equally applicable to mammalian cells in tissue culture. One of the problems of dealing with all biological material is its variability and capacity to adapt to new conditions. Genetics makes use of the variability to investigate how organisms function. Cultured mammalian cells may be regarded as organisms, and their variability makes them amenable to genetic study.

FIGURE 1

The purine salvage pathways in BHK21/C13/PyY cells

AK Adenosine kinase.

IGK Inosine/guanosine kinase.

APP Adenosine monophosphate pyrophosphate phosphoribosyl transferrase.

IPP Inosine monophosphate/guanosine monophosphate pyrophosphate phosphoribosyl transferrase.

1. 5'-nucleotidase.

2. Nucleoside phosphorylase,

3. Adenosine deaminase.

4. Adenylosuccinate synthetase.

5. Adenylosuccinate lyase.

6. AMP deaminase.

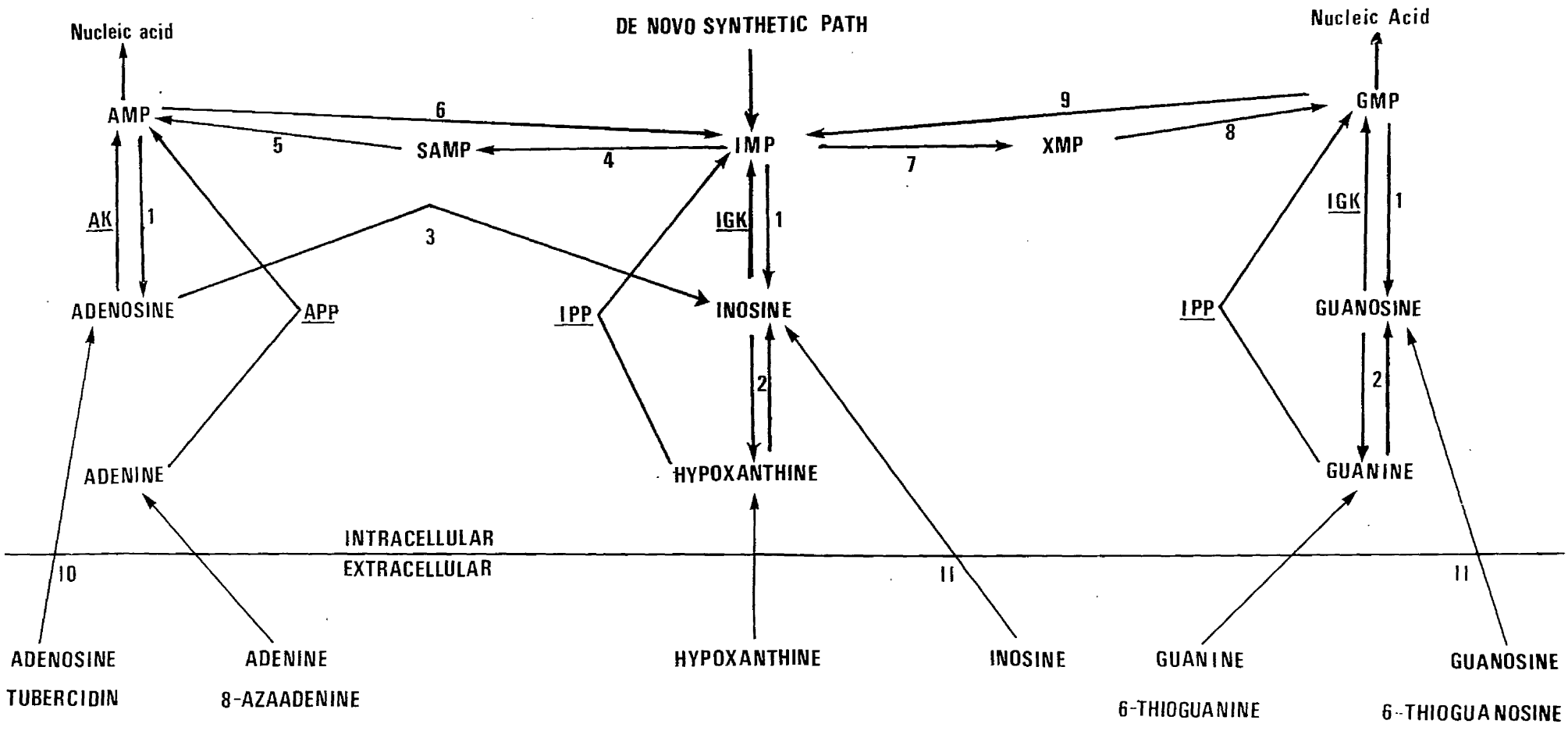
7. IMP dehydrogenase.

8. XMP aminase.

9. GMP reductase.

10. Adenosine permease.

11. Inosine/guanosine permease.


Nucleic acid
DE NOVO SYNTHETIC PATH
Nucleic Acid
AMP
6
5
SAMP
4
IMP
9
7
XMP
8
GMP
AK
1
3
IGK
1
IGK
1
ADENOSINE
APP
IPP
INOSINE
IPP
GUANOSINE
2
2
ADENINE
HYPOXANTHINE
GUANINE
INTRACELLULAR
EXTRACELLULAR
10
11
11
ADENOSINE
TUBERCIDIN
ADENINE
8-AZAADENINE
HYPOXANTHINE
INOSINE
GUANINE
6-THIOGUANINE
GUANOSINE
6-THIOGUANOSINE

1. SELECTIVE SYSTEMS INVOLVING THE PURINE SALVAGE PATHS

Mammalian cells are normally able both to utilize performed purine bases or nucleosides from the growth medium and also to synthesise purines de novo. Figure 1. Williams and Buchanan (1953) first demonstrated the phosphoribosyltransferrase reaction by which free purine bases condense with 5-phosphoribosyl 1-pyrophosphate (PRPP) to form ribonucleotides. They showed that inosinic acid was formed from hypoxanthine, ATP and ribose 5-phosphate without the formation of inosine as an intermediate. Enzymes catalyzing this reaction were characterized in bacteria (Kornberg, Lieberman and Simms, 1955, and Korn, Remy, Wasilejko and Buchanan, 1955) and in mammalian tissue (Flaks, Erwin and Buchanan, 1957). Two separate pyrophosphorylases have been described in mammalian cells. One is specific for adenine, adenosine monophosphate pyrophosphate phosphoribosyl transferrase (APP, adenine phosphoribosyltransferrase, APRT) E.C. 2.4.2.7. The other is specific both for hypoxanthine and guanine, inosine monophosphate pyrophosphate phosphoribosyl transferrase (IPP, hypoxanthine/guanine phosphoribosyltransferrase, HGPRT) E.C. 2.4.2.8. The equilibria of the reactions favour nucleotide synthesis and the pyrophosphorylases have been considered to form a salvage pathway for the reutilization of free purines (Kornberg, 1957).

The lack of IPP activity is inherited as an X-linked recessive trait in man (Seegmiller, Rosenbloom and Kelley, 1967). It is associated with a profound disturbance of purine metabolism with increased de novo synthesis and hyperuricaemia, presenting clinically as mental retardation, spastic cerebral palsy, choreoathetosis and behavioural disturbance known as the Lesch-Nyhan syndrome (Lesch and Nyhan, 1964). Since the absence of IPP causes such profound disturbances in purine metabolism, the term salvage pathway, which implies a non-essential function, is not satisfactory. However, this nomenclature will be retained.

An alternative salvage path for the formation of ribonucleotides exists. Free base may be converted to the ribonucleoside by nucleoside phosphorylase.

The ribonucleoside may subsequently be phosphorylated to the ribonucleotide by a kinase. Figure 1. Mammalian cells studied to date all lack a nucleoside phosphorylase acting on adenine (Krenitsky, Elion, Henderson and Hitchings, 1968). The presence of a kinase specific for inosine and guanosine has only been reported in a few mammalian cells (Gadd and Henderson, 1970, and Edwards, 1970). There is evidence to suggest that specific permeases may exist in the cell membrane of BHK 21/C13/PyY cells facilitating the entry of purine nucleosides from the growth medium (Edwards, 1970).

Each of the mononucleotides can be acted on by a 5'-nucleotidase to form the corresponding nucleoside. Adenosine may then either be deaminated to inosine or rephosphorylated to AMP. A kinase acting on inosine or guanosine is rare in mammalian systems, but unlike adenosine, these nucleosides can be converted to free bases by a phorphorylase.

Purine bases are catabolized via xanthine to end products which differ between species. Uric acid is produced in primates, birds and some reptiles, but a further oxidised product, allantoin, in mammals other than primates.

The de novo pathway will not be discussed in detail. There are two steps, both of which require folic acid, that are of importance as far as the selective systems are concerned. In the first glycinamide ribonucleotide is converted to formylglycinamide ribonucleotide. In the second amidoimidazole carboxamide ribonucleotide is converted to formyl aminoimidazole carboxamide ribonucleotide. The two steps are blocked by folic acid antagonists, converting normal cells to purine-requiring auxotrophs. Cell survival under these conditions requires both an external purine source and an intact purine salvage pathway capable of utilizing the external source of purines.

Inosine monophosphate (IMP) is the first nucleotide synthesised on the de novo path, and is interconvertible with the other mononucleotides. Adenosine monophosphate (AMP) is formed in two stages catalyzed by adenylo-succinate synthetase and adenylosuccinate lyase via the intermediate adenylo-succinate. Conversion to guanosine monophosphate also takes place in two

steps, the first forms xanthosine monophosphate (XMP) by IMP dehydrogenase, the second guanosine monophosphate (GMP) by XMP aminase. In each case the reverse reactions take place in a single step catalyzed by AMP deaminase and GMP reductase respectively. Since all nucleotides are interconvertible, the total purine requirements of the cell can be met by providing a single purine source after the de novo path is blocked by a folic acid antagonist. The de novo synthesis of thymidine and glycine is also affected by the folic acid antagonist, and these compounds have to be provided in the medium in addition to a purine source. Hakala (1957), Littlefield (1964) and many other workers have used selective systems based on the block of de novo synthesis of purines and thymidine by a folic acid antagonist. Depending on the external supply of precursors, different phenotypes can be selected. Commonly hypoxanthine and thymidine have been supplied, selecting for cells expressing IPP and thymidine kinase (TK). This medium has been termed HAT. Adenine has also been used as a purine source, selecting for cells expressing APP and TK.

Kidder and Dewey (1949) postulated that purine analogues would be found incorporated into the nucleic acids of sensitive cells after having been converted to the nucleotide derivatives. Confirmation of this was obtained by Remy and Smith (1957) who demonstrated that APP was able to use the analogue 2,6 diaminopurine as a substrate. It was also shown that IPP could use 6-mercaptopurine (Lukens and Herrington, 1957) and 8-azaguanine (Way and Parks, 1958) as substrates. Structural analogues of a normal metabolite which interfere with its function or use are termed antimetabolites. Many have been developed with a view to the chemotherapy of bacterial, viral and malignant diseases. Among these have been a large number of purine analogues, some of which have also been used to develop resistant cells in vitro. Purine analogues are non-toxic as the base or nucleoside, only after metabolism to the nucleotides are they able to exert an inhibitory effect on the cell.

Peters (1952) has termed this lethal synthesis. Nucleotides are unable to enter cells from the medium in significant quantities (Liebman and Heidelberger, 1955). Nucleotide analogues are therefore of no value for use as antimetabolites.

Nucleotide analogues produced within a sensitive cell may exert their inhibitory effects in several ways (Roy-Burman, 1970).

1. They may bind to the active site of an enzyme and mimic the normal metabolite. The type of inhibition may be competitive or non-competitive, depending on the affinity of the inhibitor for the active site. An analogue that is converted to several different derivatives may produce inhibitory effects on several different enzymes.

2. After incorporation into DNA, some analogues may cause errors in the process by which the DNA replicates, resulting in an increased mutation frequency. The defective DNA will also increase the errors in RNA transcription causing abnormalities in all species of RNA.

3. The analogue may interfere with cellular control mechanisms. A derivative of an inhibitor may accumulate in a cell, or cause accumulation of normal metabolic intermediates, and activate a control system of feedback inhibition or stimulation through allosteric effects or repressor mechanisms. A single analogue may be metabolized to several different abnormal products and affect the activity of a number of different enzymes by feedback effects.

Cells selected for resistance to purine analogues are often found to lack purine pyrophosphorylase enzymes. For example 8-azaguanine resistance has been found to correlate with loss of IPP activity in several mammalian cell lines (Brockman, Kelley, Stutts and Copeland, 1961, Davidson, Bradley, Roosa and Law, 1962 and Littlefield, 1963). Such cells avoid the effects following lethal synthesis of nucleotide analogues and survive in the presence of the non-toxic base analogue. Littlefield (1963) was able to correlate the level of resistance of mouse fibroblasts to 8-azaguanine with their content of IPP activity. Szybalski, Szybalska and Ragni (1962) however

obtained a partially resistant line of D98 cells in which the IPP activity was unchanged in comparison with the wild type. It was suggested that there might be a reduction in the permeability of the partially resistant D/98 cells to 8-azaguanine.

The emergence of a drug-resistant cells takes place by the selection of a variant individual from a heterogeneous population. The variant cells may pre-exist within the population, or arise during the course of selection by mutation or adaptation. Independently isolated variants may escape the inhibitory effects of a particular set of selective conditions in different ways. Theoretically, a resistant cell could utilize one or more than one of the following mechanisms to enable it to survive under selective conditions:

1. Decreased conversion of the inhibitor to an active form.
2. Increased degradation of the inhibitor to an inactive compound.
3. Increased synthesis of the inhibited enzyme.
4. Modification of the enzyme.
5. Decreased permeability to the inhibitor.
6. The emergence of alternate pathways to bypass the metabolic block.
7. Increased synthesis of the normal metabolite to compete out the inhibitor.

Different sets of selective conditions, the presence of purine analogues or folic acid antagonists for example, will favour various mechanisms of resistance being used by the surviving cells. Purine analogues are metabolized to compounds capable of exerting inhibitory effects at more than one site. 6-Thioguanine (TG) is converted to its active metabolite 6-thioguanylic acid (TGMP) by the enzyme IPP. TGMP has been quoted as inhibiting three enzymes (Roy-Burman, 1970), phosphoribosylpyrophosphate amidotransferrase which catalyzes the first step in the de novo pathway of purine synthesis, IMP dehydrogenase and a nucleotide monophosphokinase specific for the phosphorylation of GMP to GDP. TGMP is also incorporated in small amounts into both RNA and DNA via the deoxyribonucleotide triphosphates, where it

may exert deleterious effects. Lethal synthesis of TGMP may be prevented by a solitary modification at either of two steps. Entry of TG into the cell could be blocked, or there could be a decreased conversion of the inhibitor to the active form by a reduction in the activity of IPP. After lethal synthesis has taken place, adaptation by a cell to avoid inhibition would entail overcoming all the separate inhibitory effects simultaneously. It is more likely that a single modification leading to the development of resistance would occur than several. Cells resistant to purine analogues would thus be expected to have adapted in ways that prevent lethal synthesis taking place. This is found to be the case, a single line of BHK 21/C13/PyY cells resistant to both TG and 6-thioguanosine (TGR) lacks IPP activity and appears to lack a specific permease for TGR (Edwards, 1970).

Folic acid in the reduced form is necessary, among other things, for the de novo synthesis of purines. Aminopterin and amethopterin, folic acid analogues, inhibit one enzyme, dihydrofolate reductase, preventing the synthesis of reduced folic acid. No lethal synthesis is required, the folic acid analogue itself being the inhibitor. Hakala, Zakrzewski and Nichol (1961) reported that two amethopterin resistant lines of sarcoma 180 cells in culture contained increased amounts of dihydrofolate reductase. The properties of the enzymes were unchanged with respect to kinetic characteristics, molecular weight, electrophoretic mobility and the rate of reaction. It was suggested that the excess dihydrofolate reductase immobilized intracellular amethopterin by binding it tightly. Since the amethopterin could only enter the cells slowly, enough dihydrofolate reductase remained free within the cell to maintain the levels of reduced folic acid, allowing the cells to survive.

The above examples demonstrate diametrically opposite adaptations being used by variant cells selected for growth in different conditions. In one case an enzyme activity was lacking, in the other an enzyme activity was increased.

2. BIOCHEMICALLY VARIANT MAMMALIAN CELLS

The ideas outlined in the introduction were developed in an attempt to explain the alterations that had taken place within species during their evolution and the variations that were observed between individuals of a single species. They have since been tested on biological material under controlled conditions within the laboratory and form a cornerstone of present attempts to understand living organisms. Ideally, an organism to be used for genetic analysis should have:

1. A short generation time.
2. The capacity to grow in a chemically defined medium.
3. A haploid genome.
4. The ability to yield numerous nutritional mutants.
5. The capability of being handled in large numbers in the laboratory.
6. Mechanisms by which genetic material can be exchanged between different individuals.
7. A simple structure.

Bacteria and viruses approach these ideals most closely. Many of the most dramatic advances in the field of genetics have been made using these as experimental material. Mammals satisfy few of the ideals. In particular, the diploid nature of their genomes complicates the genetic analysis. The phenotype of a diploid individual may not reflect the genotype. Only after studying the pedigree, and if necessary performing breeding experiments, may the genotype of a diploid organism be determined. Most experimental genetics using higher organisms has been carried out using Drosophila or the mouse as experimental material. Genetic studies on man are difficult, due both to a long generation time and the impossibility of carrying out defined breeding programmes. However, a large amount of information has accumulated during the study of inherited abnormalities and a good deal is

now known about human genetics.

The study of mammalian genetics will be facilitated by the use of somatic cells growing in culture. These can now be obtained in large numbers and analyzed using approaches developed during the investigation of the genetics of micro-organisms. However, while it may be useful to formulate experiments on the basis of results obtained during the study of micro-organisms, there exist great differences in complexity and organization between these and mammalian cells.

In at least one case the somatic cells of a higher organism have been shown to carry all the information needed to specify a complete individual. Gurdon (1962) and Gurdon and Uehlinger (1966) showed that after the transfer of nuclei from the intestinal epithelial cells of Xenopus feeding tadpoles to enucleated eggs, normal individuals could be obtained. The retention of the complete genetic information by somatic cells after differentiation is probably a general state of affairs in higher organisms. Only part of this information is expressed at any one time by a cell within the organism or growing in vitro. Cell lines in culture exhibit few specialized features. Occasionally a cell line continues to express some of the differentiated characteristics of the tissue from which it was derived. One example is a melanoma cell line capable of producing pigment (Moore, 1964). Transplantation antigens in general continue to be expressed in cultured cells. L cells have all the specificities of H-2k in the C3H mice from which they were derived more than 25 years ago (Gangal, 1966). Tissue specific antigens however quickly disappear after explantation. Weiler (1959) showed that kidney specific antigens were absent from primary kidney cells two days after their establishment in culture.

The mechanisms which control the expression of differentiated characteristics are not fully understood. There are isolated examples of enzyme induction. In a hepatoma cell line the enzyme tyrosine aminotransferrase (TAT) is induced by steroid hormones (Tomkins, Thompson, Mayashi, Gelehrter, Granner, Peterkofsky, 1966). Interaction between cells has been shown to

be necessary for differentiation to take place in some systems. Grobstein (1961) using millipore filters of varying sizes showed that induction of kidney tubules by mesenchyme or dorsal spinal cord inducer took place even though the cell surfaces were separated by up to 50μ. The substance(s) causing induction appeared to be macromolecular because their effects were impeded by a millipore filter with a pore size of 100 Å and blocked by cellophane. Extracellular material, part of which was mucopolysaccharide, was present in the gap between the cells and may have been important in the transmission of information during induction.

The state of differentiation of an established cell line in tissue culture is uncertain. The common inability of an established cell line to express differentiated characteristics may be the result of:

1. Irreversible loss of the capability to express differentiated characteristics during adaptation to tissue culture conditions.
2. Growth in an unsuitable environment. The cells remain capable of redifferentiation on exposure to the correct conditions. Weiss (1939) has termed this modulation.
3. The selection of non-differentiated cells pre-existing within the explanted tissue. Those cells growing out to form the cell line may never have been differentiated, being derived from stem cells.

Whatever the situation, it is clear that mammalian cells in tissue culture have genetic information potentially available to them which is greatly in excess of what is required for their survival and growth in tissue culture. An alteration in their environment, for example exposure to selective conditions, may result in the expression of new characteristics or the suppression of characteristics previously expressed. A change in the way a cell expresses its genetic information is termed epigenetic. The induction of TAT by steroid hormones is one example. At the present time it is impossible to determine for certain whether a phenotypically changed mammalian

cell has mutated or undergone a permanent epigenetic change. Epigenetic changes are very likely to be involved if the phenotype of all the cells rapidly returns to normal on removal of the selective conditions. Mammalian cells in culture that are phenotypically different to the parental stock will be termed variants. The nonspecific nomenclature emphasises that the basis of the alteration is unknown.

Primary cell cultures of differentiated cells are short-lived, but there is outgrowth of cells which are usually fibroblastic in type. The outgrowing cells divide and may become established in culture and passaged for months. They are often referred to as diploid cell lines because their karyotype usually resembles that of the animal from which they were derived. In most cases diploid cell lines die out after fifty or more generations (Hayflick and Moorhead, 1961). Rarely, cells of a diploid line become altered in such a way as to permit indefinite growth in vitro. The modified cells are termed an established cell line. The change may be rapid, which is called transformation, or take place gradually over many generations. In some cases transformation appears to take place spontaneously, in others it may be caused by a virus or a chemical carcinogen.

BHK 21/C13 cells transformed by polyoma virus undergo several alterations. Transformed cells grow on a solid substrate in a random manner to form a monolayer many cells thick. Non-transformed cells cease dividing at much lower densities and are arranged in parallel arrays. Transformed cells grow in suspension in an agar medium containing 10% calf serum but non-transformed cells do not. If 10% swine serum is used instead of calf serum, both transformed and non-transformed cells are capable of growing in suspension in agar (Otsuka, 1972). Transformed cells have an increased plating efficiency and rate of glycolysis compared to non-transformed cells. Transformed cells can be subjected to selective procedures and biochemical variants obtained. After transformation, BHK 21/C13 cells have a greatly increased capability to produce tumours after injection into hamsters. Transformed cells also

carry an increased surface charge and a new surface antigen, the T antigen. After transformation, the karyotype alters from diploid to aneuploid. It is not known how the alterations in the behaviour of transformed cells are brought about. Changes in the cell membrane and in the response of the cells to factors in serum may play a part. Some established cell lines do not exhibit all of the altered characteristics of BHK 21/C13 cells after transformation by polyoma virus.

Biochemically variant mammalian cell lines may be obtained either by initiating a cell line from a genetically defective individual or by selecting a phenotypically variant cell line from wild type cells already established in culture. The range of genetic defects capable of being studied in vitro is restricted to those of characteristics that continue to be expressed by wild type cells in culture. Krooth (1970) has listed some spontaneous genetic abnormalities affecting specific molecules present in cultured human cells. Cell lines initiated from individuals suffering from these diseases are genetically defective. Cells derived from patients with the Lesch-Nyhan syndrome lack IPP activity for example. Such cell lines will initially be diploid. Techniques have been developed which select biochemical variants from many transformed cell lines. It has proved very much more difficult to obtain biochemical variants of diploid cell lines by subjecting them to selective procedures. This difference between diploid and transformed cell lines may be linked in some way with the altered in vitro growth characteristics of transformed cells, but is not fully understood.

An intermediate situation exists between growth in vivo and in vitro. Lines of tumour cells may be passaged within animal populations, one example is the Ehrlich ascites tumour of mice. These systems have commonly been used to test drugs thought potentially useful for cancer chemotherapy. Drug treatment of malignant disease is not usually satisfactory, and though a temporary remission may be achieved, the tumour usually recurs. Many factors are involved in tumour recurrence, but one of importance is the

development of resistance to the drug being used. Many of the drugs are analogues of vitamins and nucleic acid components. The rationale of their use has been described in the previous chapter. The development of resistant cells within a tumour was first described by Burchenal, Robinson, Johnston and Kushida (1950) during the use of folic acid antagonists. Initially the tumour, a transplantable acute lymphocytic leukaemia of mice, was sensitive to the drug. After serial transfers in animals treated with amethopterin, sublines of the tumour developed where survival of the animals after inoculation was not prolonged by treatment. Law (1951) produced resistant variants of a mouse lymphoid leukaemia after treatment with the purine analogue 8-azaguanine. Many other examples have been described. It is not clear from these studies whether the resistance pre-exists within the population of cells or arises during selection.

Klein (1959) investigated the acquisition of amethopterin resistance using cells derived from a tumour grown from three L-1210 cells, a mouse lymphocytic leukaemia. Tumour cells were inoculated into a series of mice treated with amethopterin. A rapid and uniform resistance developed. Reconstruction experiments were carried out by mixing wild type and sensitive cells prior to inoculation. The survival time of the mice after inoculation was reduced at the first passage even when one resistant cell was inoculated with a million sensitive cells. The incidence of resistant variants pre-existing within the wild type population must therefore be less than one in a million. This low incidence is difficult to equate with the rapid and uniform resistance developed by cells passaged in the presence of the drug. It could be explained by a high reversion rate or the rapid production of resistant cells poorly adapted to the conditions of growth under non-selective conditions. There was no evidence for the existence of a high reversion rate and resistant cells persisted for fourteen generations under non-selective conditions when mixed with wild type cells. Alternative suggestions

have been offered by Herzenbert (1962). Several changes may have to take place simultaneously within a single cell for it to become resistant, or the changes leading to resistance may be epigenetic.

Reversion of resistant cells to a sensitive state was first reported by Potter and Law (1957). They noted the progressive loss of resistance of a plasma cell tumour to azaserine on removal of selective conditions. In cases where reversion takes place it is likely that the underlying change producing the phenotypic alteration is epigenetic in nature. If a mutation is the cause it is almost certainly a single base change. Some reversible epigenetic changes may be part of the normal physiology of the cell. In these cases a high proportion of the variants will be able to revert after removal of the selective conditions. Back-mutations are chance events and appear at low frequencies in occasional individuals. Some epigenetic changes may therefore be distinguishable from mutations by having high reversion rates.

Drug resistant cells often show cross-resistance to structurally related compounds. Law (1956) showed that a mouse leukaemia cell line resistant to amethopterin was also resistant to analogues of pteroylglutamic acid but remained sensitive to other inhibitors, for example purine analogues. Sometimes cross-resistance to structurally unrelated compounds is present. Subak-Sharpe (1965) reported that BHK 21/C13/PyY variants selected for resistance to actinomycin D gained some resistance to puromycin. They had not previously been exposed to puromycin. The biochemical basis of this unexpected cross-resistance is uncertain, but there may be an alteration in the permeability of the variant cells reducing the entry of both puromycin and actinomycin D.

As has been described, much of the early work on resistant mammalian cells was carried out using tumour cells growing in experimental animals. This is less satisfactory than systems using cells in tissue culture which

the recent advances in equipment and techniques have made available to a large number of workers. The growth medium for mammalian cells in tissue culture is chemically defined, apart from the need to add serum proteins. A wide variety of selective procedures can be employed to isolate cells with varying phenotypes in tissue culture. Mammalian cells can be cloned in vitro (Sanford, 1948, and Puck, 1955) enabling a population originating from a single cell to be grown up and used for genetic and biochemical studies. However, cells in tissue culture are existing in an artificial environment and their behaviour must be regarded as only a model of that in the intact organism. Every step in the handling of cells is a selective procedure favouring the growth of some but not others. Many factors exert continuous, cyclic or short term selection and will have long term effects on the behaviour of the cells. If cells are passaged as monolayers in bottles, at each passage the cells in suspension being discarded, only those cells that stick to glass remain in the population. It is often possible to convert a cell line from growing as a monolayer to suspension culture by passaging only those cells free in the medium. Many selective factors are not rigorously controlled during the growth of cells in tissue culture. The variation between batches of the serum added to the growth medium is one example. Selective conditions created intentionally are superimposed on this continual background of selection.

Several different approaches have been used to derive variant cells in culture:

1. A metabolite not normally utilized by the cells may be substituted for a normal metabolite.
2. The testing of large numbers of clones isolated at random from wild type cells without selection for the variant phenotype desired.
3. The isolation of drug-resistant cells.

4. The isolation of auxotrophs.

5. The isolation of general conditional lethal variants.

The first approach was used by Chang (1957) who substituted D-xylose for D-glucose and obtained HeLa cells able to use D-xylose as a carbohydrate source.

Very few variant mammalian cells have been obtained without using a selective procedure. A great deal of work is needed because it is probable that many clones of cells have to be tested individually before one having the required phenotype is obtained. It is a most important approach to use because epigenetic modifications caused by the selective conditions are eliminated as a cause of the variant phenotype. Thompson, Mankowitz, Baker, Wright, Till, Siminovitch and Whitmore (1971) have obtained temperature-sensitive variants of mouse L-cells, using this method.

Drug resistance has been much used as a selective system for obtaining mammalian cell variants. The biochemical adaptations that may take place during acquisition of drug resistance have been indicated in the previous chapter. The first reports of drug resistant cells being obtained in vitro were by Szybalski and Smith (1959a) using 8-azaguanine and Lieberman and Ove (1959) using puromycin. These variants were selected by exposing wild type cells to toxic levels of the drug and growing up the survivors. Many different drugs have been used to isolate variants in this way: some of them are listed in Table 1.

The details of the conditions used for selection are highly important in determining the properties of the variant cell produced. Some variants have been isolated using a single-step procedure by one exposure of a large number of cells to the inhibitor at a high concentration. In other cases cells were exposed to increasing levels of the inhibitor in a series of selective procedures gradually raising the level of resistance. A single stage selective procedure is most likely to isolate variants whose resistance is caused by a single phenotypic change. A gradual selection procedure will

TABLE I. SOME EXAMPLES OF DRUG RESISTANT MAMMALIAN CELLS DERIVED IN VITRO.

Folic acid antagonists		Parental cell	Selection for resistance to:
Vogt	1959	HeLa	Aminopterin
Arnow	1959	mouse fibroblasts	Amethopterin
Harris & Ruddle	1960	PK-15	Aminopterin
Hakala et al.	1961	mouse fibroblasts	Amethopterin
Antipurines			
Szybalski & Smith	1959a	D 98	8-azaguanine
Harris & Ruddle	1960	PK-15	2:6 diaminopurine, 8-azaguanine
Tomizawa & Arnow	1960	mouse fibroblasts	6-mercaptopurine
Brockman et al.	1961	human epidermoid carcinoma	6-mercaptopurine
Szybalski et al.	1962	D 98	6-mercaptopurine 8-azahypoxanthine
Davidson et al.	1962	P-388	8-azaguanine
Littlefield	1963	L	8-azaguanine
Subak-Sharpe	1964	BHK 21/C13/PyY	6-thioguanine 8-azaadenine 6-thioguanosine
Albertini & DeMars	1970	human fibroblasts	8-azaguanine
Arlett & Potter	1971	Chinese hamster cells	8-azaguanine
Antipyrimidines			
Pasternak et al.	1961	L-5178Y	6-azauridine
Roosa et al.	1962	P-388	5-fluorouridine
Kit et al.	1963	L	5-bromodeoxyuridine
Kit et al.	1966	HeLa	5-bromodeoxyuridine
Subak-Sharpe et al.	1972	BHK 21/C13/PyY	5-bromodeoxyuridine cytosine arabinoside
Amino acid analogues			
Harris & Ruddle	1960	PK-15	allylglycine

TABLE I. (continued)

Actinomycin D			
Subak-Sharpe	1964	BHK 21/C13/PyY	
Antibiotics			
Liebman & Ove	1959	L?	puromycin
Metzgar	1963a	Mox(L?)	streptomycin
Subak-Sharpe	1964	BHK 21/C13/PyY	puromycin
Harris	1967	pig kidney cells	puromycin

encourage the production of cells depending on multiple changes for resistance. Each new change increases the level of resistance further.

Some mammalian cells in tissue culture are capable of interacting. One type of interaction is metabolic co-operation and it is particularly relevant to the selection of drug-resistant cells in vitro. Metabolic co-operation was originally demonstrated using BHK 21/C13/PyY cell variants (Subak-Sharpe, Bürk, and Pitts, 1966, Bürk, Fitts and Subak-Sharpe, 1963, Subak-Sharpe, Bürk and Pitts, 1969 and Subak-Sharpe, 1969). Two different variant cell lines were used. One was resistant to both 8-azaadenine and 8-azaadenosine and did not incorporate adenine from the growth medium into nucleic acid when grown alone. The other, resistant to both 6-thioguanine and 6-thioguanosine, was unable to incorporate hypoxanthine from the growth medium into nucleic acid when grown alone. While in contact with a cell capable of incorporating a radioactively labelled purine base supplied in the medium, the phenotypes of the variants were changed. They both became wild type with respect to purine incorporation. This was termed metabolic co-operation. Variant cells in the same preparation but not in contact with cells capable of incorporating the base retained their variant phenotype.

Cell contact during the procedures used to select variants of BHK 21/C13/PyY cells was avoided by exposing a thinly seeded monodisperse layer of wild type cells to the selective conditions. Since there was no cell contact, metabolic co-operation did not influence the selection process. During selection in dense culture, a potential variant cell would arise while in contact with a wild type cell. The phenotype of the potential variant would continue to be wild type by virtue of metabolic co-operation. The cell would therefore remain susceptible to the effects of the analogue and be killed. To arise under dense culture conditions, a BHK 21/C13/PyY cell variant resistant to a purine analogue would have to both prevent lethal synthesis and be unable

to take part in metabolic co-operation. Alternative ways of avoiding cell contact and overcoming the effects of metabolic co-operation during selection exist. The wild type cells may be exposed to the selective conditions either during growth in liquid suspension culture or suspended in agar. Not all cells are capable of participating in metabolic co-operation. The A9 variant of mouse L cells resistant to 8-azaguanine (Littlefield, 1963) is unable to co-operate either with wild type L cells or with wild type BHK cells (Pitts, 1971).

Several systems have been developed for the isolation of auxotrophic mammalian cells. The technique of isolation is to place wild type cells in a medium lacking the metabolite for which auxotrophy is desired. Auxotrophic cells are unable to grow under these conditions, while wild type cells continue to divide. An agent to kill dividing cells is then added to the culture, either tritiated thymidine or 5-bromodeoxyuridine with exposure to visible light have been used (Puck and Kao, 1967). Only auxotrophs survive this treatment and can be isolated from the culture by first removing the lethal agent and then adding back the metabolite for which auxotrophy has been selected. DeMars and Hooper (1960) isolated HeLa cells auxotrophic for glutamine. Puck and his collaborators have isolated Chinese hamster cells with a wide spectrum of auxotrophic requirements, glycine, hypoxanthine, inositol and thymidine (Kao and Puck, 1968, 1969). Chu, Brimer, Jacobson and Merrium (1969) have isolated a hamster V 79 cell auxotrophic for glutamine. These types of variants have been used for the study of metabolic pathways and their control.

There remains a class of variants derived in vitro which are generally referred to as general conditional lethals. General conditional lethals of mammalian cells isolated to date have all been temperature-sensitive. The configuration of a polypeptide chain may be affected by changes in temperature. When the function of a protein is altered by such a change,

it is temperature-sensitive. Mutations causing its function to become temperature-sensitive may affect any protein. The polypeptide chain affected by the temperature-sensitive mutation functions normally at one temperature (termed the permissive temperature) but abnormally at a second temperature (termed the non-permissive temperature). If the behaviour of a polypeptide chain is very severely altered at the non-permissive temperature and the normal function is essential for viability, the temperature-sensitive mutation will be a conditional lethal. Growth of the cells will take place at the permissive temperature but they will be unable to grow at the non-permissive temperature. Most mammalian cells are normally capable of growth over a temperature range of 6 or 7°C. enabling the permissive and non-permissive temperatures to differ by up to this amount.

Temperature-sensitive conditional lethal mutations of mammalian cells provide flexibility not available using other phenotypic variants. Stocks of cells can be produced under permissive conditions but studied under non-permissive conditions. Proteins carrying out essential functions can therefore be investigated. Initial selection of conditional lethal cells does not require prior knowledge of the nature of the function selected for as is the case with selection for auxotrophs. Cells growing under the permissive conditions supply a good control for experiments carried out on the same cells under non-permissive conditions. There have been reports from two groups of temperature-sensitive mammalian cells being obtained in tissue culture. Naha has obtained temperature-sensitive monkey kidney cells (1969, 1970). The defect was in some way able to prevent DNA incorporation by the nucleolus at the non-permissive temperature. Thompson, Mankovitz, Baker, Till, Siminovitch and Whitmore (1970, 1971) obtained temperature-sensitive L cells by selective killing of the wild type cells at the non-permissive temperature by growth in tritiated thymidine and cytosine arabinoside. The non-permissive temperature used was 38.5°C. and the permissive temperature 34.0°C.

All these variant cells are derived from aneuploid established cell lines, but there are advantages in using diploid cells. The use of primary cell lines derived from individuals having genetic defects, despite the difficulties in their cultivation, will provide much information which will complement that obtained using aneuploid cells. There have however been two recent reports of the derivation of variants from diploid cells in culture, human fibroblasts (Albertini and De Mars, 1970) and near diploid Chinese hamster cells (Chu, 1969). If more diploid variants could be obtained by selection in tissue culture the gap that exists between diploid cell and aneuploid cells would be filled.

The diploid nature of mammalian cells makes their genetic analysis difficult. To be phenotypically expressed, a mutation must be either a dominant, a homozygous autosomal recessive, a recessive present on the X chromosome of a male cell, or a recessive present on the non-inactivated X chromosome of a female cell. Phenotypes obtained by selective procedures in vitro are probably usually due to the presence of recessive alleles or large deletions. In the majority of cases the locus will be carried on an autosome. At least two independent genetic events would therefore be necessary before the recessive phenotype could be expressed. It may eventually be possible to isolate cell lines monosomic for different chromosomes by promoting non-disjunction, and perhaps also to obtain monosomic human lines from abortions with such anomalies. Haploid human cell lines may be obtainable by the manipulation of the genetic material within ova or sperm. Freed and Mezger-Freed (1970) and Mezger-Freed (1972) have reported the isolation of stable haploid cell lines from frog embryos. The study of mutation rates and reversion rates in these cells do not show the expected differences between them and diploid cells, though karyotype analysis confirms their haploid chromosome complement. Investigation of this discrepancy may throw light on the nature of the changes that underly

the development of resistance.

The analysis of variant cells is made difficult by the fact that they interact in the culture. This perhaps best demonstrated by the phenomenon of metabolic co-operation which has already been mentioned. Metzgar and Moskowitz (1963 (a)(b)) using mouse cells resistant to streptomycin demonstrated that after mixing wild type and resistant cells in a single culture, all were sensitive to the drug. Harris (1967) used clonal lines of pig kidney cells to analyze the variation in the expression of puromycin resistance with cell density at the population level. Any genetic variation present was obscured by nongenetic factors that increased with the cell density. Large colonies or clumps of sensitive cells grew in concentrations of puromycin that destroyed all the cells when exposed to the drug in a dispersed form. This data casts doubt on the estimations of "mutation rates" of mammalian cells in culture (Lieberman and Ove 1959, Szybalski, 1959).

Conflicting reports exist in the literature on the use of mutagens while selecting cell variants. Szybalski and Smith (1959b) reported that pre-treatment of D 98 cells with ultraviolet light depressed the rate of appearance of variants resistant to 8-azaguanine. Chu and Malling (1968) quantitated the frequencies with which variants of Chinese hamster cells were obtained after treatment with the mutagens ethyl methane sulphonate, methyl methane sulphonate and N-methyl-N'-nitroso-guanidine. Clones were isolated under conditions selecting either for resistance to 8-azaguanine or auxotrophy for L-glutamine. Mutagen pre-treatment increased the frequency with which both variants were obtained, however the yield varied with the conditions of the experiment. There was an inverse relationship between the number of cells resistant to 8-azaguanine and the cell density during selection. The mutation expression time, the interval between the removal of the mutagen and the application of selective conditions, also affected the yield. If the selective agent 8-azaguanine was added immediately after treatment with the mutagen there was no increase in the frequency with which

variants were produced. After delaying the addition of the selective agent, allowing the cells to grow and divide, the frequency with which variants appeared increased with time up to 42 hours. The cause of this effect is probably a phenotypic lag during the time taken for the variant phenotype to be expressed. At 54 hours there was a decline, perhaps due to the cells becoming crowded. It was also found that, as the concentration of 8-azaguanine used to select the variants was increased, the yield of variants declined. Differences between the cell lines used and the selective conditions may explain these opposing findings. The presence or absence of metabolic co-operation during selection is a highly important factor influencing the yield of variants obtained. It is highly probable that metabolic co-operation was the cause of the inverse relationship between the cell density and yield of 8-azaguanine resistant Chinese hamster cells.

The effects of γ-irradiation on mammalian cells have been investigated in detail. Much of the work has centered on cell survival kinetics because of the relevance to cancer therapy. Two observations throw light on the problems of mutagenising mammalian cells in culture to produce variants. The first described by Elkind and Smith (1960) is the split dose effect. The same total quantity of γ-irradiation produces a much greater effect when given as a single dose than when given as two separate doses an hour or two apart. It has been suggested that there is a repair mechanism in the mammalian cell that enables it to repair sublethal damage. Confirmation of this hypothesis was obtained from the observation that the "mutation curve" for mammalian cells is biphasic. Higher doses of irradiation are more efficient at producing variants than lower doses (Arlett and Potter 1971). The second observation was that the sensitivity of Chinese hamster cells to γ-irradiation is dependent on the position they are at in the cell cycle (Sinclair and Morton, 1965). Cells in G2 are most sensitive, those in G1 intermediate and those in S least sensitive. Perhaps the repair mechanism differs in

its efficiency with the stage the cells are at in their cycle. These observations are without parallel in bacterial systems and emphasise the care that must be taken when extrapolating from these to mammalian cells.

3. THE TRANSFER OF GENETIC INFORMATION BETWEEN MAMMALIAN CELLS IN VITRO

The ability to transfer genetic information between individuals in a controllable fashion has been important in allowing rapid advances to be made in the genetic analysis of bacteria and viruses. The transfer of genetic information between genotypically different individuals results in recombination. Three general methods of transfer are available in bacteria; transformation, transduction and conjugation. Mammalian cells in tissue culture may now in some cases be investigated in an analogous manner.

Transformation describes the permanent modification of an individual by the incorporation and genetic expression of exogenously supplied purified nucleic acid. The term is used in a totally different sense both to describe the change of a diploid cell line into an established cell line and also the stimulation of lymphocytes to enter mitosis. There have been several claims to have transformed drug-resistant cells in vitro with DNA from wild type cells (Szybalska and Szybalski, 1962; Bradley, Roosa and Law, 1962 and Fox, Fox and Ayad, 1969 are examples). Many workers have had difficulty in repeating these experiments. At present the evidence that the DNA has been integrated and its genetic information expressed is unconvincing. It is certain that the nucleic acid can enter the cell in some cases, but much appears to become rapidly degraded (Ayad and Fox, 1968). A recent report of transformation using a Rous sarcoma genome as a marker is the most definite (Hill and Hillova, 1972). DNA from a line of rat cells transformed by Rous sarcoma virus (RSV) was extracted and purified. These cells were unable to produce any infectious virus. The purified DNA was used to treat chicken fibroblasts. (If infected with intact RSV particles some chicken cells would become transformed and allow the production of infectious RSV progeny.) The DNA-treated chicken fibroblasts were assayed for the production of RSV. Some were found to produce RSV particles of the strain originally used to infect and transform the rat cells. Pretreatment with DNAse abolished the

ability of the purified DNA to cause the production of RSV. This result requires the entry of a DNA copy of the RSV genome into the chicken fibroblasts and the full expression of its genetic information leading to the free replication of the viral genome and the production of infectious virus particles.

Transduction describes the transfer between cells of cellular genetic information contained within bacteriophages or viruses. A single report exists of the successful transduction of a mammalian cell by a bacteriophage (Merril, Geier and Petricciani, 1971). Human fibroblasts from a patient with galactosaemia were infected with one of two types of bacteriophage λ. Galactosaemia is the result of an autosomally transmitted recessive inborn error of metabolism. In homozygous individuals there is absence of α-D-galactose-1-phosphate uridyl (GPU) transferase activity. One of the transducing types of contained an intact galactose operon capable of specifying GPU transferase activity. The second type of λ contained a galactose operon with a mutation rendering the transferase inactive. Only after infecting the genetically defective cells with the type of bacteriophage capable of specifying GPU transferase was this enzyme activity detectable. Infected cell cultures were assayed through repeated subcultures and enzyme activity was found to remain relatively constant at the level achieved four days after infection. The λ genome was therefore not rapidly lost by segregation. It was suggested that perhaps the bacteriophage DNA may be preserved within the human cell by one of the following mechanisms: integration into the host genome, plasmid-like existence in the cytoplasm, interaction with the mitochondria, or perhaps in some totally unknown manner.

Cells infected with polyoma virus produce particles containing cellular DNA, termed pseudovirions, at low frequencies. Preparations of polyoma virus may be enriched for pseudovirions using density-gradient centrifugation. Infection with pseudovirions is a potential method of transducing mammalian

cells. No report of successful transduction using this technique has been published, though the DNA has been shown to enter some cells (Quasba and Aposhian, 1971). The frequency of successful transduction expected may be estimated. The genomes of polyoma virus and mammalian cells differ in size by about a factor of 10^6. The molecular weight of the DNA contained within mammalian cells being about 10^{12} and that of the DNA contained within a polyoma virion about 10^6. If transformation is examined with respect to one characteristic, and assuming random packaging of the cellular DNA within pseudovirions, the chance that any single pseudovirion will contain the genetic information for the characteristic will be $1:10^6$. A single cell may be infected with 10^5 virus particles of which 10% may be pseudovirions. Much of the viral DNA is known to be degraded, there may therefore be only a 1% chance of the DNA of any one pseudovirion being integrated. On the basis of these assumptions there will be a $1:10^4$ probability that an infected cell will be successfully transduced for the character being examined. Even if successful transduction using polyoma pseudovirions is achieved it will be only a rare event that allows transduction of the information for any particular characteristic.

The fusion of somatic mammalian cells results in two or more genomes being contained within a single cell membrane. The situation is analagous to bacterial conjugation. Cell fusion makes a parasexual cycle available for the study of cells in tissue culture. The applications of this technique are far-reaching and a great deal of present-day work using mammalian cells in tissue culture involves its use. Pontecorvo (1959) worked with filiamentous fungi which lack a sexual cycle in some cases but which have a parasexual cycle. He showed that the parasexual cycle could be used for genetic analysis. The complete cycle is as follows:

1. Fusion of two unlike haploid nuclei in a heterokaryon giving a diploid heterozygous nucleus. Occasionally a hypha containing this nucleus

may separate from the heterokaryotic hypha that formed it and produce heterozygous diploid hyphae.

2. Occasional mitotic crossing over may take place during the multiplication of the diploid nucleii.

3. Haploidization of the diploid nucleii may rarely take place. Crossing over describes this process of exchange of genetic material between homologous chromosomes. Crossing over may result in reassortment after haploidization occurs and allow linkage studies to be carried out. Linkage of recessive characters can be studied after haploidization. The heterokaryon formed after the fusion of mammalian cells, which are initially diploid, is tetraploid. The term heterokaryon is used to describe a cell with more than one separate nucleus and synkaryon for a multinucleate cell in which the nucleii have fused to form a single nucleus. A homosynkaryon contains nucleii originally derived from the same kind of cells and a heterosynkaryon nucleii derived from different kinds of cells (Harris and Watkins, 1965).

Multinucleate mammalian cells are produced in a variety of situations. Some exist within the body under normal conditions, striated muscle, osteoclasts and the syncytiotrophoblast are examples. Pathological conditions can often give rise to multinucleate cells. Foreign body giant cells, some tumours, tuberculous tissue and the inflammatory lesions caused by some viruses are all examples. These have been reviewed by Harris (1970) and Poste (1970).

Multinucleate cells may also be formed in tissue culture, the first report was that of Lambert (1912). Fell and Hughes (1948) observed the synchronous mitosis of binucleate mouse cells in culture. All the chromosomes from both nucleii collected along a single equatorial plate and, after cell division, produced two mononucleate daughter cells with large nucleii. Enders and Peebles (1954) showed that measles virus caused the formation of multinucleate cells in tissue culture. Many other viruses have

similar effects, these have been reviewed by Poste (1970). The virus that has been most used for cell fusion studies is the Haemolytic Virus of Japan (HVJ), otherwise known as Sendai virus. It is a parainfluenza type I virus and its ability to fuse cells was first studied by Okada (1958, 1962).

Prior to the widespread use of cell fusion induced by Sendai virus, several examples of spontaneous fusion in tissue culture were reported. Barski, Sorieul and Cornfert (1960, 1961) found that after co-cultivating two different lines of mouse cells for prolonged periods, a new cell type arose which overgrew the original cells. The new cell type had a different morphology and contained the characteristic chromosomal complements of both parental cells. It was postulated that the new cells were hybrids formed by the fusion of two different parental cells, not caused by autopolyploidization. Perhaps cell fusion continually occurs within cell cultures, but only in the unusual event of the hybrid having a selective advantage and outgrowing the parents is this apparent. However, the above cells were later found to be carrying SV5 virus which can induce cell fusion (Barski, 1968). Ephrussi (1962) also observed the production of hybrid cells after the co-cultivation of two mouse cell lines. Littlefield (1964) used HAT selective medium and two different biochemically defective parental cells in an attempt to induce controlled fusion. Phenotypically wild type hybrid cells were selected due to complementation after spontaneous fusion between two of the different parental cells had taken place. A generally applicable technique for producing hybrid mammalian cells was still not available.

Harris and Watkins (1965) provided such a technique by using ultraviolet inactivated Sendai virus to fuse Ehrlich ascites cells and HeLa cells, producing heterokaryons containing both mouse and human nucleii. The heterokaryons were shown to be able to synthesise protein, RNA and DNA and continued to grow for long periods, in addition nuclear fusion was observed. Harris then extended the study to differentiated cells (1965). Okada (1970) and Harris (1970) have reviewed what is known of the mechanism of cell fusion. The fusing ability of the virus lies in the viral envelope and not

in the nucleic acid. The RNA may be inactivated by ultraviolet light (Okada and Tadokoro, 1962 and Harris and Watkins, 1965) or β-propriolactone (Neff and Enders, 1968) at doses which have little effect on the fusing ability. In the closely related Newcastle disease virus the important component of the viral particle for cell fusion lies in the viral envelope (Kohn, 1965). The fusion ability is resistant to trypsin but removed by lipid solvents and sensitive to the action of phospholipase. Sendai virus propagated in mouse lung cells (Matsumoto and Maeno, 1962) or pig cells (Hosaka, 1962) lacked its fusion ability. This was regained after the virus was propagated again in eggs. The host-specified lipid component of the virus is therefore important in the process of cell fusion. Okada has produced a model of the process intended to be descriptive rather than mechanistically correct.

1. Virus is absorbed to the surface of the cells causing them to agglutinate. This may take place either at 4°C. or 37°C.

2. The presence of the virus alters the cell membrane in some way. This alteration may be a local or a general effect. Okada imagined the virus punching holes in the membrane.

3. The cell then repairs the disrupted site requiring energy. If two such sites on different cells are in contact during the repair process the membranes of the two cells may fuse locally.

4. Finally the extent of fusion increases to form a heterokaryon. None of the last three steps can take place at 4°C. They occur at 37°C. The details of the process of cell fusion are still not clear. It may eventually become possible to induce controllable cell fusion chemically. Fusion of cells using lysolethecin has been reported, but the hybrids were not viable and the process was difficult to control (Poole, Howell and Lucy, 1970 and Lucy, 1970).

Harris (1970) has reviewed the results obtained utilizing the process of cell fusion. A comprehensive review will not be attempted here. Only

work on the expression of genetic information by higher organisms and on mammalian cell genetics will be discussed.

Avian erythrocytes, unlike those of mammals, remain nucleated when mature. Mature avian erythrocyte nucleii are normally dormant with respect to RNA, DNA and protein synthesis (Cameron and Prescott, 1963, and Fraser, 1964). The reactivation of avian erythrocyte nucleii after their fusion with a metabolically active cell was first observed by Harris (1965). Erythrocytes from adult hens and older embryos undergo haemolysis after treatment with Sendai virus at the concentrations required to produce cell fusion. Fusion therefore takes place between an erythrocyte ghost and the other cell, (Schneeberger and Harris, 1966). Little if any erythrocyte cytoplasm is present within the heterokaryon during the reactivation process. Frog erythrocyte nucleii have also been demonstrated to undergo reactivation after fusion with HeLa cells (Harris 1966). The reactivation of inactive erythrocyte nucleii allows the study of control mechanisms involved in the replication and transcription of DNA in higher organisms.

The avian erythrocyte has undergone reactivation in all dividing cells studied to date. The volume of the erythrocyte nucleus increased at least 20- to 30-fold during reactivation (Harris, 1967 and Bolund, Ringertz and Harris, 1969). The condensed chromatin of the erythrocyte nucleii became dispersed as the volume increased (Harris, 1967). The ability of the reactivating chromatin to bind the intercalating dyes ethidium-bromide and acridine orange increased during reactivation. The increase in binding was initiated prior to commencement of DNA replication. The melting profile of the chromatin also changed during reactivation, there being an increase in the susceptibility of the DNA to thermal denaturation. There was a 5-8 fold increase in the dry mass of the reactivated erythrocyte nucleii. The increase in dry mass occurred largely prior to DNA replication and was considered to be due to the entry of proteins from the cytoplasm (Bolund et al., 1969). Reactivating erythrocyte nucleii replicated their DNA. By 24 hours after fusion 15% of the erythrocyte nucleii were incorporating

thymidine in heterokaryons formed between HeLa cells and adult hen erythrocytes (Harris, 1965 and Harris, Watkins, Ford and Schoefl, 1966). The Feulgen-DNA value of many reactivated erythrocyte nuclei increased to the tetraploid level by 48 hours after fusion, indicating that they had completely replicated their DNA (Bolund et al., 1969). DNA synthesis in the two sets of nuclei within the heterokaryons rapidly became synchronous (Johnson and Harris, 1969).

The initial cytochemical and morphological changes and increase in dry mass also occurred in nuclei from erythrocytes that had been U-V irradiated prior to fusion. U-V irradiated nuclei had a greatly reduced ability to synthesise RNA and replicate their DNA. It was considered that very little, if any, chick-specified protein could be produced in such heterokaryons (Harris, 1967 and Bolund, Darzynkiewtcz and Ringertz, 1969). The increase in dry mass during reactivation of the erythrocyte nuclei was therefore due to the entry of proteins from the foreign cytoplasm that surrounded them. Chick erythrocyte nuclei have been shown to incorporate human proteins during their reactivation within HeLa cells (Ringertz, Carlsson, Ege and Bolund, 1971).

An increase in acridine-orange and ethidium bromide binding, an altered melting profile and a change in staining with the Feulgen technique were also demonstrable in the nuclei of intact erythrocytes and isolated erythrocyte nuclei after washing in serum-free salt solutions (Ringertz and Bolund, 1969).

The enlargement of erythrocyte nuclei was temperature dependent, it ceased after the heterokaryons were transferred to a temperature of 26°C (Harris, 1967). The reactivation process took place more rapidly when embryonic erythrocytes were used (Harris et al. 1969, Harris, 1970).

The complete reactivation process did not take place after fusion of erythrocytes with rabbit macrophages. Macrophages are normally capable of synthesising RNA but not DNA. Reactivating erythrocytes within macrophages enlarged and synthesised RNA but did not synthesise DNA (Harris et al. 1966).

The erythrocyte nucleus therefore responded to the environment within a foreign cell in a co-ordinated and apparently normal way. The behaviour

of the erythrocyte nucleii was controlled by that of the metabolically active cell. DNA replication in the erythrocyte nucleus was observed only after fusion with a cell normally capable of division.

Two general conclusions can be drawn from this work. Firstly the behaviour of a differentiated nucleus may be altered. Secondly, the mechanisms by which nuclear events are influenced are not highly species-specific and may in some cases be nonspecific. Changes in the ionic environment of the chromatin play an important part in determining its configuration and also the expression of the genetic information carried by it. The entry of foreign proteins into the reactivating nucleus appears also to be important in the process.

The ability of the reactivated erythrocyte nucleii to express their genetic information has been investigated. Heterokaryons formed between X-irradiated IPP deficient mouse A9 cells (Littlefield, 1963) and a reactivated chicken erythrocyte nucleus are able to synthesise chicken surface antigen (Harris, Sidebottom, Grace and Bramwell, 1969) chicken IPP (Harris and Cook, 1969 and Cook, 1970) and become sensitive to the effects of diphtheria toxin (Dendy, 1972). The appearance of chicken-specified functions has been correlated with the presence of a functional nucleolus in the reactivated erythrocyte nucleus (Harris et al. 1969, Sidebottom and Harris, 1969, Harris and Cook, 1969 and Deák, Sidebottom and Harris, 1972).

Hererokaryons formed from A9 cells and chicken erythrocyte nucleii have also been used for long term studies. When mouse nucleii within such a heterokaryon undergo mitosis the erythrocyte nucleus was commonly fragmented by a process which has been termed "chromosome pulverization" or "premature chromosome condensation" (Johnson and Rao, 1970). The nucleii can undergo fusion but much of the chicken chromatin may be lost. Some synkaryons continue to grow after this process. Schwartz, Cook and Harris (1971)

obtained such a line of cells after selection in HAT medium that could synthesise chicken IPP but not chicken surface antigen. No chicken chromosomes could be found in these cells. Presumably a small piece of genetic material from the erythrocyte nucleus was present either as an episome or integrated into one of the mouse chromosomes. The chicken genetic material carried the gene for IPP but not that for surface antigen. This technique potentially allows high resolution linkage studies to be carried out on mammalian cells.

Segregation of characters is an essential prerequisite for the study of any system using genetics. In filiamentous fungi segregation could be effected through the occurrence of natural haploidization. In mammalian cell hybrids the phenomenon of chromosome loss produces segregation of characters. In some intra-species hybrids one parental set of chromosomes is lost preferentially. A preferential loss of rat marker chromosomes was observed from rat-mouse hybrid cells (Weiss and Ephrussi, 1966). Human chromosomes were shown to be lost preferentially from man-mouse hybrids (Weiss and Green, 1967). A system was then available that permitted the genetic analysis of human somatic cells. The gene controlling thymidine kinase production was assigned to an E group chromosome (Migeon and Miller, 1968, and Matsuya, Green and Basilico, 1968). Genes controlling the production of lactate dehydrogenase B and Peptidase A were shown to be linked (Santichiara, Nabholz, Miggiano, Darlington and Bodmer, 1970 and Ruddle, Chapman, Chen and Klebe, 1970). Chinese hamster-human cell hybrids also lose human chromosomes and have been used for genetic analysis (Kao and Puck, 1970). Chromosome loss in both these intra-species hybrids, although confined to the human chromosomes, was not controllable. Cells with a greatly reduced number of human chromosomes suitable for linkage studies were spontaneously produced at random during prolonged passage. The chromosome loss may be influenced by growth of the hybrids in selective medium. Growth in HAT selects for the retention of loci enabling IPP to be produced (Engel, McGee

and Harris, 1969 and Miller, Cook, Meera Kahn, Shin and Siniscalco, 1971). The general factors governing the set of chromosomes from which loss occurs in any particular intra-species hybrid are unknown. Kao et al. (1970) suggested that the chromosomes of the more slowly growing parental cell may be preferentially eliminated. This is not universally found however. Hybrid cells formed between Chinese hamster cells resistant to 8-azaguanine and mouse cells resistant to 5-bromodeoxyuridine (BUdR) were grown in HAT. Preferential loss of the Chinese hamster chromosomes was found and these cells had the longer doubling time (Handmaker, 1971). Pontecorvo (1971) suggested that pretreatment of one of the parents of a hybrid cell with X-irradiation, Y-irradiation or BUdR may induce preferential loss from that set of chromosomes.

The mechanisms involved in cell differentiation can be investigated using cell fusion. The pattern of differentiation of the avian erythrocyte is changed after fusion with a metabolically active cell and has already been discussed. It is an example of a function being reactivated that had been switched off. Specialized functions continue to be carried out in tissue culture by some cells retaining a measure of differentiation. Initial experiments suggested that differentiated characters ceased to be expressed after fusion with an undifferentiated cell. Davidson, Ephrussi and Yamamoto (1966) fused pigmented melanoma cells with nonpigmented fibroblasts, the hybrids were nonpigmented. There are more recent reports of the continued expression of differentiated characters after fusion with undifferentiated cells, electrophysiological properties (Minna, Nelson, Peacock, Glazer and Nirenberg, 1971) albumin production (Peterson and Weiss, 1972) and immunoglobulin kappa chains (Mohit and Fan, 1971) for example. The situation is thus not reducible to simple terms. Each combination of cells needs to be examined individually. Perhaps when more data are available general rules governing the expression of differentiated characters in vitro will be established. These will undoubtedly throw

light on the mechanisms by which cells differentiate in vivo. One factor that is known to be of importance in some cases is gene dosage. Davidson and Benda (1970) observed that while the inducibility of glycerol-1-phosphate dehydrogenase by hydrocortisone was lost in 1s glial cells after hybridization with fibroblasts, there was some inducibility in hybrids between 2s glial cells and fibroblasts. Davidson (1972) showed that if near-tetraploid pigmented Syrian hamster cells were fused with unpigmented mouse fibroblasts, half of the hybrids formed were pigmented. All of the other hybrids involving diploid pigmented cells and nonpigmented cells failed to express the pigmented chracteristics.

Much information on the behaviour of mammalian cells has rapidly been obtained by reassorting their genetic material. The controlled fusion of somatic cells in culture in particular has enabled critical experiments to be carried out. Further advances are required if the technique of cell fusion is to be utilized to its full extent. A wider spectrum of biochemically variant mammalian cells should be selected, particularly of diploid cell lines. A generally applicable method of inducing controlled chromosome loss from hybrids is essential for genetic analysis. Lastly, culture conditions are needed which allow the continued expression of more differentiated characters by cell lines in culture.

4. MATERIALS AND METHODS

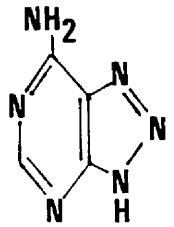
Cells

Wild type and biochemically variant polyoma transformed baby hamster kidney cells were used throughout. Originally derived from Syrian baby hamster kidney cells (Stoker and Macpherson, 1964) the BHK 21/Cl3 line is widely used for many purposes. BHK 21/Cl3 cells can be transformed by polyoma virus to produce what will be termed BHK 21/Cl3/PyY cells (PyY). PyY cells are suitable for use in biochemical selection procedures and have been selected for resistance to a number of inhibitors and metabolic analogues (Subak-Sharpe 1965, 1969, and Subak-Sharpe, Gentry and Jamieson, 1972).

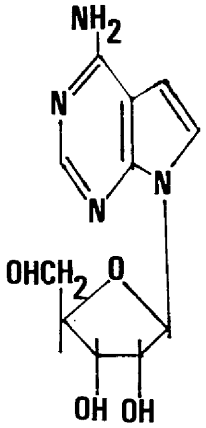
The cells were grown as monolayers on glass. Clones of cells growing out of a culture exposed to selective conditions were grown up and retained the favourable growth properties of the parent. Many lines of cells have been sequentially and independently selected for resistance to purine analogues in various combination, producing multiply marked cells. The biochemically marked variants are identified by letters denoting the inhibitors used in the initial selection procedure. When a cell is resistant to more than one analogue, the abbreviations are added in the order that selection took place. For example, PyY AA/AAR was originally selected using 8-azaadenine, initially producing a variant resistant to 8-azaadenine termed PyY AA. This variant was then subjected to a second selective procedure by exposure to 8-azaadenosine, producing a variant resistant to both 8-azaadenine and 8-azaadenosine termed PyY AA/AAR. Many of the systems became revertant-variant mixtures if cultured for long periods in the absence of the analogues to which they were resistant. At intervals during the passage the cells were exposed to the analogues to remove any revertants present.

FIGURE II

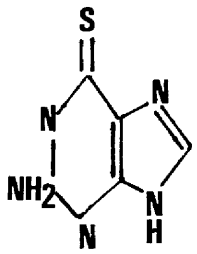
The structures of the purine bases, nucleosides and their analogues that have been used to select variants of BHK 21/C13/PyY cells.



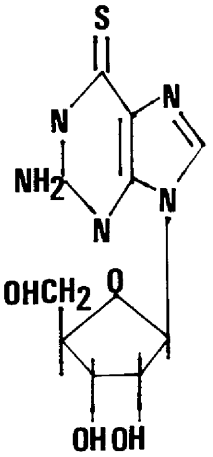
8--Azaadenine



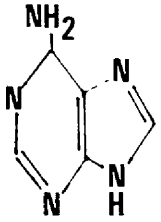
Tubercidin



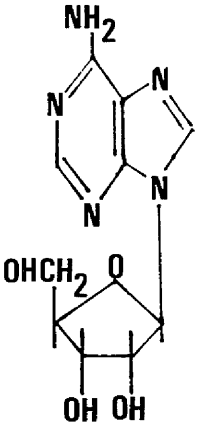
6--Thioguanine



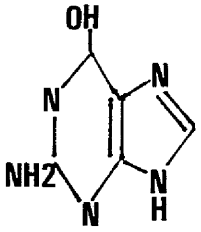
6--Thioguanosine



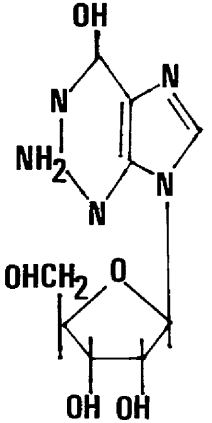
Adenine



Adenosine



Guanine



Guanosine

Staining for mycoplasma was carried out by the method of Foch,J. and Foch,H. (1964, Proc. Soc. Expt1. Biol. and Medicine 117 899-901). I have pleasure in thanking Miss E.McKay, Mr. W.R.Thomson and the staff the Cytology laboratory for carrying out this test.

The cell variants used in this study were:

PyY (Wild type)

PyY AA/AAR

PyY TG/TGR

PyY AA/AAR/TG/TGR

PyY TG/Car/BUdR.

Table III (opposite page 64) lists the levels of inhibitor at which each of the variants used formed a confluent sheet of cells. The abbreviations stand for:

AA	8-Azaadenine
AAR	8-Azaadenosine
TG	6-Thioguanine
TGR	6-Thioguanosine
TUB	7-Deazaadenosine
Car	Cytosine arabinoside
BUdR	Bromodeoxyuridine.

The structures of the purine bases, nucleosides and their analogues are shown in Figure II. 8-Azaadenosine is now no longer available. Tubercidin, also an analogue of adenosine, was used instead. As the cells used were originally selected for resistance to 8-azaadenosine, the symbol AAR has been retained in their nomenclature.

Initially each of the variants were cloned in agar and stocks grown up and stored as aliquots at -70°C. in storage medium. (See page 44.) Fresh stock could be recovered at will when required.

Testing for Contamination

All cells being grown were periodically tested for contamination by mycoplasma. Contaminated stock was discarded. Bacterial contamination was screened for by plating exhausted medium onto blood agar plates and

incubating aerobically overnight. Any stock found contaminated was discarded.

Cell Passage

Cells being used for experimental purposes were passaged for long periods. Cell splitting was performed in an enclosed cubicle with positive pressure ventilation. To minimize the risk of cross contamination between different cells each variant was split alone. The cubicle was left vacant for ten minutes or more with an ultraviolet lamp on before use with a further cell type. Cells were removed from the glass with either 0.25% trypsin in tris buffer, or a 1:4 mixture of trypsin and 0.02% EDTA in pH 7.5 phosphate buffer. The cells were then resuspended in growth medium to neutralize excess trypsin, counted in a haemocytometer if necessary, inoculated in the required numbers to bottles containing growth medium and gassed with a 5% mixture of CO_2 in air. The usual procedure for continuous propagation in 8 oz. bottles was a 1/10 split twice a week. Glass baby feeding bottles were used during the application of selective procedures. Baby bottles provided a flat surface on which the cells grew uniformly exposed to the inhibitor. No cells grew on the steeply sloping walls of the bottles as happened when they were grown in medicine bottles. At intervals the plating efficiency of the cells was determined (page 47).

Cell Storage

Cell lines were stored frozen at $-70^{\circ}C$ in vials. A confluent bottle of cells was trypsinized and resuspended at 1×10^6/ml. in a storage medium containing 5% glycerol, 25% foetal calf serum and 70% Eagles medium. 1 ml. aliquots of the medium were then added to 5 ml. screw-cap vials. The cells were then cooled to $-70^{\circ}C$. at the rate of one degree a minute. During recovery of the cells thawing was rapid.

Chicken Erythrocytes

Erythrocytes were obtained from eggs of varying ages and from adult hens. The shell over the air sac was removed, the allantoic vessels cut and the embryo allowed to bleed. The allantoic fluid was then harvested, spun at 1000 rpm. for five minutes to pellet the erythrocytes which were then resuspended in PBS. This was then repeated and the erythrocytes re-suspended at the concentration required. Adult erythrocytes were obtained by venepuncture from a wing vein. The blood was heparinized to prevent clotting.

Inhibitors

Inhibitors were prepared in stock solutions in distilled water. Poorly soluble inhibitors when required at high concentrations were prepared in alkaline solution by the addition of small quantities of 10N NaOH. After preparation, the solutions were filtered using a sterile Swinny adaptor and stored at -20°C. The inhibitor was added to the growth medium in the ratio of 0.1 ml. inhibitor to 10 ml. medium, a 1/100 dilution of inhibitor. Usually inhibitors were added after the cells had become established in baby bottles. If the solution of inhibitor was very alkaline it was necessary to bring the pH of the medium back to about 7.2 by gassing with 5% CO_2 in air. The selection procedure lasted for one week, during which no fresh inhibitor was added and the medium was not changed.

Media

Cells were grown as monolayers in medium consisting of 9 parts of Glasgow modified Eagles medium (containing twice the normal concentration of amino acids and vitamins) and one part of unheated calf serum. Usually foetal calf serum was used, obtained either from Flow Laboratories or Biocult.

However when large quantities of cells were required, neonatal bovine serum was used. For cloning, an initially slightly concentrated medium was used, Eagles 1.3. By the time the agar and inhibitors were added, the medium was diluted to its normal concentration.

Cloning

PyY cells and their variants may be cloned in agar. The cloning procedure was as follows:

1. 2.5% agar was melted in a bottle in a boiling waterbath.
2. 6 ml. of Eagles 1.3, 1 ml. each of foetal calf serum, inhibitor and distilled water to 1 ml. were mixed (8ml.).
3. 2 ml. of this mixture was then placed in a universal container and put in a waterbath at 45°C.
4. 2/3 agar was made up using 10 parts of the melted 2.5% agar and 5 parts of distilled water and also placed in the waterbath at 45°C.
5. (~~6 ml.~~) 1.5 ml. of the molten 2.5% agar was added to the second part of the mixture (6ml) plated to a 5 cm. petri dish and allowed to solidify. This acted as the bottom layer.
6. 0.1 ml. of the appropriate cell suspension was mixed with 2 ml. of the mixture from the waterbath and 0.5 ml. of the 2/3 agar and quickly plated onto the solid bottom agar and allowed to solidify.
7. The plates were incubated for up to two weeks in a moist incubator gassed with 5% CO_2 in air.
8. After incubation, clones were picked using a finely drawn pipette, trypsinized to disaggregate the cells, and then grown up in a bottle containing liquid growth medium.

Plating Efficiency

10^3 cells were inoculated into a glass petri dish in 10 ml. of growth medium. The plates were incubated for two weeks undisturbed. After incubation, the growth medium was removed and colonies present fixed and stained in a single step by adding neat Giemsa. The number of colonies was counted. Healthy cells in good medium gave plating efficiencies of 20-30%. This is a critical technique for cell growth and was used to detect deficiencies in the medium of cells and to check that conditions were satisfactory.

Coverslip preparation

The use of clean coverslips increased the plating efficiency of cells on them. 13 mm. diameter Chance No. 1 coverslips were used. They were first degreased by boiling in 1:20 Chloros and then allowed to soak overnight. After soaking, they were washed well in tap water before being rinsed twice with distilled water and once with absolute ethanol before being air-dried. The coverslips were sterilized by dry heat.

Growth of cells on coverslips

Cells were plated into 5 cm. Nunc plastic petri dishes having up to 9 x 13 mm. coverslips on the bottom. After the addition of cells and medium the coverslips were pressed onto the bottom of the dish with a pipette. The coverslips adhered to the plastic held by surface tension. The petri dishes were then gently transferred to an incubator, gassed with 5% CO_2 in air and incubated at 37°C. until required. Coverslips with an even covering of cells were obtained by this procedure. The density of the inoculum was varied according to the requirements of the experiment. An inoculum of 1×10^5 cells per plate produced a monodisperse layer in which very few cells touched. One of 1×10^6 cells per plate produced a confluent monolayer of cells.

Fixation

The fixative used during the initial experiments was 10% formol saline. This, though satisfactory in many respects, did not allow Giemsa-May-Grunwald stain to differentiate the nucleii and cytoplasm. For later experiments absolute methanol was used, allowing good differentiation to be achieved. The coverslips were rinsed twice in Dulbecco's phosphate buffered saline (PBS) and then placed in racks in the fixative.

Staining

Various staining procedures were tried, the best proved to be Giemsa-May-Grunwald. The stain was made up freshly before use in 0.1M. phosphate buffer pH 6.8 as 90 parts buffer, 10 parts Giemsa and 1 part May-Grunwald. The time required for staining varied between preparations.

Radioactive precursors

Tritiated nucleic acid precursors were obtained from the Radiochemical Centre, Amersham. The specific activities varied slightly from batch to batch, but were always in the same range for each precursor. The following were typical:

Adenine	A	Specific activity		4.3	Ci/mM
Adenosine	AR	"	"	12.6	"
Hypoxanthine	H	"	"	0.435	"
Guanosine	GR	"	"	13.0	"
Thymidine	T	"	"	18.4	"
Uridine	U	"	"	24.0	"

^{14}C labelled thymidine was used for the double label experiments, also obtained from the Radiochemical Centre, Amersham, at a specific activity of 430 mCi/mM. The isotopes were generally used at a final concentration

of 1 μCi/ml. in normal growth medium. They were stored at 4°C. (Diluted to 100 μCi/ml. if possible, in order to reduce degradation by self-irradiation to a minimum.)

Pulsing

Cells on coverslips were pulsed with radioactively labelled precursors. The pulse was usually carried out about one day after the cells were plated, the standard length of the pulse being 4 hours, after which the cells were fixed without chase. Depending on the number of coverslips required, either 10 μCi of the labelled precursor was added to a 5 cm. petri dish containing 10 ml. of medium and up to nine coverslips, or the coverslips were transferred to 3 cm. dishes, which could contain up to 3 x 13 mm. coverslips, and fresh medium containing 1 μCi/ml. of the isotope added. The quantity of the label incorporated per coverslip is proportional to:

1. The duration of the pulse, Figure IIIA. The plot is linear up to five hours. The line does not pass through the origin. Initial incorporation of label was slow because of the time required both for the cells to recover after being transferred to fresh medium and for the labelled precursor to enter the intracellular pools of nucleic acid precursors. One hour after initiating the pulse the incorporation rate of labelled precursor into macromolecules had stabilized because these points sit on the line.

2. The number of cells on the coverslip, Figure IIIB.

3. The quantity of labelled material added, Figure IIIC.

The radioactively labelled precursors were made up to 2.10^{-6}M. with cold carrier. 10 mM unlabelled hypoxanthine or inosine respectively were added to the medium during pulsing with adenine or adenosine to compete out any incorporation of labelled precursor after deamination had taken place.

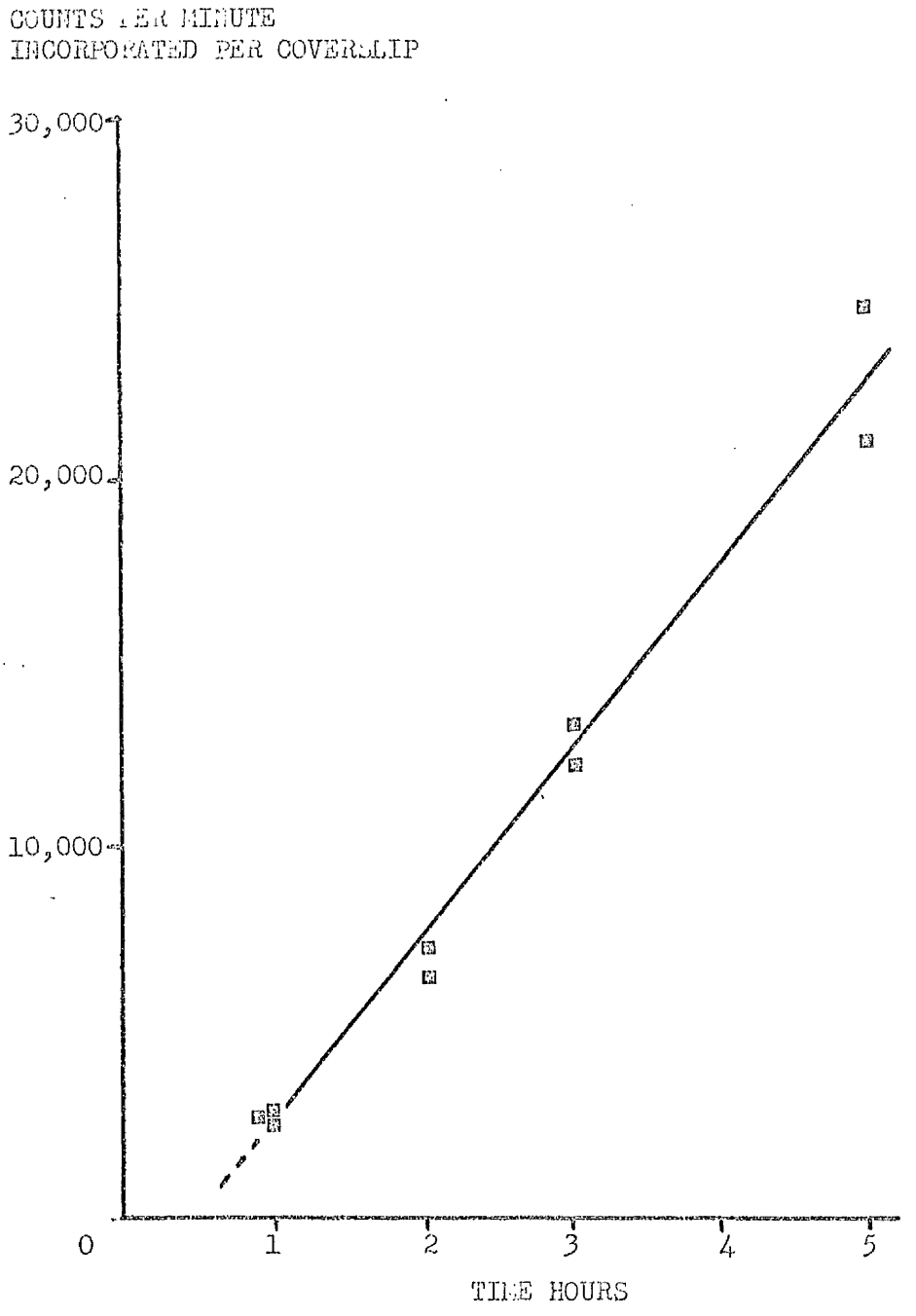


FIGURE IIIA. THE VARIATION IN THE QUANTITY OF RADIOACTIVELY LABELLED URIDINE INCORPORATED BY PyY CELLS ON COVERSLIPS WITH TIME.

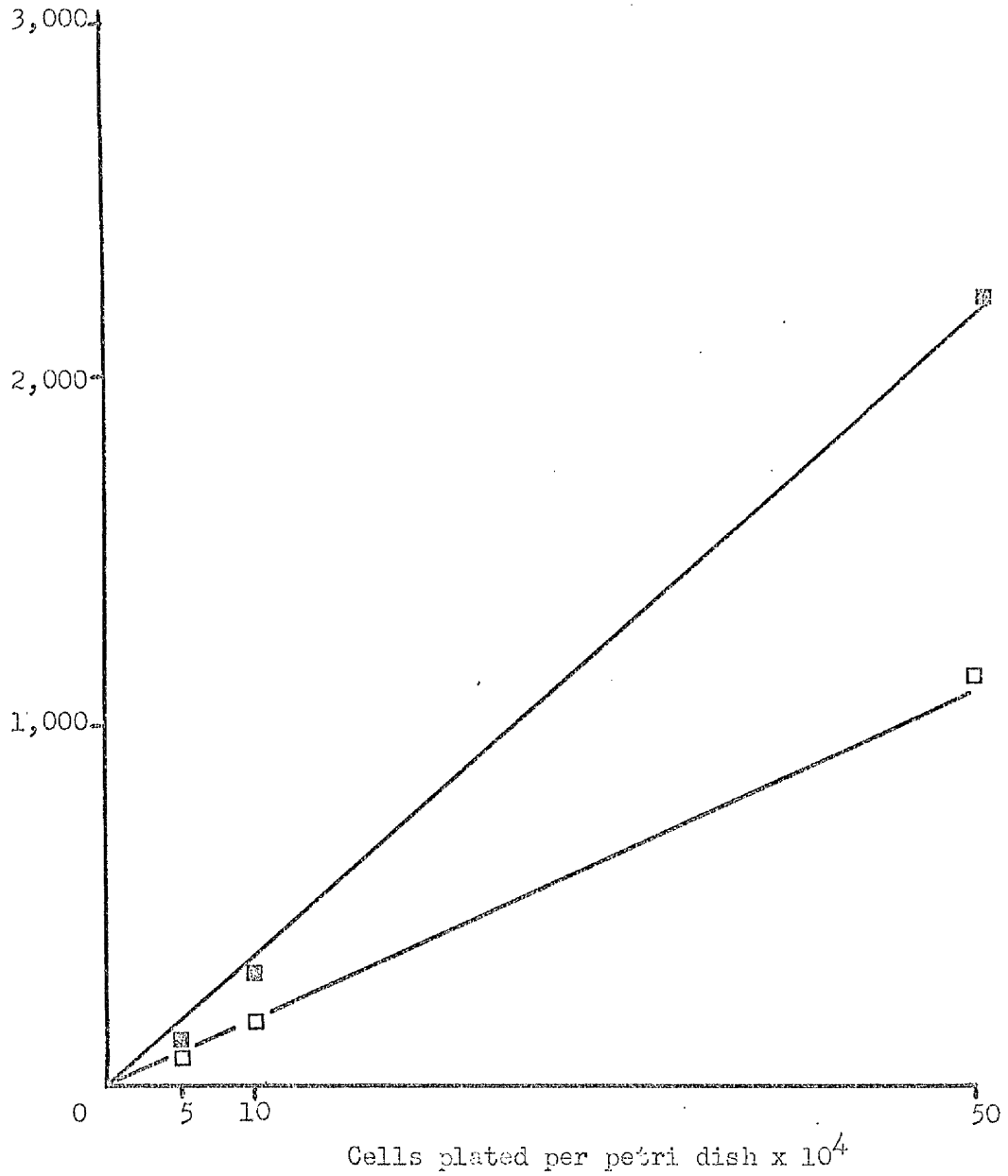


FIGURE IIIB. THE VARIATION IN THE QUANTITY OF RADIOACTIVELY LABELLED PRECURSOR INCORPORATED BY PyY CELLS ON COVERSLIPS WITH THE NUMBER OF CELLS INOCULATED.

▩ Adenine □ Hypoxanthine

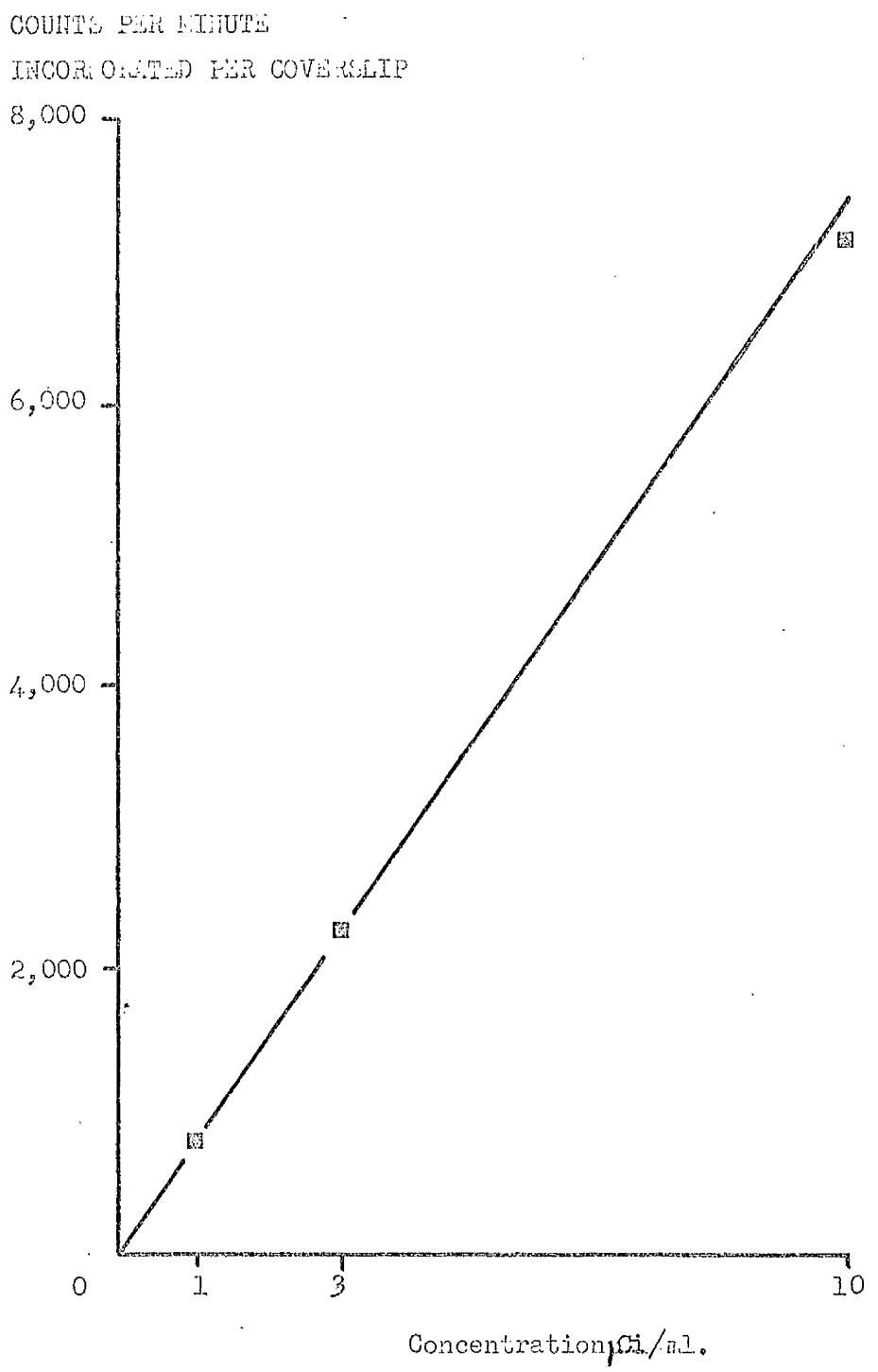


FIGURE IIIC. THE VARIATION IN THE QUANTITY OF RADIOACTIVELY LABELLED GUANOSINE INCORPORATED BY PyY AA/AAR CELLS ON COVERSLIPS WITH THE CONCENTRATION OF LABEL.

Extraction

After fixation, the pulsed coverslips were rinsed in tap water at 4°C and the acid soluble material extracted by two washes with 5% trichloracetic acid (TCA) at 4°C. They were then rinsed in running tap water overnight before being air dried from ethanol.

Scintillation Counting

The coverslips were counted intact in a liquid scintillation counting system. Each was placed individually in a vial and 5 ml. of scintillation fluid added (toluene with 4.0 G/l PPO and 0.05 G/l POPOP). The quantity of radioactive material incorporated was determined using either an Intertechnique ARAC 5640 or a Nuclear Chicago Mark 1 scintillation counter. Since all the samples were identical with respect to their geometry, self absorption and quenching, no corrections were made. Counts are expressed throughout as DPM. This method is sensitive, reproducible and the coverslips can be autoradiographed after the scintillation counting.

Mounting the coverslips for autoradiography

Coverslips that had been immersed in toluene for scintillation counting and were required for autoradiography were removed from the scintillation vial and rinsed in ethanol twice before being dried. They were then treated as usual for mounting on 3 x 1 inch slides. The coverslips were mounted individually, cells up, using DePeX mounting medium. If needed, a total of seven coverslips could be mounted on one of these slides for autoradiography as a single unit.

Autoradiography

Two methods of autoradiography were used, one based on stripping film the other on dipping emulsion. Both will be described.

1. Stripping Film

Kodak AR 10 stripping film was used with a safelight made from a Kodak Wratten Series 1 red filter and a 25 watt bulb. This light was not totally safe so care was taken to expose the film only when necessary. The film was floated onto distilled water at 20°C. in a white sandwich box, the surface of the water was kept free of dust. For exposures lasting a long time the addition of sucrose 2% and potassium bromide 0.001% to the distilled water reduced the background (C. O'Callaghan, Stevens and Wood, 1969). The stripping layer was cut around the margin of the plate with a scalpel blade. The film was then cut to produce strips of a suitable size to cover the coverslip, usually six or eight strips could be obtained from one plate. With clean forceps the margin of the film was lifted, gently stripped off the glass backing and placed emulsion side down on the surface of the water. The temperature and humidity of the darkroom were critical for this step. If the temperature was too high the film stretched and tore rather than stripping smoothly. In too low a humidity the film curled off in an uncontrollable fashion, producing many sparks due to static electricity, resulting in a high background. As it lay on the surface the film swelled by absorbing water. During this process, the film first crinkled and then stretched out tight and flat. Streaks on the film indicated that stripping had been too violent. A high background would have accumulated in the region of the streaks. After the film had expanded fully it was left for three minutes before being lifted off the water onto the slide. The slide was held at 30° to the horizontal under the water and gradually lifted so that the edge touched the emulsion first and then slowly raised from the water. The emulsion draped itself smoothly over the surface of the slide and the

excess water drained off into the trough. While the film was still wet, minor adjustments could be made to its position and any crinkles removed. The slide was then rested at an angle in the dark to drain and dry fully before being placed in a slide box in a light-tight container to expose in the presence of dry silica gel. The exposure took place at 4°C. for an appropriate time. The silica gel was changed after the first day of exposure to ensure a low humidity during exposure. A high humidity resulted in latent image fading and reduced the number of grains produced.

2. Dipping

Ilford K2 emulsion was used for tritium, and G5 for carbon 14. K2 is less sensitive and has a smaller grain size than G5. Both emulsions were handled under an Ilford 'S' safelight. The emulsion required was melted by placing in a container and stirred gently in a waterbath at 43°C. The molten emulsion was then diluted 1/3 with distilled water containing 2% glycerol. The addition of glycerol reduced the stress background caused by contraction of the gelatin during drying. The diluted emulsion was ready for use after thorough mixing and removing all bubbles from the surface. The emulsion was maintained at a temperature of 43°C. in the water-bath during dipping. Each slide was immersed in the emulsion individually, allowed to drain briefly and the back wiped with tissue. To gel the emulsion quickly the slides were cooled by being placed emulsion side up on the bottom of an inverted metal tray containing ice. After the emulsion had gelled the slides were transferred to a cool place and allowed to dry in the dark. When dry, the slides were placed in a slide box in the dark for two days during which latent image fading reduced the background produced during the dipping procedure. At the end of this period, silica gel was added to lower the humidity and allow the exposure to begin. Very low backgrounds could be produced by this procedure.

3. Double label autoradiography

Double label autoradiography involves the use of two isotopes producing particles having different energy distributions. The conditions for autoradiography are adjusted to distinguish between the disintegrations produced by the two isotoped. The two isotopes used in these experiments were carbon-14 (C-14) and tritium (3-H), both could be detected independently in the same preparation. The C-14 was used as thymidine, the 3-H as either adenine, hypoxanthine or thymidine. The β particles originating from the tritium have initial energies in the range of 0 to 18 KeV, those from the C-14 in most cases have higher energies in the range of 0 to 155 keV. Both isotopes give off β particles in the range up to 18 keV and there is no way of determining which isotope was the source of the disintegration in this energy range. However 85% of the particles from the C-14 have energies above 18 keV. These high energy particles can be shown to be present under conditions that exclude any particles originating from the 3-H. The presence of tritium can be demonstrated only by detecting the presence of an excess of particles having energies of 18 keV or less than is expected from the amount of C-14 present.

One approach to double label autoradiography has been to use a single thick layer of emulsion. This is an inflexible technique and does not produce good results.

The double exposure method originally developed by Baserga (1962) is much more flexible and allows exploitation of the main relevant variables which are:

1. Quantity of 3-H
2. Quantity of C-14
3. Type and thickness of first emulsion
4. Type and thickness of second emulsion
5. Exposure time of first emulsion

6. Exposure time of second emulsion
7. Thickness of inert material separating the emulsion
8. Different developing and fixing procedures can be used for the two emulsions.

Double label autoradiography involved two separate autoradiographic procedures carried out sequentially on the same preparation. The first procedure was to autoradiograph the specimen using Kodak AR 10 stripping film. The autoradiograph was developed and stained as usual. A thin layer of celloidin was then placed over the autoradiograph by dipping the slide in a mixture of celloidin, ether and alcohol (1:1:1). Excess celloidin was shaken off and the remainder allowed to dry as a thin layer over the preparation. The celloidin layer was impermeable to water and protected the first autoradiograph during the second procedure. The slide was then dipped in Ilford G5 emulsion, exposed and processed in the usual way.

The low energy β particles only produce grains in the first layer of emulsion. Their energy is insufficient to allow them to pass through and reach the second layer. The first layer therefore records all the 3-H and 15% of the C-14 disintegrations (those having energies within the 3-H range). The high energy particles pass straight through the first emulsion leaving very few grains. Their energy is sufficient to allow them to penetrate to the second layer where they produce grains. Grains in the second layer can only have been caused by particles originating from 14-C. The energy of 3-H β particles is such that they only cause solitary grains. The energy of most 14-C β particles is much higher and causes a series of grains along their paths termed tracks. Where the energy is high, at the start of the track, the grains are widely spaced. Towards the end of its track when much energy has been lost, the grains are closely packed together. The initial part of the track is within the first layer of emulsion, the final part in the second emulsion. The distribution of grains along the track tends to produce few in the first emulsion and the majority in the second emulsion.

The differential grain production can be increased by using different emulsions. AR 10 is sensitive to low energy particles but insensitive to high energy β particles and it therefore records 3-H very efficiently, but the energetic 14-C disintegrations poorly. The second emulsion, Ilford G5, is sensitive to high energy β particles. Another way of increasing the discrimination between the two isotopes is to adjust the conditions so that there is a high ratio of 3-H to C-14. In this situation the contribution of grains caused by the 15% of 14-C β particles of low energy in relation to the number of grains due to the 3-H β particles becomes insignificant. These conditions can be achieved in two ways. Firstly the pulsing conditions are adjusted so that the cells incorporate a great deal of 3-H labelled material and the minimum quantity of 14-C labelled material. Only enough 14-C labelled material need be incorporated to produce grains in the second layer after a reasonable length of time. The second way of increasing the discrimination is to adjust the ratio of exposure times of the two emulsions. The first exposure should be as short as possible to reduce the spillover from the 14-C label. The second exposure time is only limited by the rate of accumulation of background and the patience of the experimenter.

The last advantage of using the two emulsion technique is that the emulsions can be chosen to have different grain sizes. This is the case with AR 10 and G5. However, in practice distinguishing between grains in the two emulsions was not a problem because under oil immersion when one set of grains were in focus the other could not be seen.

The variant to be 14-C labelled was grown for 24 hours in 14-C thymidine of specific activity at 430 mCi/mM at a final concentration of 0.05 μCi/ml. During this period almost all the cells passed through S phase at least once and incorporated the label. The labelled cells were then suspended by trypsinization, washed in warm growth medium twice and resuspended at the required concentration in growth medium. They were then mixed as required

with the unlabelled variant and plated as usual into petri dishes containing coverslips. After 16 to 20 hours, the coverslips were removed and pulsed for 4 hours with the required 3-H labelled material at 10 μCi/ml. The coverslips were then processed as usual.

One difficulty encountered was that the resolution of the 14-C grains, while being satisfactory for use with lightly seeded preparations, was not enough to use for dense culture of cells. The grains spread over a wider radius then that of the cell from which they originated. It might be possible to improve this by having the second layer of emulsion nearer to the specimen. Attempts were made to use K2 dipping film instead of the AR 10 which has a 10μ thick backing of gelatin on the sensitive emulsion. To date these have not been successful.

Each experiment involving autoradiography was planned to include a positive control to show that the autoradiographic technique had been satisfactory, usually thymidine or uridine were used for this purpose. The time of exposure was controlled by exposing a duplicate of one of the experimental slides which was developed after a suitable exposure. The experimental slides could then be developed if the exposure was satisfactory. The experimental slides were developed later if a further exposure was required. The coverslips were mounted in batches on a single slide to achieve similar conditions of exposure and to save work. If the coverslips comprising one experiment had to be mounted on several slides all were exposed in the same box and developed together to minimize differences due to variations in exposure conditions and processing.

The development procedure was similar for both techniques. The slides were developed in Ilford D 19 for 5 minutes in the case on the AR 10 and 2 minutes in the case of the dipping film. They were then rinsed in tap water and fixed in Amfix diluted 1:4 with tap water for twice the clearing time, about 2 minutes in the case of the dipping film and 5 minutes in the case of the stripping film. The slides were then washed in running tap

water for five minutes and dried. They were stained with Giemsa-May-Grunwald.

Sendai Virus

Sendai virus was obtained from Dr. H. G. Pereira, National Institute for Medical Research, Mill Hill. The virus was inoculated into 10 day old embryonated eggs and grown for three days. The allantoic fluid was then harvested after the eggs had been chilled by leaving at 4°C. for four hours. The crude allantoic fluid was spun at 1000 rpm for 10 minutes to remove debris. The supernatant was then spun at 18,000 rpm for an hour, the supernatant was discarded and the virus pellet resuspended in Dulbecco's PBS to 5% of the original volume. The titre of the virus was determined by haemagglutination. Serial doubling dilutions of the virus were prepared in 0.5 ml. volumes in WHO Salk haemagglutination trays using PBS as diluent. To all cups 4 drops of 2% guinea pig red cells were added, the tray shaken to mix and then left at room temperature for the red cells to settle. After about 2 hours the last cup to show complete haemagglutination was taken as containing 1 haemagglutination unit (HAU) of the virus. Usually the final concentrate contained 20,000 HAU of the virus per ml. The active virus was either stored as crude allantoic fluid or as the concentrate rapidly frozen to -70°C. The infectivity of the virus was well retained up to 18 months after freezing.

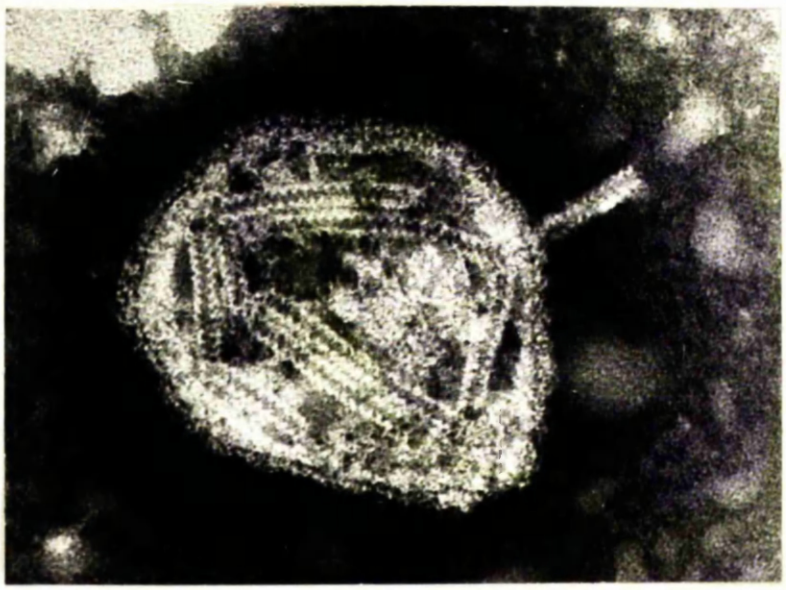
Inactivation of the Sendai virus

For the purposes of cell fusion the infectivity of the virus could be abolished without affecting its ability to fuse cells by treating with betapropriolactone (BPL), (Neff and Enders, 1968). A 10% solution of BPL in distilled water was prepared immediately before use. The 10% solution was then diluted to a concentration of 0.6% in a saline bicarbonate solution. (1.68 G. of $NaHCO_3$ and 0.5 ml. of 4% phenol red solution were added to 100 ml. of isotonic NaCl solution.)

FIGURE 1V

a) Sendai virus fixed with 4% ammonium molybdate. The particle is intact showing the envelope with its surface covered by small projections surrounding the coiled ribonucleoprotein core.

b) Sendai virus fixed with 2% phosphotungstic acid. The particle is disrupted and the helical structure of the core shown.



Diluted BPL was added in the proportion of 1 part to 9 parts of the Sendai virus concentrate to make a final concentration of 0.06% BPL. All the dilution procedures were carried out quickly at $4^{\circ}C$ in precooled glassware. The mixture of BPL and Sendai virus was shaken tightly in a closed container for 10 minutes at $4^{\circ}C$. to mix completely before placing at $37^{\circ}C$. for two hours with intermittent mixing. The mixture was then cooled to $4^{\circ}C$. and kept overnight to totally hydrolyse the BPL to betaproprionic acid which has no effect on the virus or the cells. The dilutions were prepared quickly to prevent premature hydrolysis of the BPL. There are reports that BPL is oncogenic, so care was taken to ensure complete hydrolysis before disposal. Each batch of inactivated virus was tested for infectivity in fertile 10 day old eggs. Serial ten-fold dilutions of noninactivated and inactivated virus were prepared and inoculated as 0.1 ml. aliquots into pairs of eggs. These were incubated for a further two days and the allantoic fluid harvested. The neat fluid was then tested for haemagglutinating activity. The results shown in Table I are typical. The untreated virus infected all the eggs at every dilution until 10^{-6} when one egg was haemagglution negative. The original fluid therefore contained about 10^{7} 50% egg infecting doses/ml. The BPL treated virus however caused haemagglutination only in one of the eggs infected with neat virus. This haemagglutination may have been due to the virus of the original inoculum rather than its growth. The BPL treatment abolished the infectivity of the virus. Inactivated virus was also tested for haemagglutination. Usually the haemagglutination titre dropped after inactivation by up to a factor of two, but never more.

Figure IV shows two electron micrographs of the Sendai used. The particles consist of a lipoprotein envelope surrounding a coiled ribonucleoprotein core. The BPL treatment abolished the infectivity of the virus but at the concentrations used had little effect on the ability of the envelope to promote cell fusion. Inactivated batches of virus were rapidly frozen in aliquots and retained fusing ability for at least 18 months.

TABLE I. TITRATION OF THE INFECTIVITY OF SENDAI VIRUS IN EGGS

Log Dilution	Untreated	BPL treated
Neat	ND	+ -
-1	ND	- -
-2	+ +	- -
-3	+ +	- -
-4	+ +	- -
-5	+ +	- -
-6	+ -	- -
-7	- -	- -

\+ Haemagglutination positive allantoic fluid

\- Haemagglutination negative allantoic fluid

ND Not Done

0.1 ml. volumes

Two eggs per dilution per treatment.

Cell fusion

The technique of cell fusion was basically that of Harris and Watkins (1965) but modified to suit the hamster variant cells. It was necessary to obtain fusion of 50% or more of the hamster cells with erythrocyte nucleii, but reduce the fusion between the hamster cells to a minimum. To achieve this, a ratio of 100 erythrocytes to one hamster cell was used. The cells were removed from the glass with trypsin alone, the presence of EDTA, a chelating agent, reduced the efficiency of cell fusion. The cells were washed and resuspended in PBS at a concentration of 5×10^6 hamster cells and 5×10^8 erythrocytes per ml. and placed at $4^\circ C$. The inactivated Sendai virus was then added and the mixture given a brief shake to ensure mixing. Usually the addition of 0.1 ml. of Sendai virus containing 1000 HAU produced satisfactory fusion of the above number of cells. There was massive agglutination of the cells as soon as the virus was added. The mixture was left at $4^\circ C$. for 15 minutes. It was then warmed to $37^\circ C$. for 15 minutes, before the addition of growth medium to dilute the cells to the concentration required. The heterokaryons plated normally and were fully spread within 12 hours of plating in most cases. In some experiments the medium was changed at one day after plating in order to remove unfused erythrocytes, cell debris and the remaining Sendai virus. This produced cleaner preparations, but did not appear to increase cell survival. The degree of fusion produced varied from experiment to experiment. The presence of serum in the fusion mixture appeared to reduce cell fusion, as did shaking of the cell mixture after addition of the virus. In general, the more virus added the better was the fusion. In particular one cause of poor fusion was the use of a narrow bore pipette to transfer the agglutinated cells to the growth medium, presumably the cells were separated by shearing forces and were unable to reagglutinate.

Irradiation of the Cells

The hamster cells were irradiated before fusion to prevent subsequent cell division. Different cell variants reacted in different ways after irradiation. In some cases there was massive micronucleation and fragmentation, in others though a large proportion of cells died, some survived in a morphologically normal state for up to a week. The optimal dose for the prevention of micronucleation was 6000 rad. The cells were irradiated either from a cobalt 60 source or a Watson 90/30 trolley set producing 80 kV X-rays.

Photomicroscopy

Autoradiographs were examined using a Reichert Zetopan microscope. Photomicrography was carried out using a Leica camera. Ilford Pan-F film was used developed either in Diafine (Acufine, Inc.) or Contrast FF (Ilford). Printing was carried out using Ilford papers. For the measurement of nuclear areas photographs of the cells were printed at a constant magnification onto uniform paper. Nucleii could then be cut out of the prints and weighed. The weight of a nucleus was proportional to its area.

Protein Estimation

The Lowry method was used. The reagents were:

1. 0.5 ml. 1% copper sulphate and 0.5 ml. 2% potassium tartrate were mixed and added slowly with stirring to 50 ml. 2% sodium carbonate in 0.1 N sodium hydroxide. (Reagent A).
2. Folin-Ciocalteau reagent, 4 ml. diluted to 10 ml. with water.
3. Standard crystalline bovine serum albumin, 0.5 mg./ml.

The cells had been fixed by methanol on 13 mm. coverslips but could be taken into solution by heating in 1 N sodium hydroxide. Usually the cells from two coverslips were taken up into 1 ml. of the sodium hydroxide. 0.2 ml.

of this was added to 3 ml. reagent A, mixed and left at room temperature for 10 minutes. 0.3 ml. of diluted Folin-Ciocalteau reagent was added, mixed well and left for one hour at room temperature. The optical density of the blue colour was read at 750 mμ. By referring to the standard curve obtained using known dilutions of the standard bovine serum albumin, the quantity of protein in the experimental samples was estimated.

5. RESULTS I. CHARACTERIZATION OF THE VARIANT CELLS

1. Cell cloning in agar

Polyoma transformed cells and their variants grow in soft agar. This property was used to clone the cells. Unlike cloning in liquid medium, there was no possibility that the cells could spread from the site of the clone and therefore no likelihood of cross-contamination between the clones. It was assumed that the clones originated from single cells. The cell suspensions used were checked during counting and less than 2% of the cells were present as clumps. The variants were cloned in the presence of each purine analogue apart from tubercidin (Table II).

There was absolute discrimination between the different variants and the wild type at the concentrations of the analogues used. The variants produced clones only in the presence of the analogues for which resistance had been selected. The wild type PyY cells were unable to form clones in the presence of any of the analogues. The morphology of the clones differed between some variants. All the clones of any one variant were of the same morphology but could differ in size. Those of PyY and the PyY TG/TGR variant were morula-like, being compact balls of cells. The PyY AA/AAR and PyY AA/AAR/TG/TGR clones were much less well-defined forming fluffy patches. Two clones of each variant were grown up and tested for their abilities to incorporate purines and thymidine. (See this Chapter, Section 5.)

2. Testing resistance in liquid medium

In addition to testing resistance in agar, the ability of the variants to grow on glass surfaces in the presence of various concentrations of the analogues was examined. The cells were inoculated into baby feeding bottles in the presence of the analogues and allowed to grow for one week. They were then trypsinized off the glass, resuspended in growth medium and counted

TABLE II. GROWTH OF CELLS IN AGAR

Cell Line	Analogue concentration µg/ml.				
	Control	AA 100	TG 2	TGR 50	TG 2+ TGR 50
PyY	*	-	-	-	-
PyY AA/AAR	*	*	-	-	-
PyY TG/TGR	*	-	*	*	*
PyY AA/AAR/TG/TGR	*	*	*	*	*

* Colonies present

\- No colonies present

Inoculum 4 x 10^4 cells/dish.

either in a Coulter counter or a haemocytometer chamber. Figures V, VI, VII and VIII show the cell numbers obtained plotted against the concentration of the analogue used as a percentage of the cell number obtained from a control bottle not exposed to inhibitor. Figure V demonstrates that the PyY AA/AAR cells continue to grow in 8-azaadenine (AA) at 250 µg/ml., the cell number produced being reduced by 40%. The PyY and PyY TG/TGR cells were unable to grow in the presence of AA at 20 µg/ml. There was a better growth of the PyY TG/TGR cells than PyY at 5 and 10 µg/ml. Figure VI shows that the PyY AA/AAR cells grew in tubercidin (Tub) at 200 µg/ml. Both the wild type and PyY TG/TGR cells [illegible] behaved similarly in Tub, being unable to survive at 10 µg/ml. Figure VII shows the survival of the variants in the presence of 6-thioguanine (TG), the PyY TG/TGR variant grew in 100 µg/ml. of the analogue. Neither PyY nor PyY AA/AAR cells were capable of growing in TG at 1.0 µg/ml. Figure VIII demonstrates that the PyY TG/TGR variant continued to grow in 6-thioguanosine (TGR) at 100 µg/ml. PyY and PyY AA/AAR cells were unable to grow in TGR at 1 µg/ml. The variant cells continued to grow in analogues for which resistance had been selected at concentrations about 100 times higher than those at which wild type cells were killed.

It was possible to test the ability of the cells to grow in the presence of the analogues in a slightly different way. The ability to form a cell sheet was used as a criterion of resistance instead of trypsinizing the cells off the glass and counting them. Each variant was exposed separately to a series of concentrations of each inhibitor. The concentration at which a good cell sheet formed was taken as the level of the analogue to which the cells were resistant. Table III indicates the highest level of the analogues at which a cell sheet formed after growth for one week in the presence of each of the analogues. The determinations of resistance by counting and by morphological criteria agree well. Higher concentrations of AA were used in the latter. The AA/AAR variant continued to form a sheet

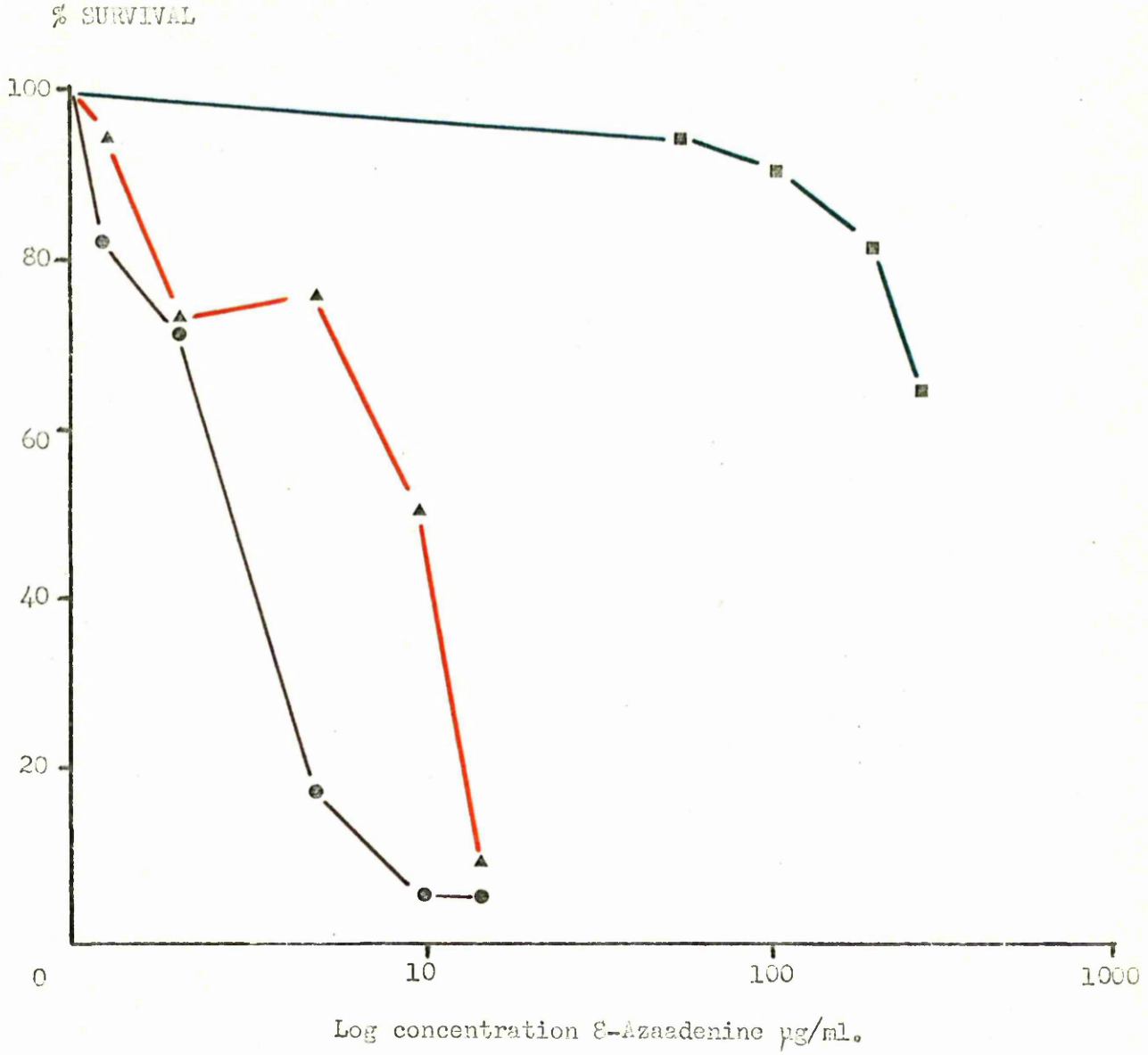


FIGURE V. THE NUMBER OF PyY, PyY AA/AAR and PyY TG/TGR CELLS AFTER GROWTH FOR A WEEK AT VARIOUS LEVELS OF 8-AZAADENINE.

% SURVIVAL

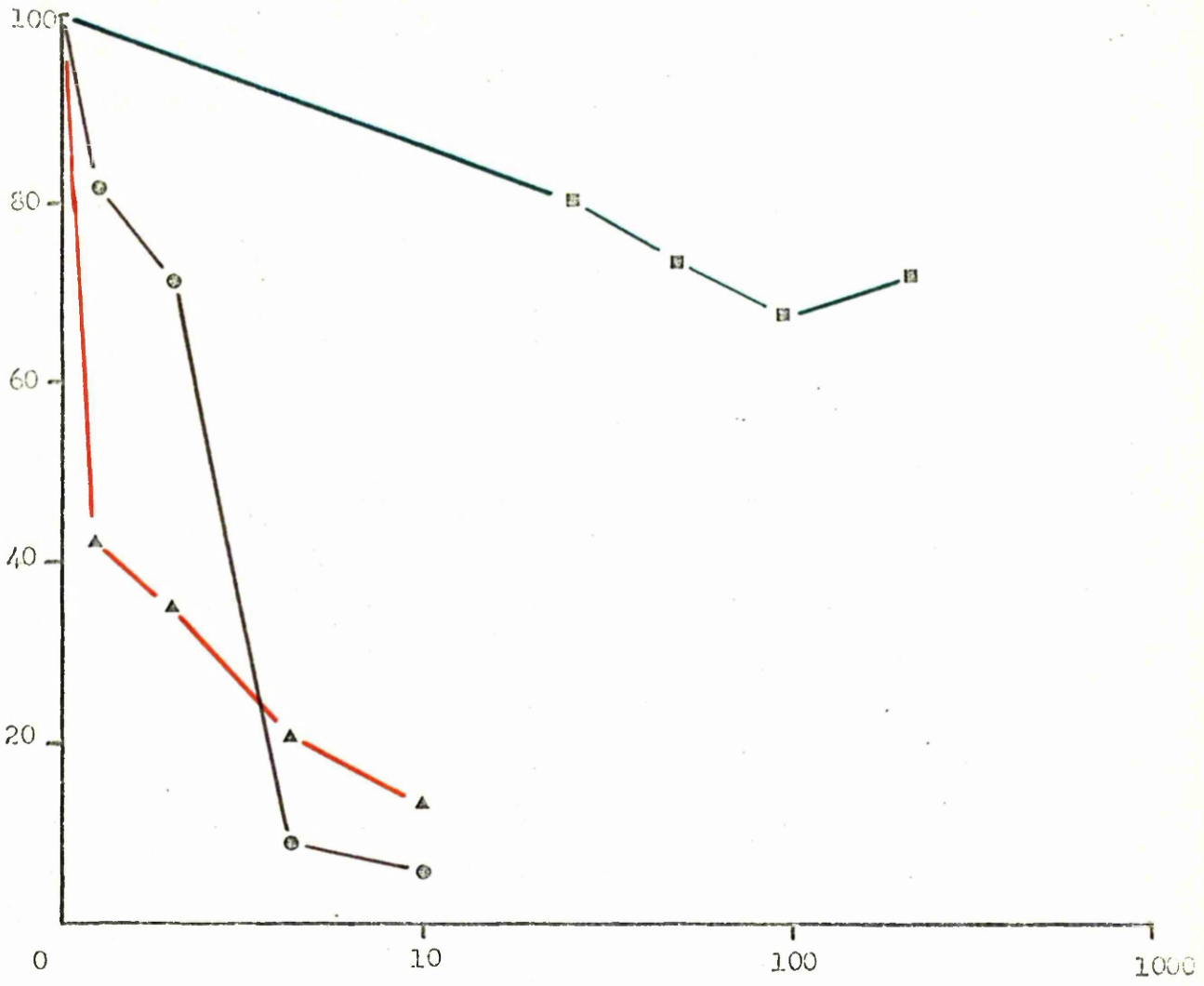


FIGURE VI. THE NUMBER OF PyY, PyY AA/AAR AND PyY TG/TGR CELLS AFTER GROWTH FOR A WEEK AT VARIOUS LEVELS OF TUBERCIDIN.

— ● — PyY

— ■ — PyY AA/AAR

— ▲ — PyY TG/TGR

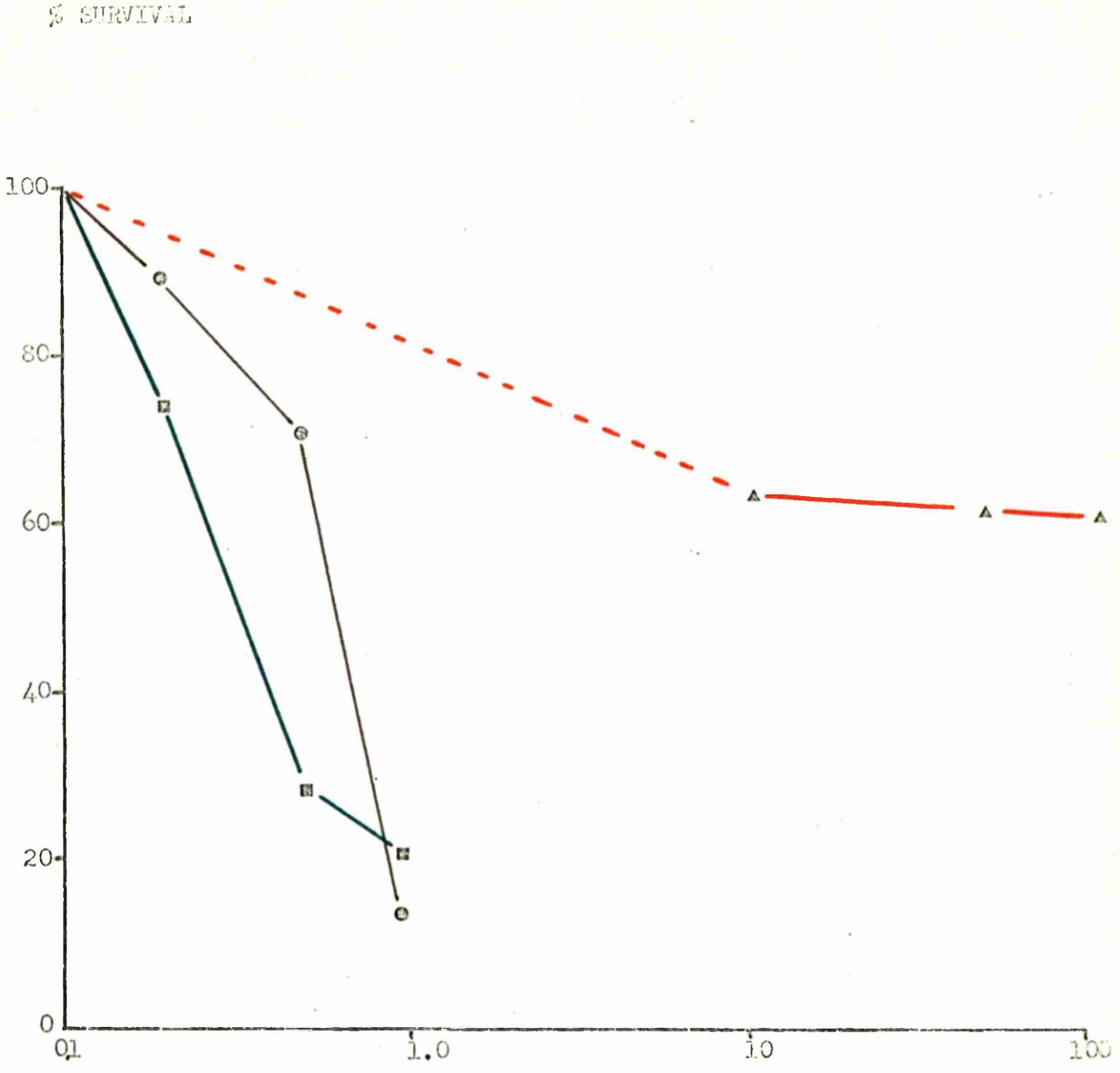


FIGURE VII. THE NUMBER OF PyY, PyY AA/AAR AND PyY TG/TGR CELLS AFTER GROWTH FOR A WEEK AT VARIOUS LEVELS OF 6-THIOGUANINE

% SURVIVAL

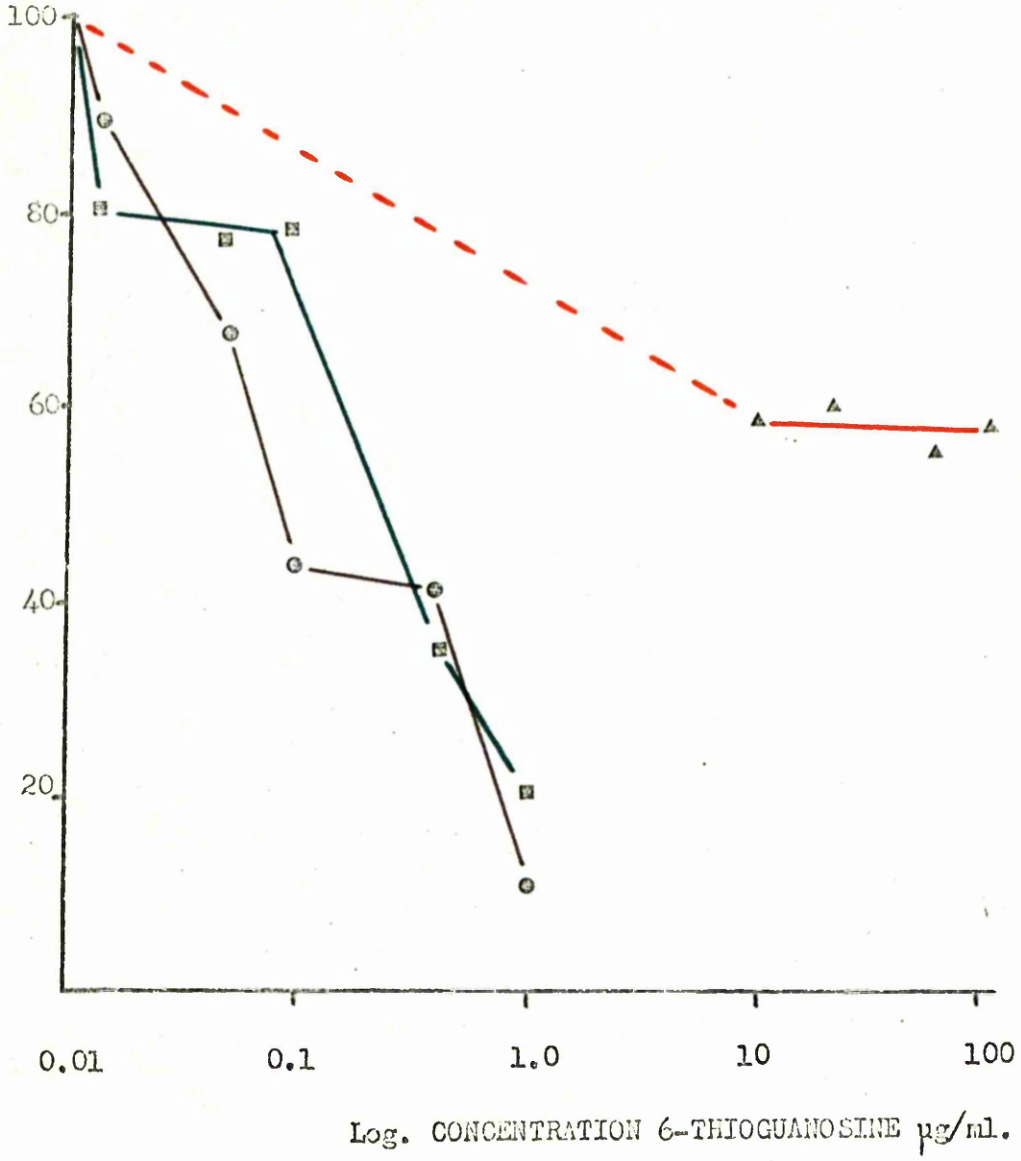


FIGURE VIII. THE NUMBER OF PyY, PyY AA/AAR AND PyY TG/TGR CELLS AFTER GROWTH FOR A WEEK AT VARIOUS LEVELS OF 6-THIOGUANOSINE.

TABLE **III.** RESISTANCE TO BASE ANALOGUES DETERMINED VISUALLY

Variant	Level of inhibitor µg/ml.				
	8-Azaadenine	Tubercidin	6-Thioguanine	6-Thioguanosine	5-bromodeoxyuridine
PyY	5	2	0.2-0.5	0.1	15-20
PyY AA/AAR	1000	200	0.2-0.5	ND	15-20
PyY TG/TGR	10	ND	100	100	15-20
PyY AA/AAR/TG/TGR	1000	200	75	100	20
PyY TG/Car/BUdR	10	ND	20	20	1000

The maximum level of inhibitor at which a monolayer of cells grew in one week. Initial inoculum of 1×10^5 cells to a baby bottle. ND indicates a combination of cell and inhibitor that was not tested.

in 1000 µg/ml. AA.

The PyY AA/AAR/TG/TGR and PyY TG/Car/BUdR variants were only tested for resistance by the criterion of their ability to form a cell sheet. PyY AA/AAR/TG/TGR cells formed a cell sheet in high concentrations of all the purine analogues tested. PyY TG/Car/BUdR cells had a very slightly increased resistance to 8-azaadenine. Their resistance to 6-thioguanine was less than that of the PyY TG/TGR and PyY AA/AAR/TG/TGR variants but still increased 100-fold over that of the wild type.

All the variants were also tested for their ability to form a cell sheet in BUdR. The wild type and those variants for which resistance had not been selected were killed by BUdR at 15-20 µg/ml. The behaviour of cells sensitive to this analogue was unusual. In all other cases where cells were grown in an analogue at concentrations higher than their level of resistance, little or no growth took place before the cells were killed. Growth appeared to be normal in BUdR for the first 5-6 days but suddenly massive cell death occurred. PyY TG/Car/BUdR cells were capable of growth in BUdR at 1000 µg/ml. while sensitive cells were killed by 15-20 µg/ml.

Referring back to the previous section, it should be noted that the levels of the analogues used for cloning the cells in agar were lower than those for cells on glass. The maximum levels of the analogues tolerated in agar were not determined, but it did appear that they would have been lower than those for growth on a glass surface. The cells were not normally grown in agar, so growth under these conditions was in a general way more stringent than on the glass surface normally used.

3. Resistance levels to aminopterin and the development of selective systems

Aminopterin, a folic acid antagonist, renders cells auxotrophic for glycine, a purine source and thymidine by blocking de novo synthesis.

Table IV shows the lethal level of aminopterin for PyY, PyY AA/AAR and PyY TG/TGR was the same. A good cell sheet formed at 1×10^{-8} M. but very few cells survived after a week at 5×10^{-8} M. These levels agree well with those obtained by Hakala using S-180 cells (1969).

It was found that the rate at which the cells were killed was proportional to the concentration of aminopterin used. Massive cell death took place within three days in the presence of 1.5×10^{-6} M. aminopterin. However in the presence of a purine source (which may either be adenine or hypoxanthine), thymidine and glycine, wild type cells grew normally at this level of aminopterin, more than fifty times the lethal dose determined above (Table IV). PyY AA/AAR, being defective in their ability to use adenine (see this Chapter, Section 5) were unable to survive when the purine source was adenine but grew normally in the presence of hypoxanthine. Likewise the PyY TG/TGR cells were unable to survive using hypoxanthine but grew in the presence of adenine. The concentration of hypoxanthine was not critical, 1×10^{-4} M. was used, however adenine at this level was toxic for all cells and had to be used at 5×10^{-5} M. It was possible to remove the cells from the selective conditions, supplementing the medium with thymidine, a purine source and glycine for the first day, after which they could be treated normally. The AAT and HAT selective systems, together with the use of the purine analogues 8-azaadenine and 6-thioguanine should allow selection for the survival or death of either of the variants PyY AA/AAR and PyY TG/TGR (Table V).

4. Determination of growth rate

The behaviour of wild type and variant cells was investigated using cells growing in baby bottles without change of medium. In the case of PyY and PyY AA/AAR/TG/TGR complete growth curves were obtained. An initial inoculum of 1×10^{5} cells was placed in a series of baby bottles with 10 ml. of growth medium. At daily intervals after inoculation one bottle was

TABLE IV. AMINOPTERIN RESISTANCE

	Control	Aminopterin M. 5.10^{-8}	1.10^{-8}	5.10^{-9}	HAT	AAT
PyY	C	-	C	C	C	C
PyY AA/AAR	C	-	C	C	C	-
PyY TG/TGR	C	-	C	C	-	C

\- Cell sheet not formed

C Confluent cell sheet produced after one week.

Initial inoculum 1×10^5 cells.

TABLE V. SUMMARY OF SELECTIVE SYSTEMS

	8-Azaadenine	6-Thioguanine	AAT	HAT
PyY	-	-	+	+
PyY AA/AAR	+	-	-	+
PyY TG/TGR	+	+	+	-

\+ Normal growth of cells.

\- No growth of cells.

AAT Adenine 5×10^{-5} M., Aminopterin 1.5×10^{-6}M., Thymidine 2×10^{-5} M. Glycine 3.10^{-6}M.

HAT Hypoxanthine 1×10^{-4} M., Aminopterin 1.5×10^{-6} M., Thymidine 2×10^{-5} M., Glycine 3.10^{-6} M.

8-Azaadenine 1000 µg/ml.

6-Thioguanine 100 µg/ml.

taken, the cells removed by trypsinization and counted in a Coulter counter. Figure IX shows that the cell number increased by 40 to 60 fold after a week, but that the initial growth rate was not sustained beyond the fifth day. The growth curves of PyY and PyY AA/AAR/TG/TGR were very similar despite the prolonged selection the variant cells had been subjected to. Single determinations of the numbers attained by the PyY AA/AAR and PyY TG/TGR variants indicated that their growth rates were also identical with that of the wild type. After mixing these two cell variants and allowing them to grow to confluence, there should be no preferential growth of one of them, and the initial cell ratio should be maintained. In one experiment using PyY cells, also shown in Figure IX, the medium was changed on the sixth day, resulting in a sustained increase in the growth rate for at least three days afterwards. The final cell number achieved exceeded that obtained in bottles without a change of medium. This stimulation of growth suggested that the number of cells was limited by exhaustion of some factor or factors in the medium needed for growth. The prolonged stimulation of growth is unlike the short-lived stimulation reported after addition of fresh serum. It is also unlike what would be expected if the cells were producing an inhibitor which on reaching a critical concentration prevented cell growth. Such an effect would also be transitory since the large number of cells present at the time of the change would be able to rapidly replace the inhibitor discarded with the used medium.

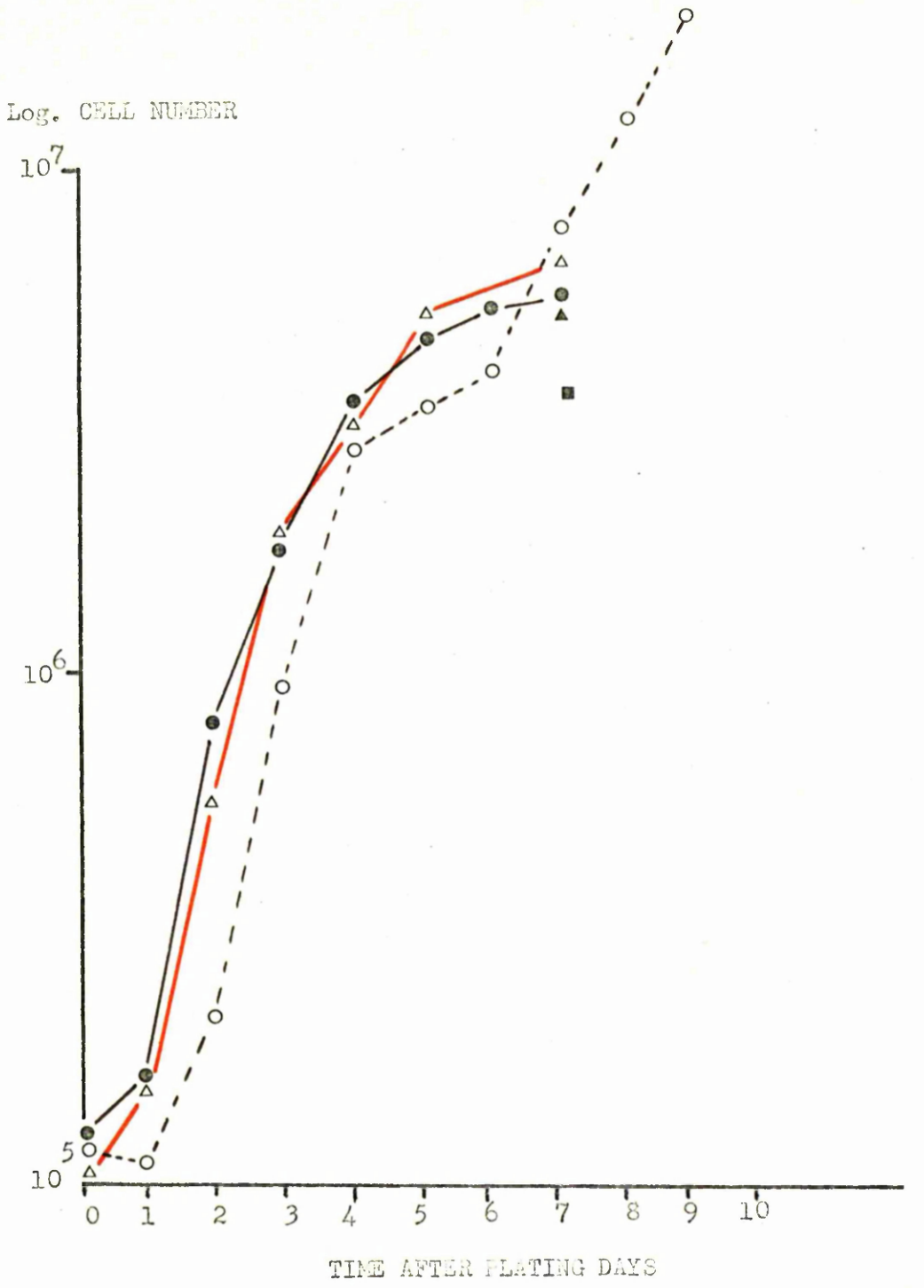


FIGURE IX. GROWTH CURVES FOR PyY and PyY AA/AAR/TG/TGR.

—●— PyY

--○-- PyY (change of medium at day 6)

■ PyY AA/AAR

▲ PyY TG/TGR

—△— PyY AA/AAR/TG/TGR

5. Testing the incorporation of nucleic acid precursors by scintillation counting

The incorporation of tritiated nucleic acid precursors supplied in the medium into nucleic acid by the wild type cells and the variants was assayed by scintillation counting. The cells to be tested were inoculated into petri dishes containing coverslips and incubated. After the cells had spread, usually 12-24 hours after plating, replicate coverslips were exposed to the labelled precursors for four hours. At the end of the period of incorporation, the cells were fixed, the TCA soluble material extracted, and the quantity of labelled material incorporated determined by scintillation counting.

Table VI shows the incorporation of purines and thymidine by clones of the variants. Pairs of clones were independently isolated by growth in agar in the presence of purine analogues. (See this Chapter, Section 1.) The clones were then grown up in liquid medium to produce cell lines. In each case, pairs of clones derived from the same variant had closely similar patterns of incorporation. PyY AA/AAR cells incorporated a reduced quantity of adenine. PyY TG/TGR cells incorporated negligible quantities of hypoxanthine and guanosine. PyY AA/AAR/TG/TGR cells incorporated negligible quantities of all purines tested. All incorporated large quantities of thymidine. One of each of these clones was continued as a stock cell line.

At intervals, checks similar to that described above were carried out on the cell lines being passaged. Table VII presents the results of one such test. The patterns of incorporation were very similar to those shown in Table VI, but the experiments are impossible to compare in detail because of differences in the concentrations and specific activities of the labelled precursors. The conditions of pulsing for the experiment shown in Table VII were as described in Chapter 4. The overall standard deviation of these counts for their means is 7.9%. The counts obtained from whole cells on coverslips were therefore reproducible enough to allow the technique to be used to investigate the defects in the abilities of the cells to incorporate purine and pyrimidine bases and nucleosides.

TABLE VI. THE INCORPORATION OF TRITIATED NUCLEIC ACID PRECURSORS BY CLONED CELLS.

Variant	CPM tritium incorporated				
	Precursor				
	A	AR	H	GR	T
PyY AA/AAR 1	275	1,608	585	4,460	29,348
	208	977	781	5,854	32,195
PyY AA/AAR 2.	223	1,004	804	4,407	18,455
	185	738	839	4,154	22,664
PyY TG/TGR 1	2,689	532	7	27	11,431
	3,003	564	1	9	7,688
PyY TG/TGR 2.	2,384	563	6	23	15,321
	2,581	562	23	30	9,282
PyY AA/AAR/TG/TGR 1	34	71	35	64	20,770
	47	65	30	85	15,810
PyY AA/AAR/TG/TGR 2	39	41	29	40	34,419
	26	42	13	40	31,187

A adenine
AR adenosine
H hypoxanthine
GR guanosine
T thymidine

TABLE VII. THE INCORPORATION OF TRITIATED NUCLEIC ACID PRECURSORS BY PyY CELLS AND VARIANTS

Variant	CPM tritium incorporated						
	Precursor						
	A	AR	H	GR	dC	T	U
PyY	29,186 23,346	57,404 45,764	20,044	11,824 11,385	13,010 12,210	25,499 26,523	28,122 27,936
PyY AA/AAR	886 834	21,369 25,698	21,437 24,150	11,312 10,609	10.709 10,169	52,680 54,825	32,025 25,556
PyY TG/TGR	20,896 12,724	776 644	236 232	248 260		17,840 17,152	6,164 7,392
PyY AA/AAR/ TG/TGR	103 96	176 211	211 215	114 109	4,048 4,153	11,360 10,368	23,568 18,085
PyY AA/AAR/ TG/TGR (R)	291 188	2,397 2,397	2,345 2,254	705 574	7,306 6,969	11,615 9,556	25,359 26,047
PyY TG/Car/ BUdR.	6,509 7,228	1,634 1,556	108 98	91 96	46 52	96 122	15,582 17,581

A adenine
AR adenosine
H hypoxanthine
GR guanosine
dC deoxycytidine
T thymidine
U uridine

TABLE VIII. NORMALIZATION OF RESULTS IN TABLE VII WITH RESPECT TO URIDINE INCORPORATION

Variant	Precursor						
	A	AR	H	GR	dC	T	U
PyY	94	184	73	40	45	93	100
PyY AA/AAR	3.2	82	79	38	37	185	100
PyY TG/TGR	250	10.5	2.9	2.6		110	100
PyY AA/AAR/ TG/TGR	0.9	1.8	2.0	1.0	39	100	100
PyY AA/AAR/ TG/TGR (R)	0.9	9.0	8.9	4.0	35	41	100
PyY TG/Car/BUdR	42	9.9	0.6	0.6	0.3	0.7	100

To allow the results to be interpreted more easily and facilitate comparison between the incorporation by different variants, each pair of counts was averaged and then normalized with respect to the uridine incorporation of that variant. Table VIII.

The PyY cells incorporated large amounts of every precursor tested. The incorporation by the variant cells will be compared to the incorporation by the PyY. PyY AA/AAR cells had a markedly reduced incorporation of adenine, and a slightly reduced incorporation of adenosine, but very similar incorporations of hypoxanthine and guanosine. PyY TG/TGR cells had an increased incorporation of adenine, a reduced incorporation of adenosine and negligible incorporation of hypoxanthine and guanosine. PyY AA/AAR/TG/TGR cells incorporated negligible quantities of all purine precursors. PyY TG/Car/BUdR cells had a slightly reduced incorporation of adenine, a somewhat reduced incorporation of adenosine, but negligible incorporation of hypoxanthine and guanosine. PyY AA/AAR/TG/TGR (R) cells had been passaged continually for five months without exposure to any analogues. In comparison with the PyY AA/AAR/TG/TGR cells there was increased incorporation of adenosine, hypoxanthine and guanosine but no increase in incorporation of adenine. The PyY AA/AAR/TG/TGR (R) cells had "reverted" in three systems, but not the fourth. All the variants were PPLO negative.

The pattern of incorporation of the PyY AA/AAR/TG/TGR (R) cells cannot be explained by contamination between the cell lines during passage. None of the cells had the ability to incorporate adenosine, hypoxanthine and guanosine, but not adenine. This pattern of reversion was found consistently in every line of PyY AA/AAR/TG/TGR cells carried for long periods without selection. A different reversion pattern was never observed.

All lines of cells not resistant to Car incorporated similar quantities of deoxycytidine. PyY TG/Car/BUdR cells incorporated negligible quantities of deoxycytidine. The incorporation of thymidine by cells not resistant to BUdR was similar in all cases. The elevated incorporation of thymidine

by the PyY AA/AAR cells and depressed incorporation by the PyY AA/AAR/TG/TGR (R) cells in this experiment were not consistent findings. PyY TG/Car/BUdR cells incorporated negligible quantities of thymidine.

These results are as predicted from the abilities of the variants to grow in the purine and pyrimidine analogues. Variants were unable to incorporate labelled nucleic acid precursors in cases where they were resistant to an analogue of the precursor. There were some unexpected findings however:

1. PyY TG/TGR cells had an increased incorporation of adenine.
2. PyY AA/AAR cells incorporate adenosine.
3. PyY TG/TGR and PyY TG/Car/BUdR do not incorporate adenosine.
4. PyY TG/Car/BUdR cells do not incorporate guanosine.

The normalization of the incorporation to that of uridine assumes that all of the variants incorporate similar quantities of uridine.

Table IX shows another experiment; PyY, PyY AA/AAR, PyY TG/TGR and PyY AA/AAR/TG/TGR cells were pulsed with adenine, adenosine, hypoxanthine, guanosine and uridine. Table X shows these results normalized to the uridine incorporation as before. The patterns of incorporation seen in Table VIII are repeated. The results in Table VIII and Table X are directly comparable since the conditions of the pulses and the specific activities of the precursors were the same. The cells behaved in a similar manner in both cases.

The quantity of protein on the coverslips was estimated. The cells were dissolved off the coverslips in hot 1 N. NaOH and protein estimations carried out by the Lowry method. The cells from four coverslips were pooled and the estimation carried out twice. The average of these values in mg/coverslip are shown in Table IX. Table XI presents the results from Table IX, but normalized to the protein estimation, this allows the uridine incorporation by the cells to be compared. There was a significant reduction in the incorporation of uridine by the PyY TG/TGR cells. The incorporation

TABLE IX. TESTING INCORPORATION OF TRITIATED NUCLEIC ACID PRECURSORS OF PyY CELLS AND VARIANTS.

Variant	COUNTS PER MINUTE INCORPORATED					
	Precursor					Protein mg/coverslip
	A	AR	H	GR	U	
PyY	18,741	26,779	13,335	8,611	16,699	0.125
	14,652	25,493	11,794	9,128	17,193	
	19,742	29,699	12,434	8,529	17,767	
Average	17,711	27,657	12,521	8,759	17,219	
PyY AA/AAR	730	19,263	8,720	6,764	15,309	0.150
	655	18,049	8,120	8,499	15,565	
	604	14,121	7,120	8,131	17,001	
Average	663	17,144	7,953	7,795	15,958	
PyY TG/TGR	9,365	454	68	100	3,099	0.130
	7,383	461	84	104	2,197	
	10,841	429	187	86	2,181	
Average	9,196	448	113	96	2,492	
PyY AA/AAR/TG/TGR	277	244	157	142	14,815	0.180
	122	254	140	104	16,376	
	189	208	199	91	9,141	
Average	196	235	165	112	13,442	

A adenine
AR adenosine
H hypoxanthine
GR guanosine
U uridine

TABLE X. NORMALIZATION OF INCORPORATION OF RESULTS IN TABLE IX WITH RESPECT TO URIDINE INCORPORATION.

Variant	Precursor				
	A	AR	H	GR	U
PyY	103	160	73	51	100
PyY AA/AAR	4.1	109	50	49	100
PyY TG/TGR	340	18	4.5	3.8	100
PyY AA/AAR/TG/TGR	1.4	1.7	1.2	1.1	100

TABLE XI. INCORPORATION OF PRECURSORS RELATED TO PROTEIN ESTIMATION

Variant	Precursor				
	A	AR	H	GR	U
PyY	14,200	22,150	10,000	7,000	13,800
PyY AA/AAR	442	11,420	5,300	5,190	10,650
PyY TG/TGR	7,070	346	87	74	2,265
PyY AA/AAR/TG/TGR	109	131	92	62	7,450

Quantity of tritiated precursor incorporated by the wild type and three variants expressed as counts per minute per 0.1 mg. protein.

TABLE XII. NORMALIZATION OF INCORPORATION OF RESULTS IN TABLE IX WITH RESPECT TO PROTEIN ESTIMATION AND INCORPORATION OF URIDINE BY THE WILD TYPE CELLS

	A	AR	H	GR	U
PyY	103	160	73	51	100
PyY AA/AAR	3.1	83	38	37	77
PyY TG/TGR	51	2.5	0.6	0.5	16.5
PyY AA/AAR/TG/TGR	0.8	0.95	1.2	0.8	54

was reduced to 16% of that of the PyY cells. The uridine incorporation of the PyY AA/AAR cells was 78% and that of the PyY AA/AAR/TG/TGR cells was 54% when compared to the wild type. The reduction of incorporation in the PyY AA/AAR cells was probably not significant. The reduction in incorporation by the PyY AA/AAR/TG/TGR cells was probably significant, but the experiment should be repeated.

The variation between the variants in their abilities to incorporate uridine makes its use as a base for normalization invalid. This affects one of the unexpected results obtained. The increase in the relative incorporation of adenine by the PyY TG/TGR when normalized to the uridine incorporation is due to a reduction in the incorporation of uridine, not an increase in the incorporation of adenine. The adenine incorporation appears, in fact, to be reduced by 50% in comparison to the PyY cells.

To enable comparisons to be made more easily, these counts/0.1 mg. protein were normalized to the uridine incorporation of the wild type cells, Table XII. There was good incorporation of all the precursors by the PyY cells. The incorporation of uridine by the PyY AA/AAR and PyY AA/AAR/TG/TGR variants was reduced slightly, but much less then that of the PyY TG/TGR. The incorporation of hypoxanthine and guanosine was as expected, the PyY AA/AAR cells continuing to incorporate high levels but the cells resistant to their analogues, PyY TG/TGR and PyY AA/AAR/TG/TGR incorporating 1% or less in comparison to the wild type. The incorporation of adenine and adenosine by the PyY AA/AAR/TG/TGR variant was reduced to less than 1% in each case, but their incorporation by the PyY AA/AAR and PyY TG/TGR variants was not quite as expected. The incorporation of adenine by the PyY AA/AAR cells was reduced to 3%, that of adenosine was reduced by 50% when compared to the PyY cells. The incorporation of adenine by the PyY TG/TGR variant was reduced to 51% and that of adenosine to 2.5%.

Experiments are in progress to assay the activities of the enzymes involved in the purine and pyrimidine salvage pathways of these cells using

cell extracts. It will be interesting to see if there is a correlation between enzyme activity in vitro and the incorporation of precursor by intact cells. This part of the investigation is being carried out in collaboration with A. Jamieson.

6. The incorporation of radioactive precursors determined by autoradiography

In addition to scintillation counting, the incorporation of radioactively labelled purine and pyrimidine precursors by the variant cells was detected by autoradiography. Figures IX'-XIII. The variants were pulsed with tritiated precursor for 13 hours, processed for autoradiography, and exposed to stripping film for 19 days.

Figure IX' demonstrates the incorporation after pulsing with adenine. PyY AA/AAR and PyY AA/AAR/TG/TGR cells are unlabelled while PyY TG/TGR and PyY TG/Car/BUdR cells are heavily labelled.

Figure X shows the incorporation after a pulse with adenosine. PyY AA/AAR, PyY TG/TGR and PyY TG/Car/BUdR cells are lightly labelled, PyY AA/AAR/TG/TGR are unlabelled.

Figure XI shows the incorporation of hypoxanthine. PyY TG/TGR, PyY AA/AAR/TG/TGR and PyY TG/Car/BUdR cells are unlabelled, PyY AA/AAR are heavily labelled. The same pattern of labelling is also present after pulsing with guanosine, Figure XII.

Figure XIII demonstrated the incorporation of both deoxycytidine and thymidine by PyY AA/AAR which are heavily labelled. PyY TG/Car/BUdR are very lightly labelled in both cases.

PyY cells are heavily labelled by all these precursors.

PyY AA/AAR, PyY TG/TGR and PyY TG/Car/BUdR variants are all of similar morphology and size. They are mainly large fibroblastic cells but some epitheloid cells are also present. The PyY AA/AAR/TG/TGR cells are generally smaller and very few cells are seen that do not have a fibroblastic morphology.

The PyY TG/Car/BUdR cells commonly had a "bubbly" appearance. The bubbles protruded from the cell surface and stained heavily with Giemsa. Their nature is at present not understood. Filtered medium from confluent cultures of PyY TG/Car/BUdR cells failed to produce tumours after injection into newborn hamsters.

This experiment was carried out independently of those previously discussed in which the incorporation was assayed by scintillation counting. The agreement between the patterns of incorporation determined by scintillation counting and autoradiography was good in all cases.

FIGURE IX'

Adenine incorporation by BHK 21/C13/PyY cell variants

a) PyY AA/AAR

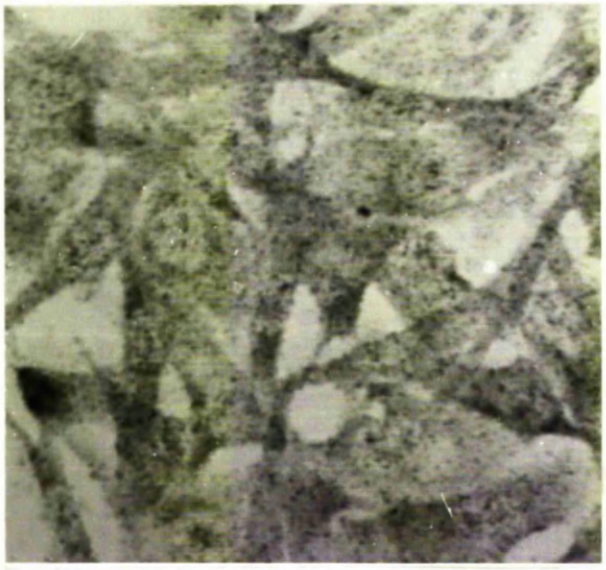
b) PyY TG/TGR

c) PyY AA/AAR/TG/TGR

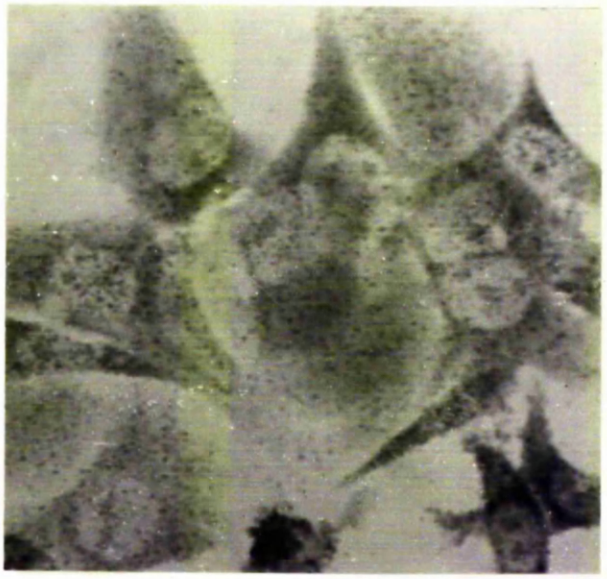
d) PyY TG/Car/BUdR

FIGURE X

Adenosine incorporation by BHK 21/C13/PyY cell variants.

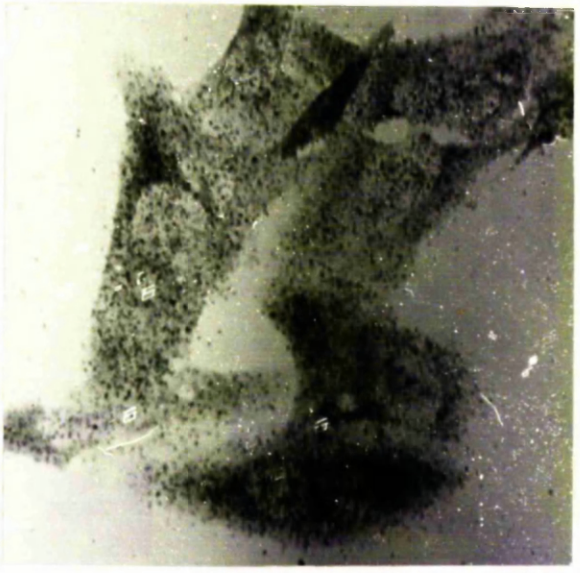


a) PyY AA/AAR



b) PyY TG/TGR

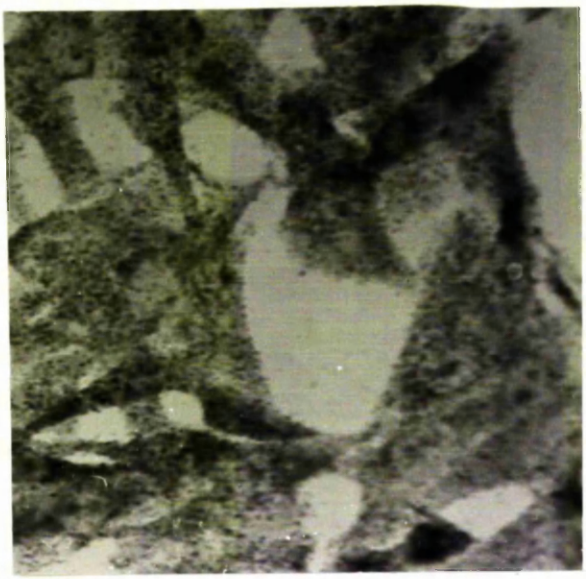
c) PyY AA/AAR/TG/TGR



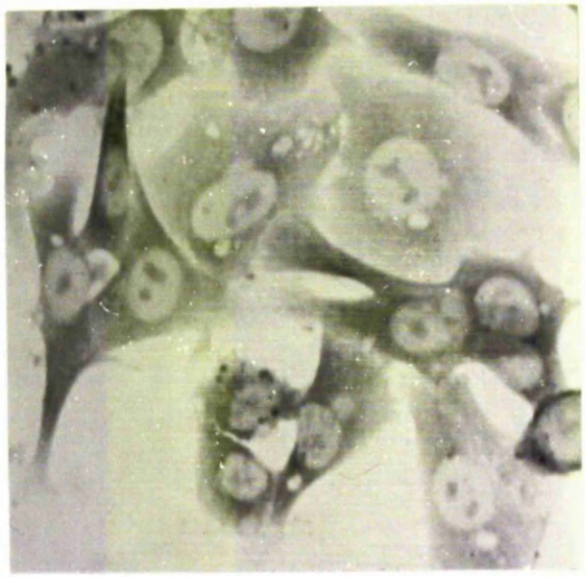
d) PyY TG/Car/BUdR

FIGURE XI

Hypoxanthine incorporation by BHK 21/Cl3/PyY cell variants

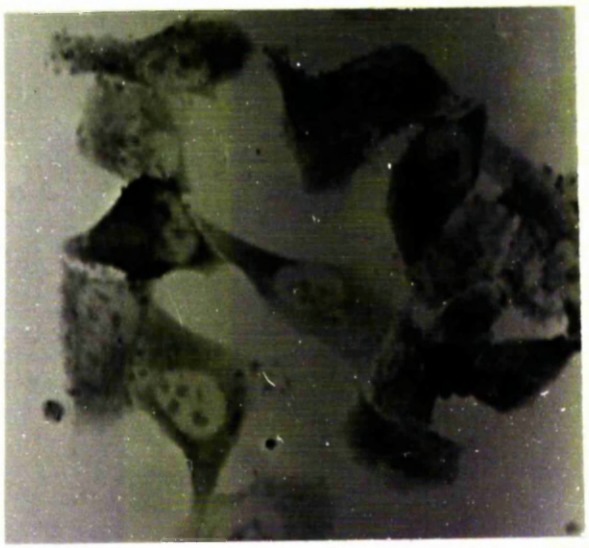


a) PyY AA/AAR



b) PyY TG/TGR

c) PyY AA/AAR/TG/TGR

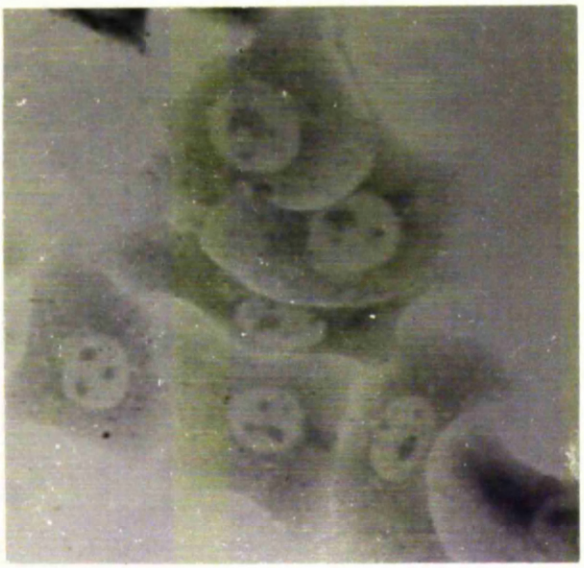


d) PyY TG/Car/BUdR

FIGURE XII

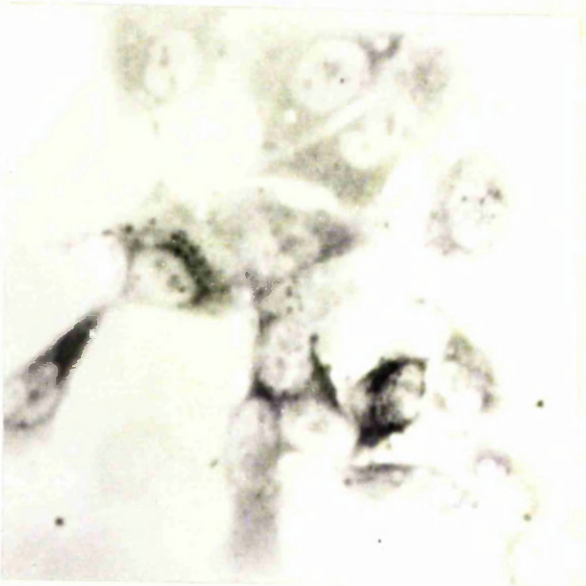
Guanosine incorporation by BHK 21/C13/PyY cell variants.

a) PyY AA/AAR



b) PyY TG/TGR

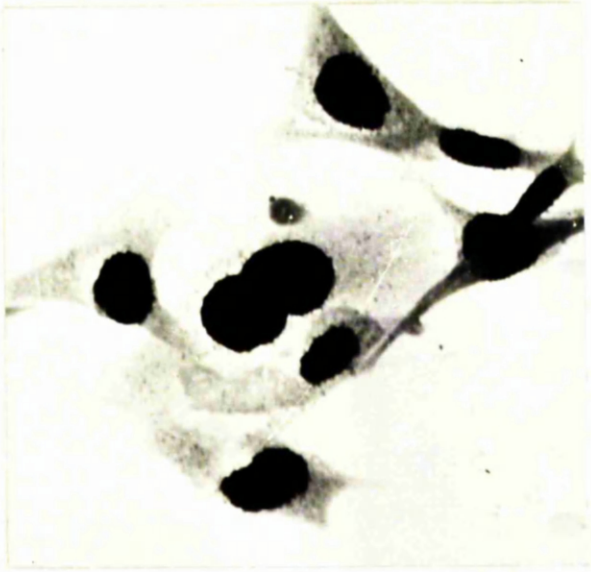
c) PyY AA/AAR/TG/TGR



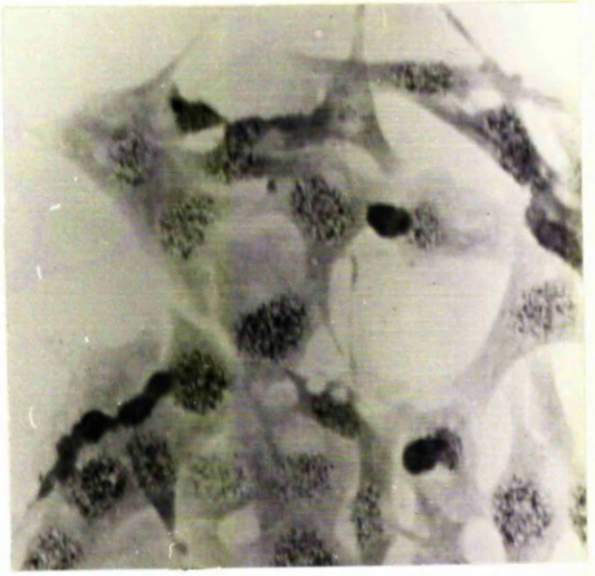
d) PyY TG/Car/BUdR

FIGURE XIII

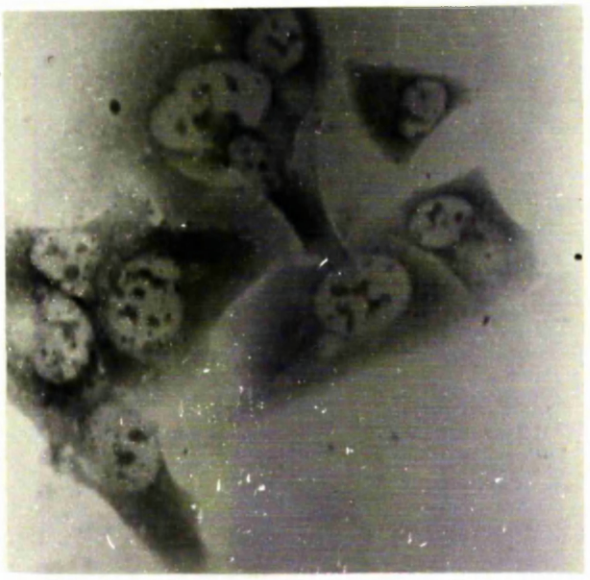
Deoxycytidine and thymidine incorporation by BHK 21/C13/PyY cell variants.



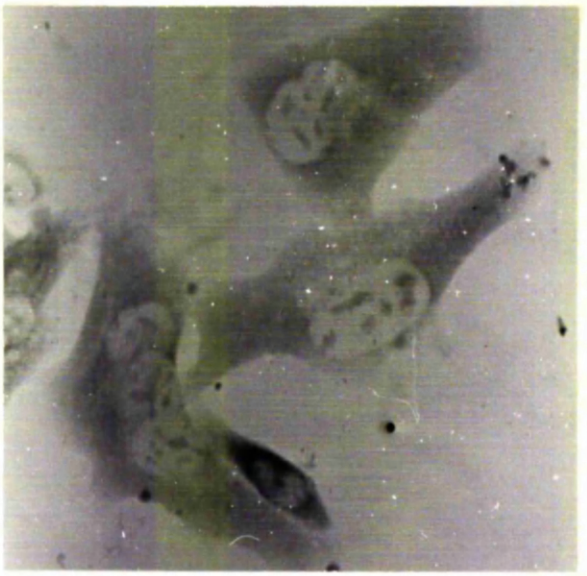
a) PyY AA/AAR pulsed with thymidine



b) PyY AA/AAR pulsed with deoxycytidine



c) PyY TG/Car/BUdR pulsed with thymidine



d) PyY TG/Car/BUdR pulsed with deoxycytidine.

6. RESULTS II. DOUBLE LABEL AUTORADIOGRAPHY USED AS A MARKER TO DISTINGUISH BETWEEN TWO VARIANT CELLS IN A MIXTURE

Metabolic co-operation was first described by Subak-Sharpe, Bürk and Pitts (1966) occurring between PyY TG/TGR cells that were unable to incorporate hypoxanthine, and wild type cells able to incorporate hypoxanthine. The phenotype of the PyY TG/TGR cells in contact with the incorporating cells was altered. The PyY TG/TGR cells became able to incorporate label presented as preformed hypoxanthine into their nucleic acid. The phenomenon was confined to those cells that could be seen to be in contact when using an optical microscope. PyY TG/TGR cells close to, but not actually in contact with incorporating cells retained their variant phenotype, so the possibility of complementation by the exchange of metabolic intermediates via the medium was eliminated. When fibroblasts derived from patients with the Hunter and Hurler syndromes were co-cultivated, there was mutual correction of their defects. The exchange of metabolic intermediates between the cells took place via the medium and the cells did not have to be in contact for their defects to be corrected (Fratantoni, 1968).

The initial observations on metabolic co-operation were confirmed and extended (Bürk, Pitts and Subak-Sharpe, 1968; Subak-Sharpe, Bürk and Pitts, 1969 and Subak-Sharpe, 1969). All the cells were shown to be potentially capable of metabolic co-operation and the phenomenon could also be demonstrated with another purine salvage pathway. When in contact with PyY TG/TGR cells, PyY AA/AAR cells became able to incorporate adenine. A donor-recipient relationship is implicit in all instances of metabolic co-operation. The cell normally capable of incorporating the precursor being the donor and the variant normally incapable of incorporation being the recipient. Since metabolic co-operation was demonstrated between PyY AA/AAR and PyY TG/TGR cells using both adenine and hypoxanthine, one type of cell may either act as the donor or the recipient depending on the precursor available.

Using fibroblasts derived from a patient suffering from the Lesch-Nyhan syndrome and therefore lacking the enzyme IrI, Friedman, Seegmiller and Subak-Sharpe (1968) and later Cox, Krauss, Earl Balis and Dancis, (1970), demonstrated that metabolic co-operation took place between mutant cells and normal human fibroblasts. When Lesch-Nyhan cells were grown in contact with normal human fibroblasts, which are able to incorporate hypoxanthine, their defect was corrected and they became able to incorporate hypoxanthine into their nucleic acid. Thus metabolic co-operation is not a unique property of the PyY laboratory variants. It has been shown to occur between cells of heterologous species, for example the hamster and rat or mouse, so is not species specific. The capability of taking part in metabolic co-operation is not universal to all cell types. Mouse L cells are unable to co-operate either with each other, or with BHK 21/C13/PyY Cells (Pitts, 1971).

One of the problems in conducting experiments on metabolic co-operation is that of distinguishing between the two types of cell after they have been mixed together. Morphological criteria are of limited reliability, in the initial experiments different proportions of the two cell types were used, and the proof depended on stastical considerations. Stoker (1967) using carbon and carmine particles to prelabel the cells, demonstrated that the defect of the PyY TG/TGR cells was corrected on contact with freshly isolated mouse fibroblasts. The marking technique did not allow the unambiguous identification of individual cells. It was found impossible to remove all the material adhering to the outside of the cells. This remained available for phagocytosis by the other cells after mixing, so criteria were chosen to distinguish the cells on a stastical basis.

In my own work I attempted to repeat these experiments using PyY AA/AAR cells prelabelled with carbon granules and PyY TG/TGR cells prelabelled with carmine granules. Many of the granules were phagocytosed by the cells

and concentrated in a ring around the nucleus. More than 95% of the cells became labelled after one day in the presence of the granules. The system was found to be limited by transfer of particles between the cells, so a double-label autoradiographic technique was developed in an attempt to distinguish the cells with more certainty.

A preparation labelled only with 14-C thymidine was autoradiographed to demonstrate the degree of labelling in the first layer. Figure XIV shows such a preparation. There are very few grains in the AR 10 layer over the cells. However in the G 5 layer there is a very heavy labelling over the cells, showing that good differential labelling between the layers had been achieved. It can also be seen that the grains due to the 14-C extend far beyond the boundary of the cells from which the B particles originated.

A good comparison of the resolution of autoradiography using 3-H and 14-C is obtained from Figure XV. The autoradiograph is of a preparation containing cells pulsed with 14-C thymidine for twenty-four hours before plating to petri dishes containing coverslips. Then after the cells had spread, the culture was pulsed for four hours with 3-H thymidine. The grains in the lower layer due to tritium are confined to the nucleus, those in the upper layer due to the carbon 14 spread far beyond the cell limits. The source of these tracks was also the nucleus of the cell. All the cells in the preparation were labelled with 14-C thymidine. The presence of grains in one layer is independent of grains in the other, confirming the previous observation on 14-C only labelled material and extending it to show that tritium had no effect on the second layer of film. It is also a good demonstration of the difference in resolving power of autoradiography using 14-C labelled and 3-H labelled material. The photographs were taken in different focal planes and distinguish between the grains in the two layers well.

The technique was next applied to cell mixtures. In an effort to reduce the spread of grains in the G 5 layer the emulsion was made thinner and the exposure time shorter.

FIGURE XIV

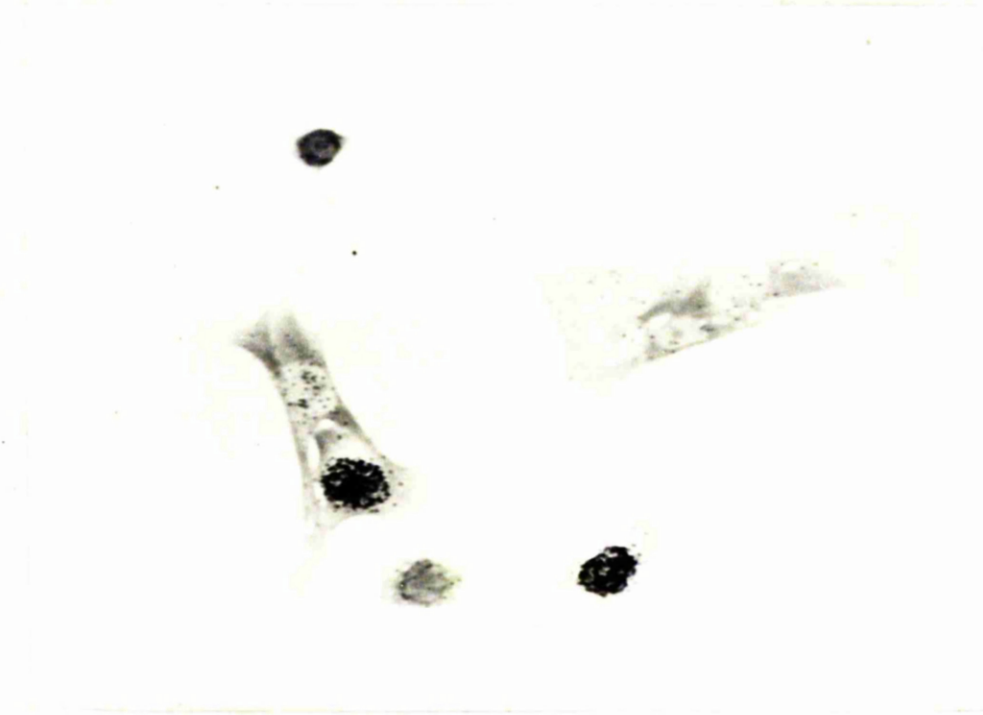
PyY AA/AAR cells labelled with 14-C thymidine for 24 hours.

AR 10

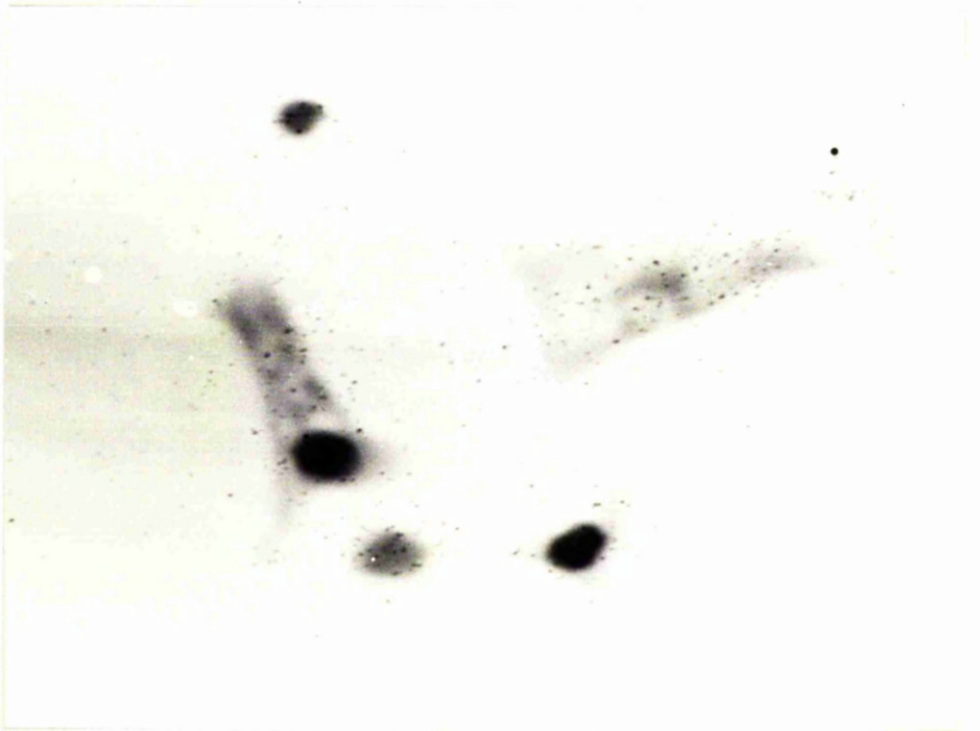
G 5

FIGURE XV

PyY AA/AAR cells labelled with 14-C thymidine for 24 hours before plating and 4 hours with 3-H thymidine after they were fully spread.



AR 10



G 5

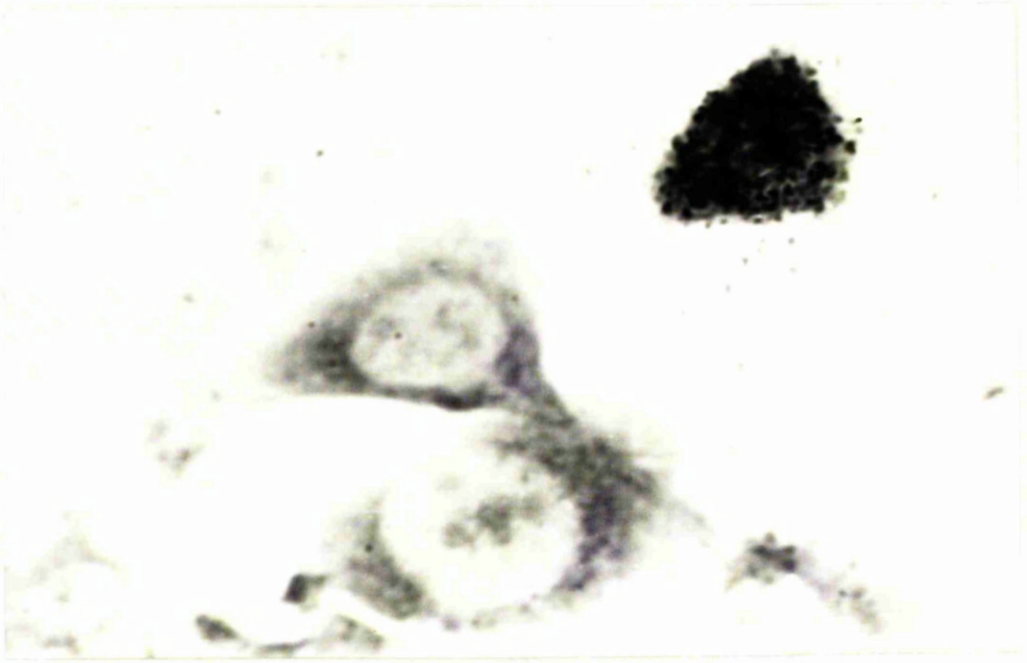
Autoradiographs were prepared from preparations of mixtures of PyY AA/AAR and PyY TG/TGR cells in which one of the variants had been prelabelled with 14-C thymidine. After the cells had been mixed and allowed to spread they were pulsed with either tritiated adenine or hypoxanthine. Figure XVI shows a clump of unlabelled cells and a solitary labelled cell. Since the preparation had been pulsed with hypoxanthine the unlabelled cells are PyY TG/TGR cells and the labelled cell is a PyY AA/AAR cell. This interpretation can be confirmed by examining the photograph taken in the focal plane of the second emulsion. Only the solitary cell is labelled and so identifiable as a PyY AA/AAR cell since these were prelabelled with 14-C thymidine. Figure XVII shows the converse situation in which the PyY TG/TGR cells were prelabelled and the preparation pulsed with adenine. The same result was obtained. It confirms that metabolic co-operation does not ~~take~~ occur when the cells are not in contact.

When cells from the same preparations were examined where there was contact between the two variants metabolic co-operation was demonstrable. Figure XVIII shows such a situation where the PyY TG/TGR cells were prelabelled with 14-C thymidine and the mixture pulsed with adenine. Figure XIX shows a situation where the PyY AA/AAR cells were prelabelled with 14-thymidine and the mixture pulsed with hypoxanthine. In both cases, cells in contact with the 14-C labelled cell which are not themselves labelled with 14-C, have incorporated the tritiated precursor. Non-14-C labelled cells in the same preparations not in contact with 14-C labelled cells are unlabelled. The incorporation of the tritiated purine precursors by the non-14-C labelled cells in contact with the 14-C labelled cells is therefore due to metabolic co-operation.

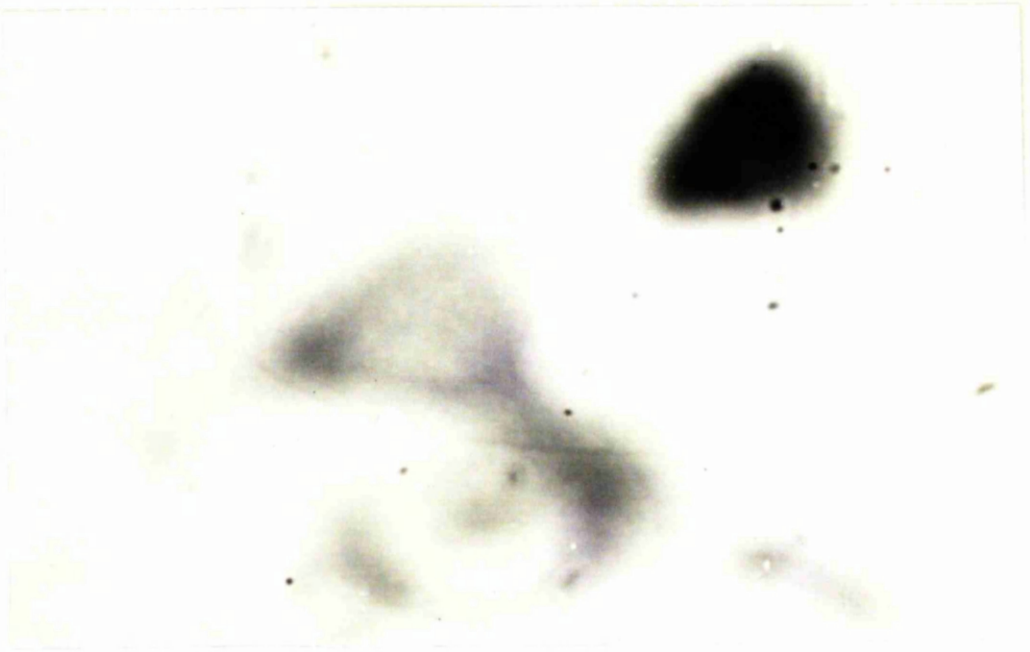
The double label technique provides a visual demonstration of metabolic co-operation between PyY AA/AAR and PyY TG/TGR cells using both adenine and hypoxanthine as precursors. Both variants can act either as the donor or the recipient depending on the precursor provided.

FIGURE XVI

PyY AA/AAR cells prelabelled with 14-C thymidine then resuspended and mixed with PyY TG/TGR cells. After the cells had spread the preparation was pulsed with tritiated hypoxanthine.



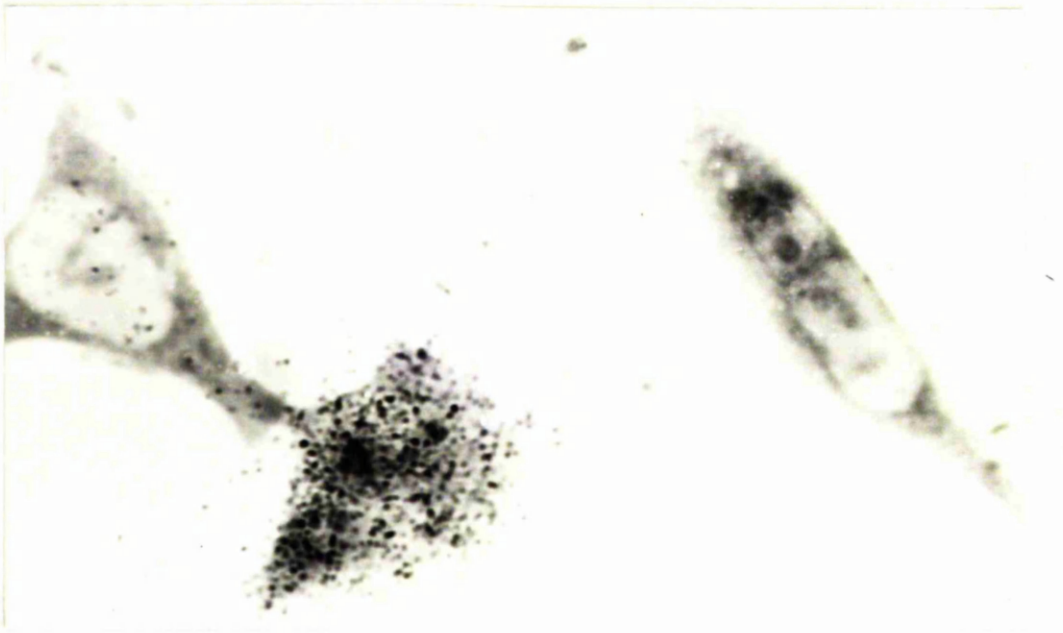
AR 10 only the solitary cell is labelled



G 5 only the solitary cell is labelled and therefore a a PyY AA/AAR cell. The other is a PyY TG/TGR cell.

FIGURE XVII

PyY TG/TGR cells prelabelled with 14-C thymidine then resuspended and mixed with PyY AA/AAR cells. After the cells had spread, the preparation was pulsed with tritiated adenine.

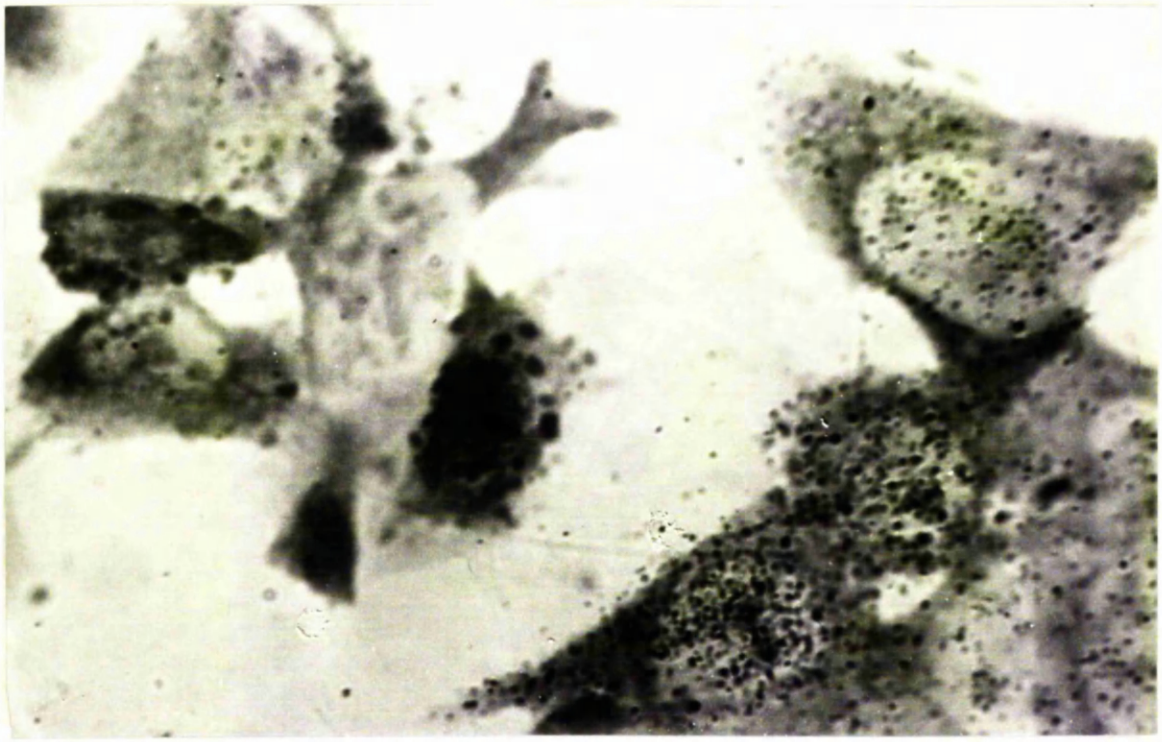


AR 10 one heavily labelled cell in contact with a lightly labelled cell. The solitary cell is unlabelled.

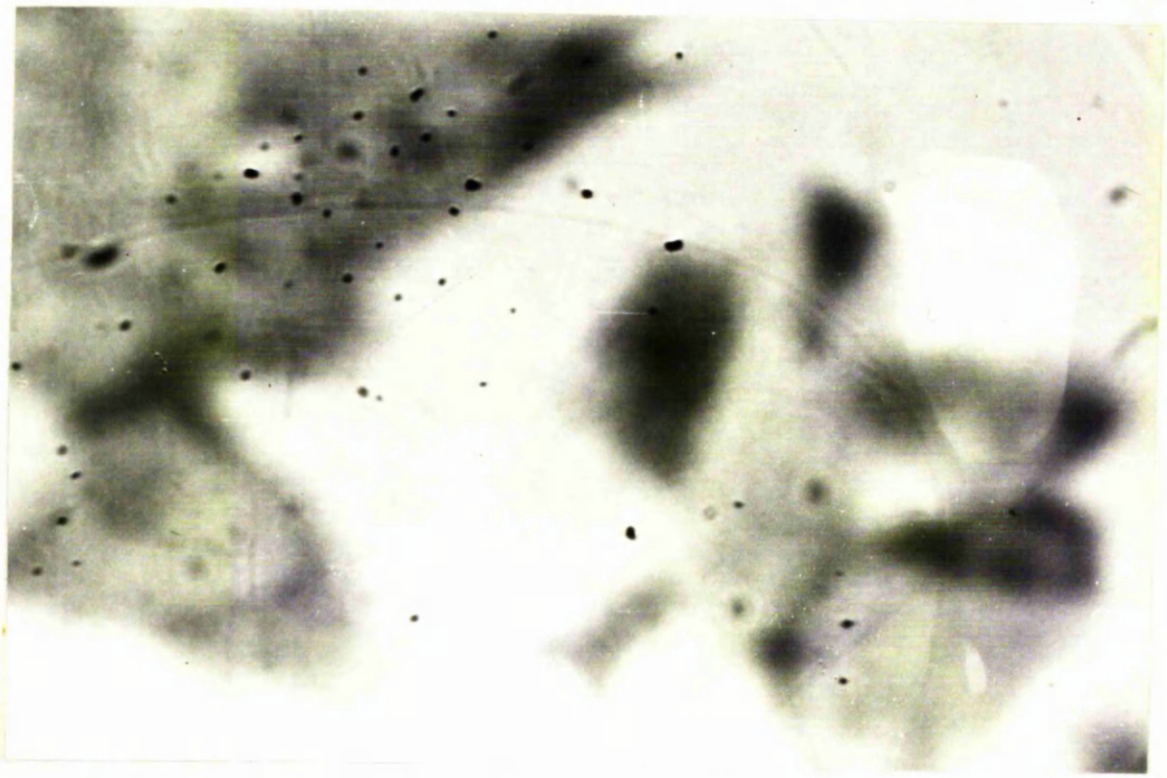
G 5 labelling only over the cell that incorporated a large amount of adenine which is therefore a PyY TG/TGR cell. The other cells are unlabelled and are PyY AA/AAR cells. The solitary one retains its phenotype, the other which is in contact with the PyY TG/TGR cell has incorporated adenine due to metabolic co-operation.

FIGURE XVIII

PyY TG/TGR cells prelabelled with 14-C thymidine then resuspended and mixed with PyY AA/AAR cells. After the cells had spread, the preparation was pulsed with tritiated adenine.



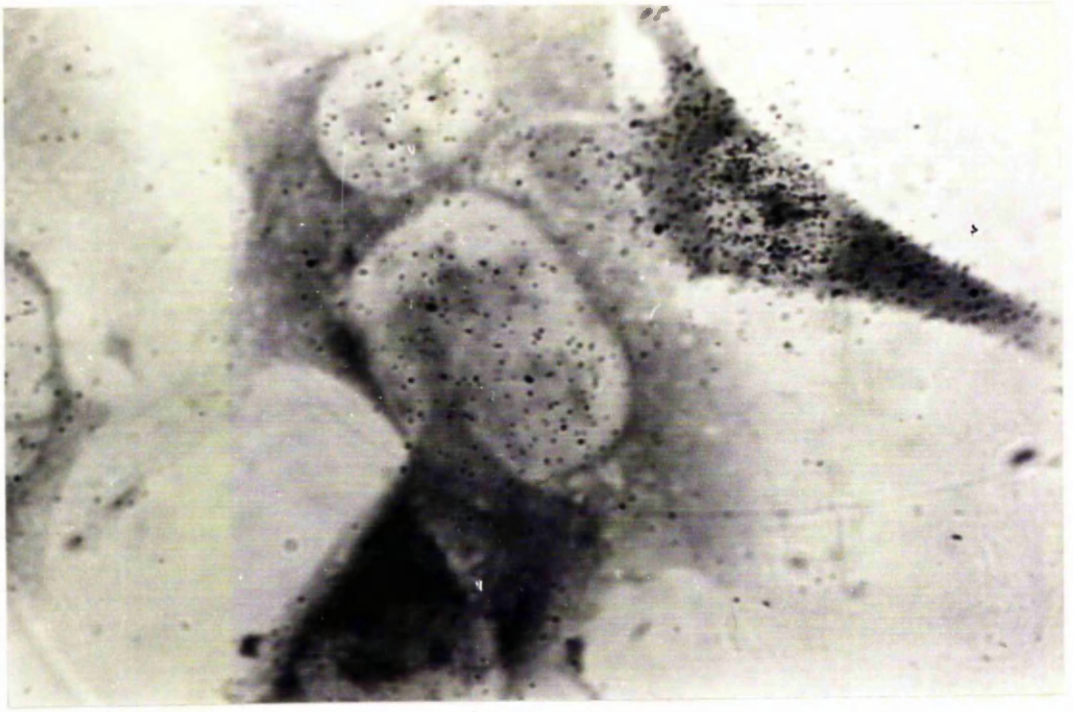
AR 10 two heavily labelled cells in contact with four lightly labelled cells. Five unlabelled cells also shown.



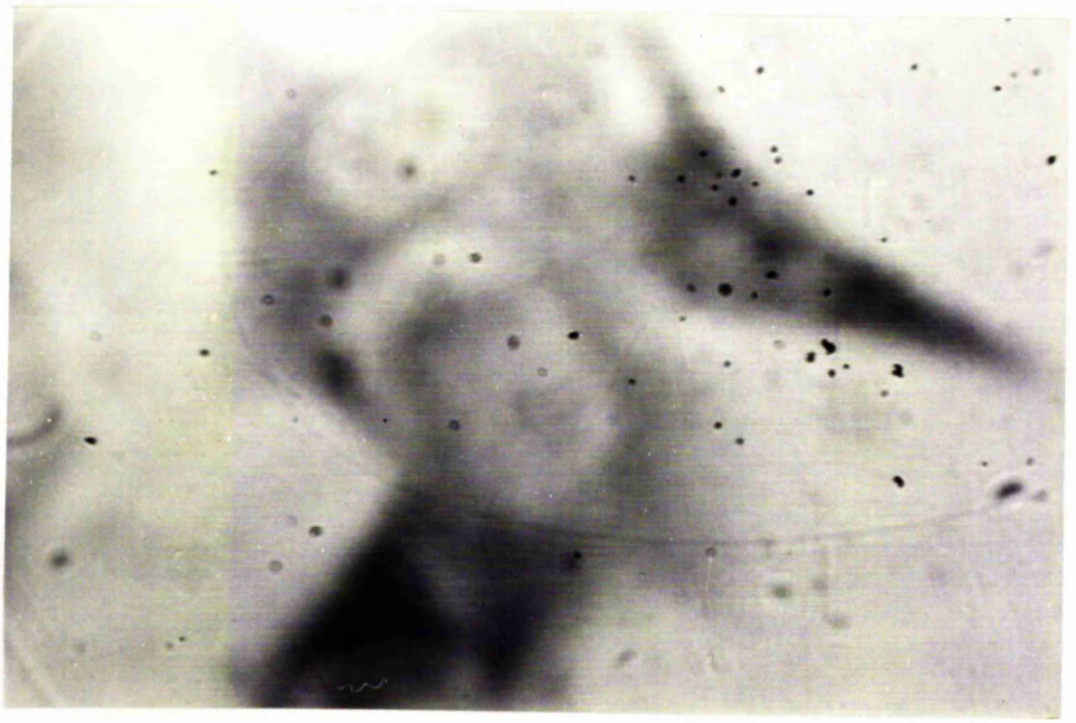
G 5 only the two cells heavily labelled by adenine are labelled and therefore PyY TG/TGR cells. All the other cells are PyY AA/AAR cells, those in contact with the PyY TG/TGR cells incorporated adenine because of metabolic co-operation.

FIGURE XIX

PyY AA/AAR cells prelabelled with 14-C thymidine and then resuspended and mixed with PyY TG/TGR cells. After the cells had spread, the preparation was pulsed with tritiated hypoxanthine.



AR 10 one heavily labelled cell in contact with two lightly labelled cells.



G 5 labelling only over the cell that incorporated a large amount of hypoxanthine which is therefore a PyY AA/AAR cell. The others are PyY TG/TGR cells that incorporated hypoxanthine by virtue of metabolic co-operation.

These experiments therefore confirm the previous work on metabolic co-operation but with individually identifiable cells.

7. RESULTS III. SELECTIVE CONDITIONS APPLIED TO CELL MIXTURES

The IPP gene in man is X-linked and fibroblasts from females carrying the recessive allele for the Lesch-Nyhan syndrome are of two types, as expected from the Lyon hypothesis (Lyon, 1961). Cells in which the X-chromosome carrying the normal allele is active have normal levels of IPP activity, and those in which the X chromosome carrying the defective allele is active lack IPP activity (Rosenbloom, Kelley, Henderson and Seegmiller, 1967). Individuals expressing the Lesch-Nyhan syndrome are males whose X chromosome carries a defective allele for IPP. The complete syndrome has been described earlier (Chapter 1), it is untreatable and is associated with severe mental retardation. No female homozygous for the defective allele has been reported as affected males are unable to reproduce. A programme for the control of the Lesch-Nyhan syndrome is possible by prenatal diagnosis and therapeutic abortion of affected foetuses. Foetal cells may be obtained by amniocentesis and assayed for IPP activity by incubating with radioactive hypoxanthine and autoradiographing. Cells from affected individuals are unable to incorporate hypoxanthine (Fujimoto, Seegmiller, Uhlendorf and Jacobson, 1968; DeMars, Sarto, Felix and Benke, 1969, and Boyle, Raivio, Astrin, Schulman, Graf, Seegmiller and Jacobson, 1970).

It is also possible to determine which female relatives of an affected individual are carriers of an allele for the Lesch-Nyhan syndrome and so at risk for giving birth to an affected individual (Rosenbloom et al. 1967). A fibroblast biopsy may be taken and the cells autoradiographed after pulsing with tritiated hypoxanthine. However, the proportion of cells capable of incorporating hypoxanthine varies between individuals and also between replicate cultures from the same individual. In one example an obligate heterozygote had only 5% of cells able to incorporate hypoxanthine (Fujimoto and Seegmiller, 1970).

The variability may be due to several factors and is not at present fully understood. The inactivation of the X chromosome may be random but an early event in development. A heterozygous individual would therefore be composed of large clones of normal and defective cells and serial biopsies from the same individual could contain different proportions of normal and defective cells. The X inactivation may not be random, one chromosome may be preferentially inactivated in some cases. The X inactivation may be random, but after inactivation there may be selection in favour of cells having a particular X chromosome active. Another complication is that of metabolic co-operation. Under culture conditions in which the majority of cells touched, the genotype of the mutant cells was obscured by metabolic co-operation (Friedman, Seegmiller and Subak-Sharpe, 1968). The ratio of wild type to mutant was thus to some extent a function of the plating density. Metabolic co-operation can cause confusion if its presence is not appreciated (Albertini and DeMars, 1970). It also explains why some groups have only been able to demonstrate the mixed phenotype of the heterozygote by cloning techniques (Migeon, der Kaloustian, Nyhan, Young and Childs, 1968, and Salzman, DeMars and Banke, 1968). They have been working with dense cultures of cells. The ability to select for phenotypically normal and mutant cells in cultures from heterozygous individuals would make diagnosis easier.

Fujimoto, Subak-Sharpe and Seegmiller (1971) demonstrated the feasibility of applying selective conditions to cultures from heterozygotes, and showed that metabolic co-operation affected the outcome of selection. Metabolic co-operation results in an alteration in the phenotype of the IPP deficient cells. They either gain the ability to incorporate hypoxanthine, guanine, or their analogues into nucleotides, or receive nucleotides formed by the wild type cells (See Chapter 8). In the presence of azaserine IPP deficient cells were killed if the sole purine source was hypoxanthine. (Azaserine, ~~like folic acid antagonists,~~ blocks a step in the de novo synthesis

of purines and converts normal cells to the equivalent of purine-requiring auxotrophs.) Through metabolic contact with the wild type cells, the deficient cells received "the kiss of life" and survived under these conditions. In the presence of thioguanine the IPP deficient cells survived if grown alone, but those in contact with the wild type incorporated the analogue and were killed. Under these conditions the effects of metabolic co-operation has been described as "the kiss of death".

The availability of biochemically marked variants PyY AA/AAR and PyY TG/TGR allowed selection to be studied using conditions which selected for the growth or death of cells through the presence or absence of APP or IPP activity. Selective systems were potentially available to select for survival or death of either of the variants in a mixed culture. These studies are complementary to and extend those described above using primary human cells where only selective systems depending on the presence or absence of IPP could be used (Fujimoto et al. 1971).

To set up the experiment 1×10^5 of the following cells were inoculated each to five baby bottles: PyY AA/AAR, PyY TG/TGR and PyY. A set of five baby bottles was also set up using a mixture of 5×10^4 each of PyY AA/AAR and PyY TG/TGR. This was analagous to the situation in some biopsies from a carrier of the Lesch-Nyhan syndrome, half the cells being IPP deficient but in addition allowed the simultaneous study of a system lacking APP. After two days growth, HAT and AAT were added at the optimal concentrations described previously, each to one separate complete set of bottles. On the fourth day, 8-azadenine 1000 µg/ml. (AA) and 6-thioguanine 100 µg/ml. (TG) were added, each to a further complete set of bottles. In two of the experiments shown AA 100 µg/ml. and TG 10 µg/ml. were used, the exposure being for 5 days. One complete set of bottles was left unrestricted as a control. After one week, the cells were trypsinized off the glass, suspended in growth medium, washed and resuspended in a known volume of PBS. The concentration of cells was then determined using either a haemocytometer

chamber or a Coulter counter.

Table XIII shows the results of four such experiments. The numbers of PyY cells produced in the control, AAT and HAT were similar, indicating that these conditions allowed a normal growth of the wild type cells. The numbers of PyY cells produced after exposure to AA and TG were dramatically reduced.

The application of the selective conditions to the variants alone produced the expected results. PyY AA/AAR cells grew in AA or HAT, but not in TG or AAT. Conversely, PyY TG/TGR cells grew in TG or AAT, but not in AA or HAT. The PyY AA/AAR surviving in TG and PyY TG/TGR surviving in AA would have died had the experiment been continued. In long-term experiments cells grew neither from 10^5 PyY AA/AAR in 10 µg/ml. of TG nor from 10^5 PyY TG/TGR in 100 µg/ml. of AA (see Chapter 5, Section 2). The "survivors" referred to above had incorporated lethal quantities of the analogues and were in a moribund state, but not actually dead.

The variants generally produced nearly as many cells under those selective conditions allowing growth as under no restrictive conditions. However, the AAT system reduced the number of cells produced, this was particularly marked with the PyY TG/TGR cells alone. As previously mentioned, the concentration of adenine used in AAT was only just below the level at which inhibitory effects were produced, which is probably the main reason for this observation. To enable comparisons to be made more easily, the numbers of cells obtained under the selective conditions are expressed as a percentage of those obtained under non-restricted conditions, Figure XX.

The numbers of cells obtained from the bottles containing a 50:50 mixture of the two variants were similar under all conditions. This would be predicted from the behaviour of the cells when grown independently. PyY AA/AAR cells continued to grow in AA and HAT, and PyY TG/TGR cells

TABLE XIII. THE NUMBER OF CELLS PRODUCED AFTER GROWTH OF THE VARIANTS UNDER A VARIETY OF SELECTIVE CONDITIONS.

1×10^5 cells plated per baby bottle. AAT and HAT selection from day 2. 8-azaadenine and 6-thioguanine selection from day 4. Read at day 7.

Cell	Experiment Number	Cell number $\times 10^6$				
		Control	AA	TG	HAT	AAT
PyY	4	8.4	0.08	0.06	7.2	5.1
	3	7.3	0.1	0.35	8.0	5.0
PyY AA/AAR	4	6.9	2.3	0.29	6.4	0.64
	3	4.3	3.4	0.1	5.8	0.3
	2	5.4	4.0*	0.5+	5.1	0.2
	1	7.8	7.6*	0.02+	8.0	0.04
PyY TG/TGR	4	2.8	0.06	1.0	0.09	0.87
	3	8.0	0.8	6.9	0.25	4.6
	2	4.8	0.05*	2.6+	0.05	1.3
	1	1.4	0.03*	0.6+	0.02	0.5
PyY AA/AAR and PyY TG/TGR	4	6.9	5.5	2.9	7.9	3.5
	3	3.5	2.5	1.1	3.1	3.2
	2	7.5	4.7*	4.2+	1.5	6.1
	1	2.4	5.1*	2.5+	7.7	2.2

AA 1000 µg/ml. (except * where 100 µg/ml. added at day 2)
TG 100 µg/ml. (except + where 10 µg/ml. added at day 2)
Four separate determinations apart from PyY where only two are shown.

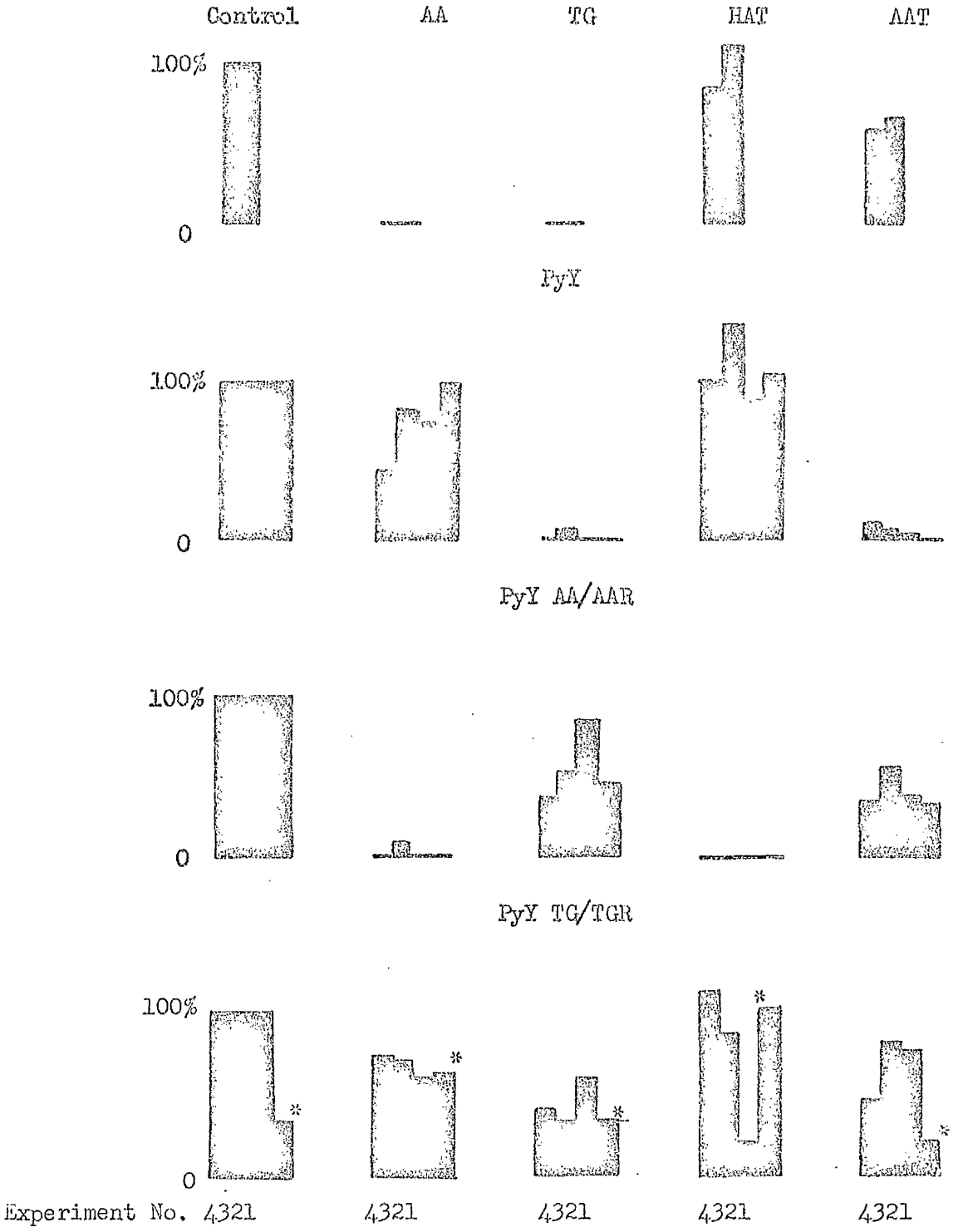


FIGURE XX. THE NUMBER OF CELLS OBTAINED UNDER SELECTIVE CONDITIONS EXPRESSED AS A PERCENTAGE OF THAT OF THE CONTROL.

*Expressed as a percentage of the cell number in HAT medium.

continued to grow in TG and AAT. However since metabolic co-operation takes place between these cells "the kiss of death" would be expected to reduce the cell number produced in the presence of AA and TG. Such a reduction was not apparent, despite the fact that only half the number of cells of each variant were seeded as the mixture than when seeded alone. A similar number of cells was produced by the cell mixture and PyY AA/AAR alone in AA, and also similar numbers were obtained from the cell mixture and PyY TG/TGR alone in TG.

The selective conditions were modified in an attempt to enhance any possible affects of metabolic co-operation. In the first two experiments AA 100 µg/ml. and TG 10 µg/ml. were added at day 2. It was thought probable that the selective conditions were being applied before sufficient growth had taken place for all the cells to be in contact, so metabolic co-operation could not affect all the resistant cells.

In the two later experiments the cultures were allowed to become semiconfluent before the addition of the inhibitors. Higher concentrations were used, AA 1000 µg/ml. and TG 100 µg/ml. were added at day 4. The modification to the selective conditions did not significantly affect the cell numbers produced. It is probable that at the higher levels of inhibitors overkill occurred. The majority of the sensitive cells were killed too quickly to allow enough of the analogues to become incorporated into the resistant cells by metabolic co-operation to kill them. The resistant cells then continued to grow and formed a confluent sheet.

The numbers of cells produced by the mixed culture after growth in HAT and AAT approached that in the unrestricted bottles of mixed cells in many cases. The numbers produced were very similar to the numbers of PyY AA/AAR in HAT and PyY TG/TGR in AAT. The phenotype of the cells growing in the mixture was determined in order to ascertain if "the kiss of life" had taken place.

The proportions of the two variants present in the mixed culture after selection was determined in two ways, by cloning in the presence of inhibitors and by autoradiography. Table XIV shows the numbers of clones produced by the cells of experiment 1 after growth in agar either without restriction, in AA 100 µg/ml. or in TG 10 µg/ml. The cells were counted and in cases where significant numbers were present, cloned in agar. 500 cells were inoculated into each dish and incubated for two weeks. The numbers of clones present in each dish was then scored visually, each estimate being the average of two dishes. The cloning efficiency of the PyY AA/AAR cells was between 5% and 25%, that of the PyY TG/TGR cells was only 2% to 4%.

When cloned in the absence of inhibitor, both variants were free to grow. In the presence of AA 100 µg/ml., only PyY AA/AAR cells grew, and in TG 10 µg/ml. only PyY TG/TGR cells grew. It was hoped that for each cell mixture the sum of the numbers of clones produced in the inhibitors would equal the number that grew under nonselective conditions, allowing some quantitation. This was not the case, indeed the PyY AA/AAR cells produced more clones in the presence of AA than in the absence of inhibitor. The numbers of PyY TG/TGR clones produced were too small to be statistically significant. A qualitative analysis can be made however. No PyY TG/TGR cells were present in the mixed bottle after selection in AA, and no PyY AA/AAR cells were present after selection in TG. After growth in HAT and AAT, the mixture retained both types of cell demonstrating that "the kiss of life" had occurred.

The proportions of cells present were also determined by autoradiography. Samples of the cells from each bottle were plated at low density to petri dishes containing coverslips. The cells were allowed to spread, pulsed with either tritiated adenine or hypoxanthine and processed for autoradiography. The numbers of labelled and unlabelled cells on each of the coverslips were counted. To eliminate metabolic co-operation only solitary cells were scored. Table XV shows the results obtained using the cells obtained

TABLE XIV. THE PHENOTYPES OF CELLS PRESENT IN THE MIXTURE OF PyY AA/AAR CELLS AND PyY TG/TGR CELLS AFTER DIFFERENT SELECTION PROCEDURES AS DETERMINED BY CLONING IN AGAR. (EXPERIMENT 1).

		Control	AA	TG	HAT	HAT
PyY AA/AAR	-	25	45		36	
	A	80	120		83	
	T	0	0		0	
PyY TG/TGR	-	14		18		7
	A	0		0		0
	T	2		6		3
PyY AA/AAR	-	37	55	8	13	13
+	A	105	130	0	13	5
PyY TG/TGR	T	4	0	5	2	3

- \- Control
- A 8-azaadenine 100 µg/ml.
- T 6-thioguanine 10 µg/ml.

Cloned in agar, each figure is the average of two plates.

TABLE XV. THE PHENOTYPES OF CELLS PRESENT IN THE MIXTURE OF PyY AA/AAR AND PyY TG/TGR CELLS AFTER DIFFERENT SELECTION PROCEDURES AS DETERMINED BY AUTORADIOGRAPHY (Experiment 4)

	Adenine		Hypoxanthine	
	% labelled (PyY TG/TGR)	% unlabelled (PyY AA/AAR)	% labelled (PyY AA/AAR)	% unlabelled (PyY TG/TGR)
Control	61	39	36	64
8-Azaadenine	1	99	98	2
6-Thioguenine	100	0	3	97
AAT	73	27	25	75
HAT	31	69	71	29

In each case 100 solitary cells were scored at random.

A two-sample t-test was used to estimate the significance of the shifts in the proportions of the two variants present after selection in HAT and AAT.

The difference in proportions between the control and the AAT bottles was significant at the 5% level ($t = 6.38$).

The difference between the control and the HAT bottles was significant at the 1% level ($t = 18.03$).

in experiment 4. 100 cells were scored on each coverslip.

The control unselected bottles contained between 36% and 39% PyY AA/AAR cells, (labelled by hypoxanthine but not by adenine) and 61% to 64% PyY TG/TGR cells (labelled by adenine but not by hypoxanthine). The two estimates using different labelled precursors were independent but agree well in their estimate of the ratio of the two variants present. Indeed they agree so well that perhaps there was some bias in the sampling. The counts were not carried out blind, and during counting a selection for solitary cells to be counted was necessary.

After exposure to AA, the proportion of PyY TG/TGR cells was reduced to 1-2%. After TG the proportion of the PyY AA/AAR cells was reduced to less than 3%. Under these conditions, the selection proceeded in the mixture as predicted by the behaviour of the variants when grown singly, there being selection for the variant resistant to the analogue present. In each case two estimates were again obtained which agreed well.

The mixture continued to contain both variants after selection in HAT and AAT. Their behaviour in the mixture was different to that when plated singly because of the phenotypic change resulting from metabolic co-operation. In each case, the proportion of each variant present was altered in favour of the one capable of growing alone under the selective conditions. In HAT the number of cells able to incorporate hypoxanthine (PyY AA/AAR) increased from 36% in the control to 71%. In AAT the number of cells able to incorporate adenine (PyY TG/TGR) had increased from 61% to 73%. In each case the independent estimate of the cell ratios determined by the second label confirmed the shift. These results demonstrate the presence of "the kiss of life", confirming the result obtained in a separate experiment by cloning in agar.

"The kiss of life" has been shown to allow survival of APP deficient cells in AAT and IPP deficient cells in HAT when in contact with cells capable of surviving alone under these conditions. "The kiss of death" could not be demonstrated.

8. RESULTS IV. THE MOLECULAR BASIS OF METABOLIC CO-OPERATION

Subak-Sharpe (1969) outlined five possible molecular bases for metabolic co-operation. The phenomenon could be due to the exchange of either:

1. Nucleotides.
2. Radioactively labelled nucleic acid.
3. Informational nucleic acid (messenger RNA or DNA) carrying the genetic information lacking in the variant cell.
4. Preformed polypeptide.
5. Regulating molecules.

By studying the half life of the phenotypic alteration caused by metabolic co-operation after dissociation of co-operating cells it is theoretically possible to distinguish between some of these possibilities (Subak-Sharpe 1969). The first two would be expected to result in instantaneous reversion of the cells to their normal phenotype. Both possibilities depend on the continued contact of the cells. The last three possibilities would be expected to result in a prolonged "phenotypic lag" after separation, during which the cells would continue to behave as if they were still co-operating. The assumption is made that the half life of the transferred molecules is detectable. Chicken APP activity was found to be stable in PyY AA/AAR/TG/TGR cells, having a half life of about one day (see Chapter 9). IPP activity in Lesch-Nyhan cells was found to be stable for 12 hours (Cox, Krauss, Earl Balis and Dancis, 1970). Using Lesch-Nyhan cells, Cox et al. (1970) showed that the half life of metabolic co-operation was short in the case of IPP. Pitts (1971) using PyY TG/TGR cells also showed that the half life of any phenotypic lag after separation was less than ten minutes. These experiments thus suggest that metabolic co-operation depends on one or both of the first two alternatives.

These observations were confirmed in the following experiment. Figure XXI. PyY AA/AAR, PyY TG/TGR and a 50:50 mixture of the two were each seeded

FIGURE XXI. FLOW CHART

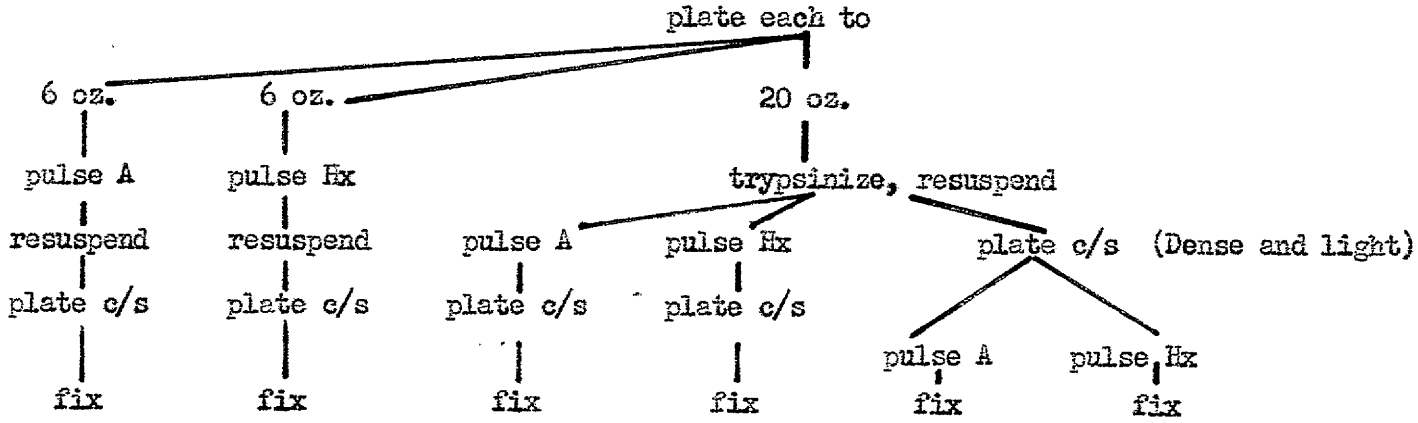
PyY AA/AAR

or

PyY TG/TGR

or

PyY AA/AAR and PyY TG/TGR



A adenine

Hx hypoxanthine

c/s coverslips

to a 20 oz. and two 6 oz. bottles and allowed to grow to confluence. The 6 oz. bottles were then pulsed in each case with either adenine or hypoxanthine 1 μCi/ml. for two hours before trypsinizing, washed in growth medium and plated at low densities in petri dishes containing coverslips. The cells from the 20 oz. bottles were trypsinized off, washed and then treated in either of two ways. First a sample of each was pulsed in suspension in growth medium immediately after trypsinization with either 1 μCi/ml. adenine or hypoxanthine for three hours at 37°. During the pulse, the cell suspension was shaken gently. At the end of the pulse the cells were spun gently, washed twice in growth medium to remove unincorporated label and plated separately to petri dishes containing coverslips. Second, cells were plated in the normal way and allowed to spread onto coverslips. Two densities of inoculation were used, 1×10^5 cells per dish and 5×10^5 cells per dish. In the lightly inoculated preparations few cells touched, the more heavily inoculated preparation contained few cells that were solitary. Twenty hours after plating, coverslips were removed for pulsing with either adenine or hypoxanthine at 1 μCi/ml. for three hours. They were then fixed, together with the coverslips carrying the cells pulsed prior to splitting and with those pulsed in suspension. All three sets of coverslips were extracted with TCA and autoradiographed as usual.

The phenotypes of the cells pulsed alone prior to splitting were as expected (Table XVI), PyY AA/AAR cells being heavily labelled with hypoxanthine and slightly labelled (less than 50 grains/cell nucleus) with adenine and PyY TG/TGR cells heavily labelled with adenine unlabelled (less than 3 grains per cell nucleus) by hypoxanthine. The phenotypes of the cells grown and pulsed as a mixture (in the 6 oz. bottles) before being plated were altered, there being heavy labelling of virtually all cells both with adenine and with hypoxanthine. This was clearly caused by metabolic co-operation. The nucleic acids labelled during the pulse remained in the cells throughout the time they were trypsinized and plated until fixation.

TABLE XVI. THE ABILITY OF VARIANT CELLS TO INCORPORATE ADENINE AND HYPOXANTHINE AFTER PULSING INDEPENDENTLY AND AS A MIXTURE

	Pulse			
	adenine		hypoxanthine	
	% > 50 grains (PyY TG/TGR)	% < 50 grains PyY (AA/AAR)	% labelled (PyY AA/AAR)	% unlabelled (PyY TG/TGR)
1. Pulse prior to split				
PyY AA/AAR	0	100	100	0
PyY TG/TGR	98	2	0	100
PyY AA/AAR+ PyY TG/TGR	97	3	99	1
2. Pulse in suspension				
PyY AA/AAR	0	100	100	0
PyY TG/TGR	100	0	0	100
PyY AA/AAR+ PyY TG/TGR	62	38	26	74
	% > 70 grains	% < 70 grains	% labelled	% unlabelled
3. Pulse after splitting and plating at 5×10^5/dish				
PyY AA/AAR	2	98	100	0
PyY TG/TGR	100	0	0	100
PyY AA/AAR+ PyY TG/TGR	95	5	98	2
4. Pulse after splitting and plating at 1×10^5/dish				
PyY AA/AAR	5	95	100	0
PyY TG/TGR	100	0	0	100
PyY AA/AAR+ PyY TG/TGR	80	20	43	57

The grain number over the nuclei of 100 cells counted in each case. In sections 1, 2 and 4 only solitary cells scored. In 3 consecutive cells were scored.
The background incorporation of hypoxanthine by the PyY TG/TGR cells was less than 3 grains/cell nucleus.
The background incorporation by the PyY AA/AAR cells was under 50 grains/cell nucleus in sections 1 and 2, but under 70 in sections 3 and 4.

In the preparations pulsed in suspension, PyY AA/AAR and PyY TG/TGR cells alone behaved as before. The mixture however produced different results from those when the cells were pulsed while confluent in the 6 oz. bottles. After pulsing the mixture in suspension with adenine, there was a mixture of 62% heavily labelled cells and 38% slightly labelled cells. After pulsing, the mixture in suspension with hypoxanthine contained labelled cells to unlabelled cells in the ratio of 26% to 74%. The ratios of the cell types present in the mixture as estimated by the independent pulsing with the two labels agree well. There were 62-74% PyY TG/TGR (labelled with adenine, unlabelled with hypoxanthine) and 26-38% PyY AA/AAR (slightly labelled with adenine, heavily labelled with hypoxanthine). It was occasionally noted that after pulsing with hypoxanthine there was slight labelling of a cell in contact with a very heavily labelled cell. Apparently a PyY AA/AAR cell in contact with a PyY TG/TGR cell taking part in metabolic co-operation. The lightly labelled PyY TG/TGR cell could have derived its label from the following sources:

1. Prolonged phenotypic change caused by metabolic co-operation having a slow rate of decay.

2. Incorporation of residual quantities of label present in the growth medium by metabolic co-operation.

3. Continued sticking initiated in suspension.

4. Incorporation of labelled material derived from the intracellular pools of the cell with which it is in contact. Some labelled material may remain in the pools of cells for some time after the end of a pulse, due both to recycling of the label and to the time taken for the pool specific activity to become diluted by de novo synthesised material.

The first possibility can be discarded because the solitary cells would be expected to behave similarly. The second seems unlikely because the cells were washed twice before plating. The last two possibilities cannot

be distinguished on this data. A reconstruction experiment is presently being undertaken in an attempt to do so. If the last interpretation is correct, metabolic co-operation can take place in the absence of external precursor, and would involve the passage of labelled material between co-operating cells.

The last section of the experiment confirmed that the cells were still capable of participating in metabolic co-operation. The cells were pulsed after plating either at a high or a low density while attached to the glass. Either adenine or hypoxanthine were used, the pulse lasting three hours. The unmixed cells behaved as before, their phenotype reflecting their pattern of resistance. The PyY TG/TGR cells had a background of less than four grains/nucleus after pulsing with hypoxanthine. The background of the PyY AA/AAR cells after pulsing with adenine was less than seventy grains/nucleus. The background was too high for this section of the experiment to be interpreted reliably. The reason for the high background is unknown.

In mixed culture at the high density 95% or more of the cells were labelled after pulsing with either adenine or hypoxanthine. The proportion of unlabelled cells in the mixture pulsed with adenine shifted from 5% in the dense preparation to 20% in the lightly seeded preparation. After pulsing with hypoxanthine the proportion of unlabelled cells increased from 2% in the dense preparation to 57% in the lightly seeded preparation. The phenotypes of the variants in dense culture were altered because of metabolic co-operation, confirming the result obtained after pulsing the mixed culture before splitting. On the basis of the result obtained after pulsing with hypoxanthine the mixture contained PyY AA/AAR and PyY TG/TGR cells in about equal proportions.

9. RESULTS V. REACTIVATION OF CHICKEN ERYTHROCYTE NUCLEII WITHIN BHK 21/Cl3/PyY CELL VARIANTS.

The PyY AA/AAR/TG/TGR and PyY TG/Gar/BUdR variants between them offer the possibility of following the reactivation of the chicken erythrocyte nucleus with respect to six enzyme activities not previously documented. The PyY AA/AAR/TG/TGR variant lacks detectable APP and IPP activity and, though this is not proven with complete rigour, the permeases involved in the passage of purine nucleosides through the cell membrane. The PyY TG/Gar/BUdR variant lacks detectable IPP, deoxycytidine kinase (dCK) and TK activity (Edwards, 1970).

The kinetics of the appearance of chicken surface antigen, IPP activity and sensitivity to diphtheria toxin in heterokaryons formed from mouse A9 cells and chicken erythrocytes are well established (Harris et al., 1969; Harris and Cook, 1969; Cook, 1970, Deák et al., 1972, and Dendy, 1972). The appearance of chick specific nucleolar material has been followed by immunofluorescent techniques in heterokaryons formed from HeLa cells and chick erythrocytes (Ringertz et al., 1971).

The BHK 21/Cl3/PyY cell variants were fused with erythrocytes obtained from eggs of varying ages using BPL inactivated Sendai virus. Experiments described here utilized erythrocytes from either ten, twelve or fifteen day old eggs. Erythrocytes from adult hens were difficult to fuse efficiently. The reactivation process proceeded within BHK 21/Cl3/PyY cell variants as described in other cells. The rate of reactivation was dependent on the age of the egg from which the erythrocytes were obtained, the younger the egg the quicker the reactivation proceeded. When used in long-term experiments the variants were pre-irradiated with 6000 rads of X-rays before fusion in order to prevent cell division which may result in the disruption of the nucleii within the heterokaryon (Harris et al., 1969). The cells were plated to a series of petri dishes containing coverslips after treatment

with the Sendai virus. The ability of the heterokaryons to incorporate radioactive nucleic acid precursors was assayed at intervals by transferring replicate coverslips to medium containing appropriate label. After terminating the pulse, the coverslips were prepared either for scintillation counting or for autoradiography.

1. Morphological changes in the erythrocyte nuclei during reactivation

The erythrocyte nuclei began a process of enlargement immediately after fusion. During this process the chromatin expanded, the erythrocyte nuclei stained less densely and the nuclear bodies (Davies, 1961) ceased to be prominent. Within one day of fusion some erythrocyte nuclei developed structures resembling small nucleoli. These structures were present in Giemsa-May-Grunwald stained material, being compact bodies having similar staining properties to the hamster nucleoli. Their number varied, some erythrocyte nuclei having one, but others having two or occasionally three. They appeared in a progressively greater proportion of erythrocyte nuclei as the reactivation process progressed and become larger and more densely staining. Sometimes erythrocyte nuclei that appeared to have reactivated normally in other respects failed to develop nucleoli.

Figure XXII demonstrates the morphological changes that took place during the reactivation of erythrocyte nuclei from fifteen day old eggs in PyY AA/AAR/TG/TGR cells that had been pre-irradiated with 6000 rads of X-rays prior to fusion. The morphological changes during reactivation were the same in both PyY TG/Cax/BUdR and PyY AA/AAR/TG/TGR cells. Nucleolar-like structures had developed by twenty four hours after fusion in 50% of erythrocyte nuclei from twelve day old eggs after fusion with PyY TG/Cax/BUdR cells. The development of the large nucleoli from the small nucleolar-like structures was a gradual process lasting several days. It took place at varying rates in different heterokaryons within the same preparation. The time of appearance of nucleoli therefore was not defined using morphological criteria alone.

FIGURE XXll.

The reactivation of nucleii from fifteen day old eggs after fusion with X-irradiated PyY AA/AAR/TG/TGR cells. All were supplied with tritiated hypoxanthine for four hours before fixation apart from a),b) and c). All printed at the same magnification.

a) Erythrocytes from fifteen day old eggs. The nucleii contain heavily staining nuclear bodies.

b) A dikaryon eight hours after fusion. No enlargement of the erythrocyte nucleus, the nuclear bodies are peripheral.

c) A dikaryon eight hours after fusion. There has been some enlargement of the erythrocyte nucleus, the nuclear bodies are peripheral.

d), e), f) One day after fusion. There has been considerable swelling of the erythrocyte nucleii. d) and e) show erythrocte nucleii that have lost their nuclear bodies, they may contain primitive nucleoli. f) shows an erythrocyte with some nuclear bodies remaining at the periphery. None of these dikaryons was labelled.

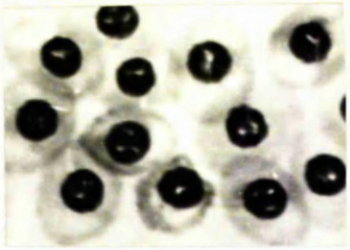
g), h), i) Two days after fusion. The nucleolar structures are more prominent. Two of the dikaryons are labelled.

j), k),l) Three days after fusion. Both the hamster and the erythrocyte nucleii have enlarged. The nucleolar structures are very prominent. The three dikaryons are labelled.

m), n), o) Four days after fusion. A similar pattern. The hamster nucleii have become larger. Two of the dikaryons are labelled.

p), q), r) Five days after fusion. A similar pattern to day three and four. All three of the dikaryons are labelled.

10μ



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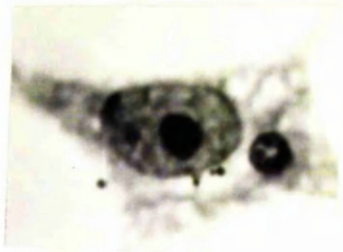
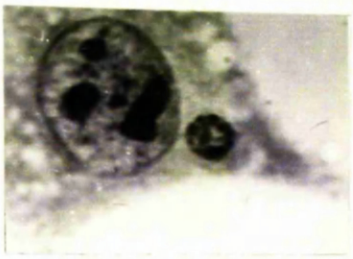
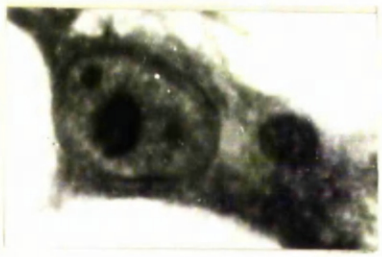


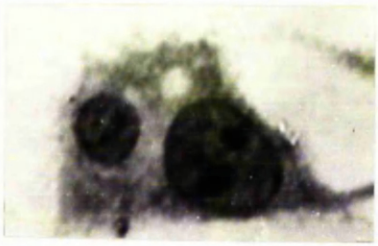
b).



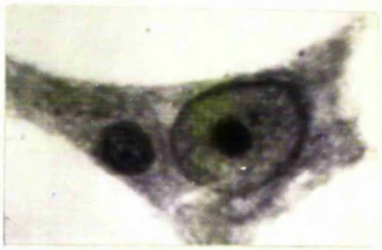
c).



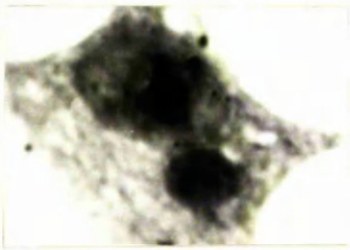
d).



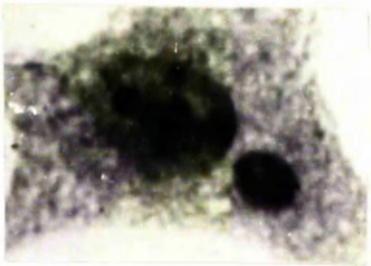
e).



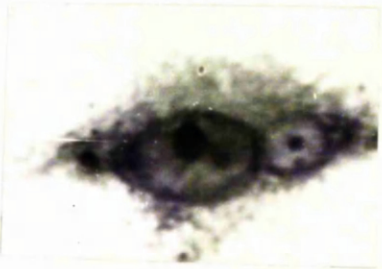
f).



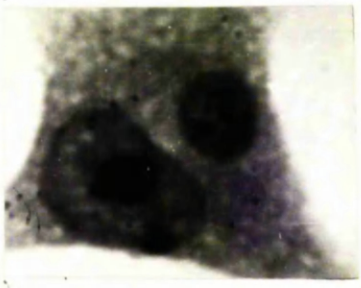
g).



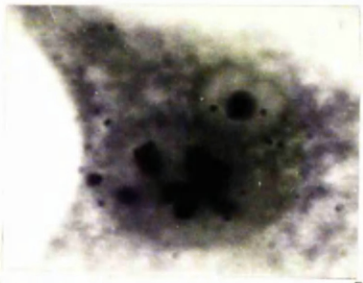
h).



i).

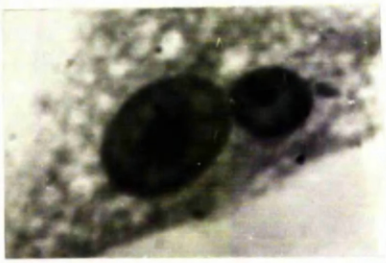


j).



k).

l).



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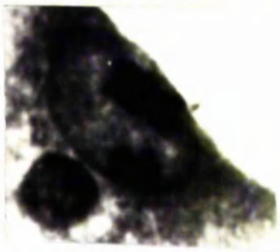
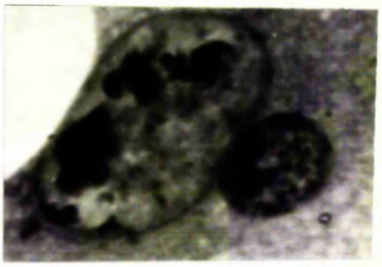
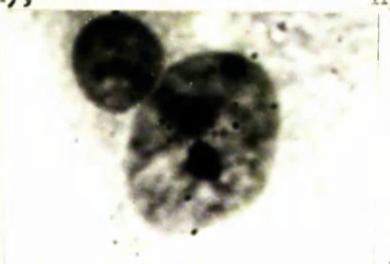


n).

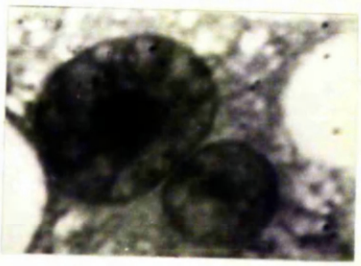


o).



p).

q).



r).

2. Increase in the area of the reactivating erythrocyte nucleii

2. Increase in the area of the reactivating erythrocyte nucleii

Both the erythrocyte nucleus and the hamster nucleus of the heterokaryon formed between X-irradiated PyY AA/AAR/TG/TGR cells and chicken erythrocytes enlarged as the time after fusion increased. Figure XXIII shows the increase in the area of the erythrocyte nucleii from fifteen day old eggs during reactivation in PyY AA/AAR/TG/TGR cells. The measurements were carried out by cutting out the areas occupied by the chicken nucleii from unselected photographs of dikaryons taken at various times after fusion and printing them at a constant magnification on uniform paper. These pieces of paper were weighed, and since their weights were proportional to their areas a measure of the increase in their area was obtained. The area of nucleii within intact erythrocytes was forty units on the arbitrary scale chosen. As soon as eight hours after fusion the process of enlargement had been initiated in some erythrocyte nucleii. The proportion of erythrocyte nucleii that had enlarged increased with the time after fusion. By two days after fusion most had undergone at least a two-fold increase in area. The process of enlargement produced up to a ten-fold increase in the area of the erythrocyte nucleii after five or six days. The process was biphasic with a lag at two to three days by which time the average area had increased three-fold.

In Figure XXIV the increase in the area of the PyY AA/AAR/TG/TGR nucleii from dikaryons is plotted. The average area of these nucleii increased two-fold during six days, the majority of the increase taking place during the latter half of the experiment by which time the heterokaryons had become radiation giant cells. The second phase of the enlargement of the erythrocyte nucleii could be correlated with the time during which most of the enlargement of the hamster nucleii took place. The enlargement of the erythrocyte nucleii may be due to two processes usually taking place sequentially, the first being due to the reactivation of the erythrocyte nucleus within the foreign cytoplasm of the hamster cell; the second being due to the

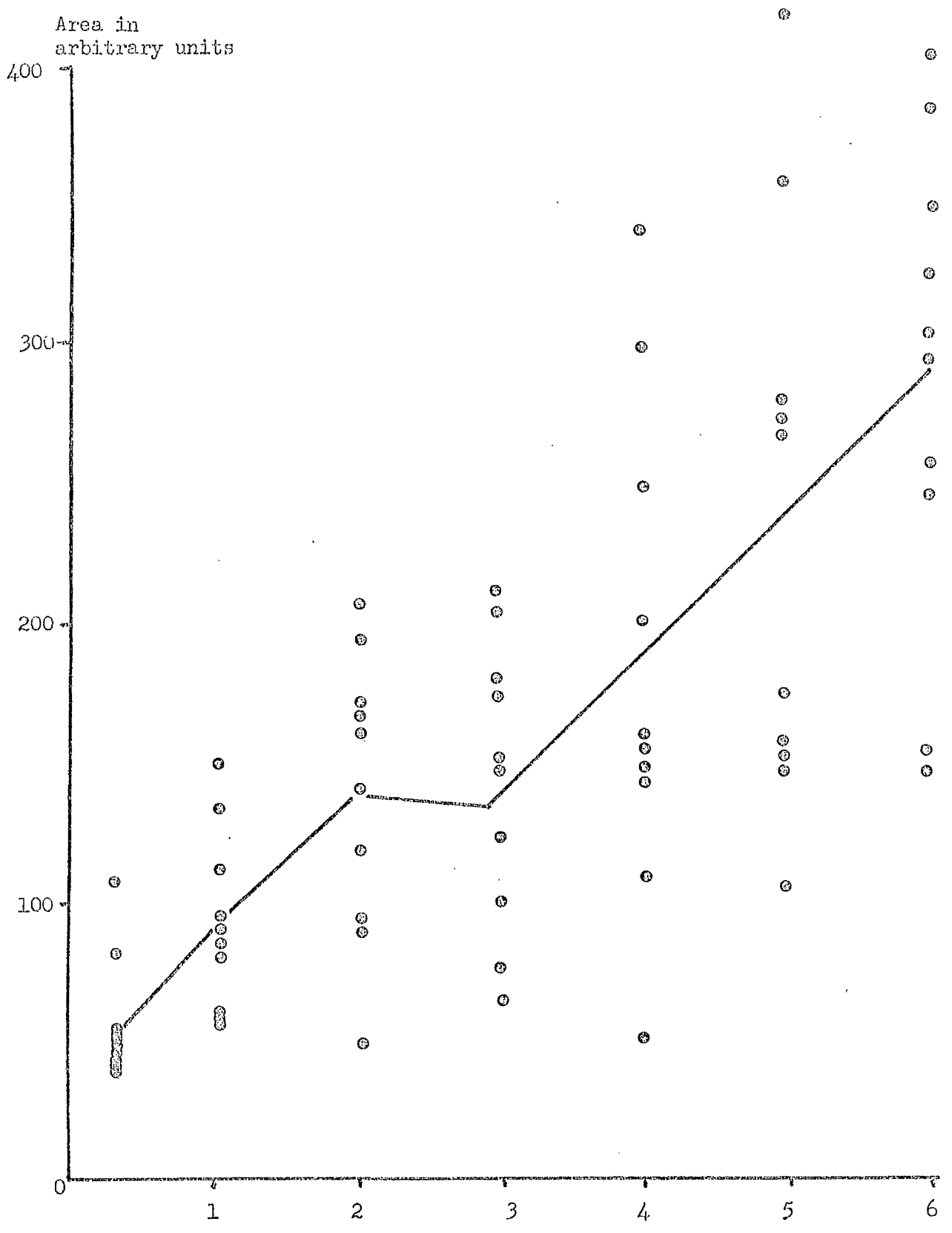


FIGURE XXIII. THE INCREASE IN AREA OF ERYTHROCYTE NUCLEII FROM FIFTEEN DAY OLD EGGS WITHIN DIKARYONS FORMED AFTER FUSION WITH PyY AA/AAR/TG/TGR CELLS. 10 ERYTHROCYTE NUCLEII SAMPLED AT EACH TIME.

——— Average value.

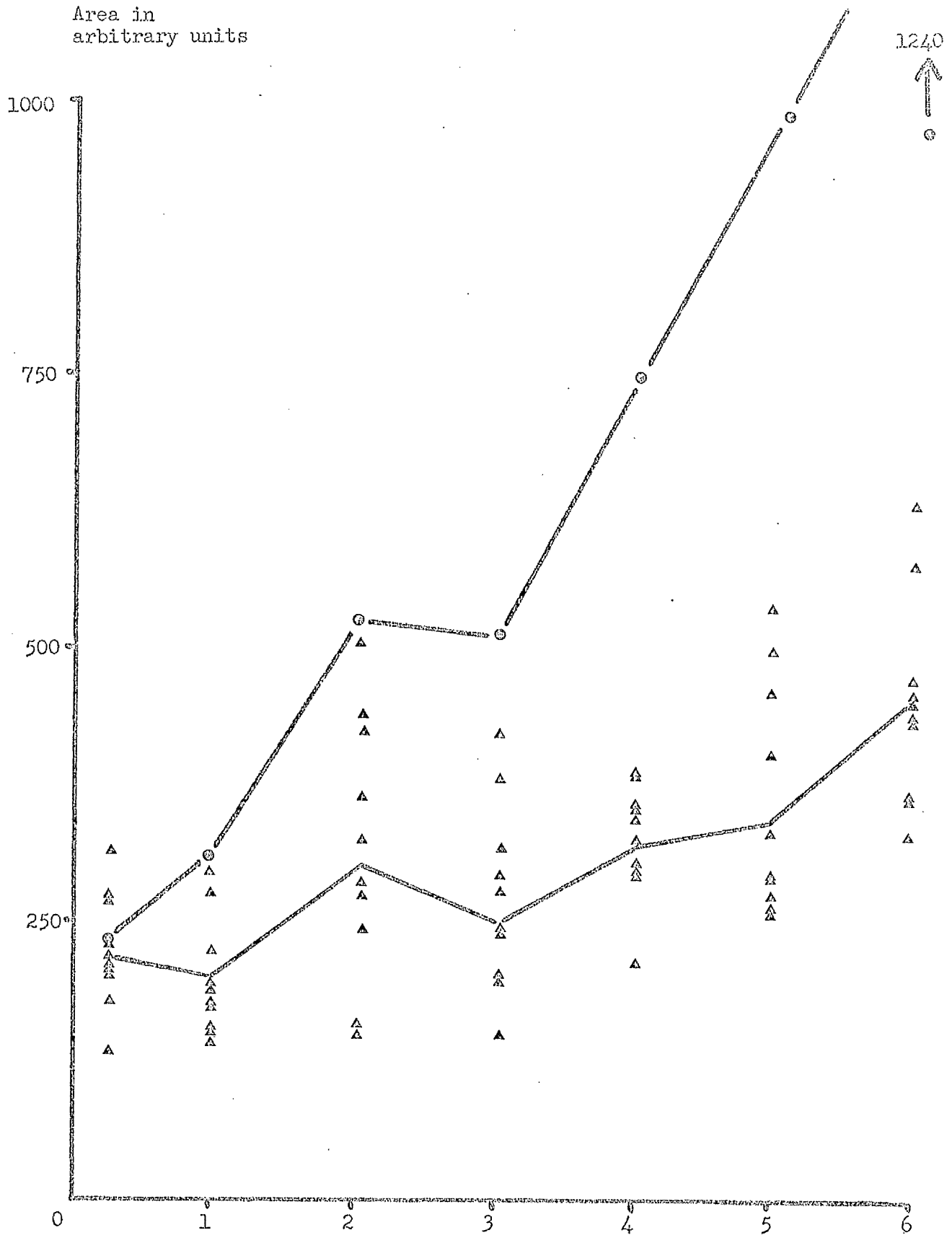


FIGURE XXIV. THE INCREASE IN AREA X-IRRADIATED PyY AA/AAR/TG/TGR CELL NUCLEII AFTER FUSION WITH ERYTHROCYTES FROM FIFTEEN DAY OLD EGGS. 10 NUCLEII SAMPLED AT EACH TIME

△ Area of PyY AA/AAR/TG/TGR nucleii

⊙ Average area of erythrocyte nucleus (x4)

presence of the erythrocyte nucleii within a radiation giant cell and was caused by whatever processes also caused the enlargement of the hamster nucleus.

The increase in the volume of the erythrocytes, if measured, would ~~probably~~ have been greater than the increase in area.

3. Thymidine incorporation

The incorporation of tritiated thymidine by the reactivating erythrocyte nucleii indicated DNA replication taking place. When present the labelling was usually heavy and evenly distributed over the reactivating nucleus, Figure XXV. Erythrocyte nucleii from twelve day old eggs fused with non-irradiated PyY AA/AAR cells after a two hour pulse were labelled in 25% of cases at fifteen hours, 51% at eighteen hours and 61% at twenty-six hours after fusion. Most often both chick and hamster nucleii in a heterokaryon were labelled, but each could be labelled independently on occasions, Figure XXV.

4. Uridine incorporation

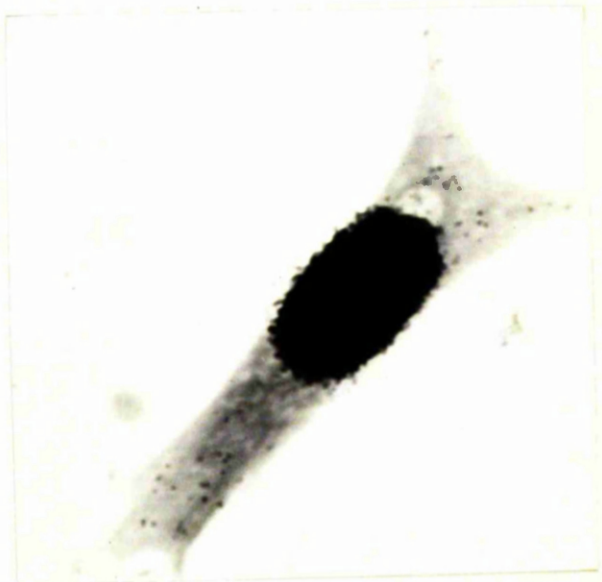
The time at which the erythrocyte nucleii commenced to incorporate uridine, and therefore make RNA was determined. After fusion with unirradiated PyY AA/AAR cells, erythrocyte nucleii from twelve day old eggs were labelled in 63% of cases by thirteen hours after a pulse lasting thirty minutes. This short time did not allow a significant proportion of the RNA synthesised during the pulse to become processed and exported to the cytoplasm. The grains produced by the decay of the tritiated uridine incorporated were therefore situated over nucleii that had been synthesising RNA.

By eighteen hours after fusion virtually every erythrocyte nucleus was incorporating uridine and therefore synthesising RNA.

FIGURE XXV

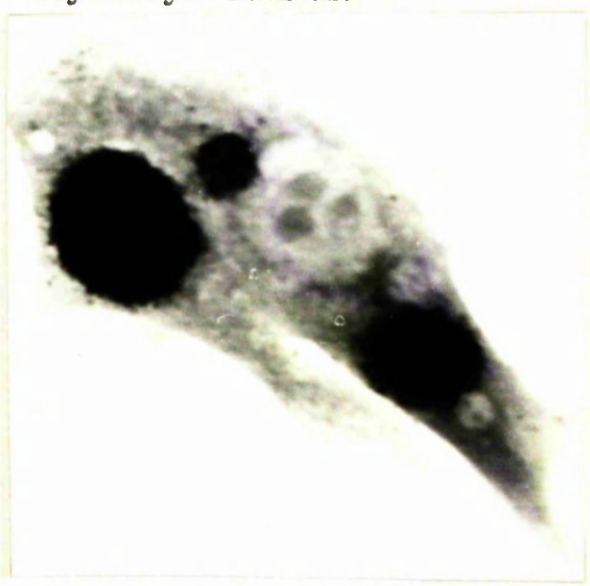
Heterokaryons formed from unirradiated RyI AA/AAR/TG/TGR cells and erythrocytes from 10 day old eggs, one day after fusion. Incubated with tritiated thymidine for four hours before fixation.

a) Both hamster and erythrocyte nucleus labelled.



b) Hamster nucleus labelled. Two small nucleolar-like structures can be seen in the unlabelled erythrocyte nucleus.

c) Erythrocyte nucleus labelled. Slight labelling of nucleolar regions of the hamster nucleus.



d) A polykaryon containing two labelled and one unlabelled hamster nucleii and three erythrocyte nucleii. Only one of the erythrocyte nucleii is labelled, two nucleolar-like structures can be seen in the lower of the unlabelled erythrocyte nucleii.

A functional criterion of nucleolar activity is heavy labelling after pulses of an hour or more with tritiated RNA precursors. Figure XXVI demonstrates heavy labelling over the nucleolar-like structures within erythrocyte nuclei from ten day old eggs twenty-nine hours after fusion with non-irradiated PyY TG/Car/BUdR cells. The preparation had been pulsed with tritiated adenine for four hours prior to fixation and then autoradiographed. In one region of the coverslip there had been slippage of the film, allowing both the heterokaryons and the grains produced by them to be viewed. The small structures referred to as nucleolar-like are seen to be labelled more heavily than the surrounding nucleoplasm of the reactivated erythrocyte. Therefore on the basis of both their morphology and their pattern of labelling at least some of these structures are functional nucleoli. Quantitation is at present lacking, but heavy labelling of a similar pattern was often seen over reactivating erythrocyte nuclei when the grain density was suitable.

5. Metabolic co-operation between heterokaryons and adjacent hamster cells

Erythrocytes from ten day old eggs were pulsed in suspension with either tritiated adenine, hypoxanthine, deoxycytidine or thymidine. Virtually all were labelled to some extent by adenine, in 5% of cases the labelling was heavy. 10% incorporated very small amounts of hypoxanthine. 1% were slightly labelled after deoxycytidine. 2% were lightly labelled and 1% heavily labelled by thymidine. Erythrocytes from fifteen day old eggs were lightly labelled with adenine but not the other precursors.

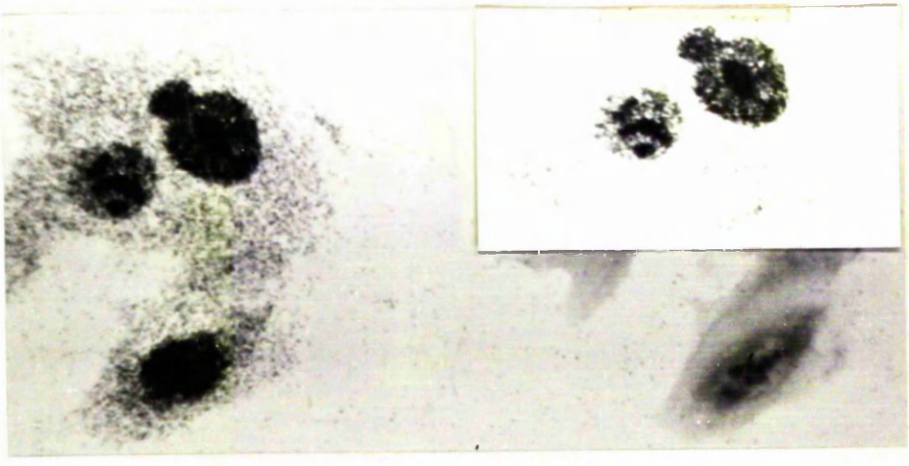
Within a day of fusion there was ~~heavy~~ labelling of heterokaryons formed from PyY AA/AAR/TG/TGR cells and ten day old erythrocytes after pulsing with tritiated adenine or hypoxanthine, Figure XXVII. This early labelling was due to the passive transfer of preformed enzyme (or perhaps mRNA) to the heterokaryon at the time of fusion.

FIGURE XXVI.

Heterokaryons formed from unirradiated PyY TG/Car/BUdR cells and erythrocytes from 10 day old eggs, fixed 29 hours after fusion. The preparation was incubated with tritiated adenine for four hours before fixation and then autoradiographed using AR 10 stripping film. Over some regions of the preparation the film had slipped after exposure, allowing the cells and the grains produced by them to be viewed separately.

Direction of grain shift.

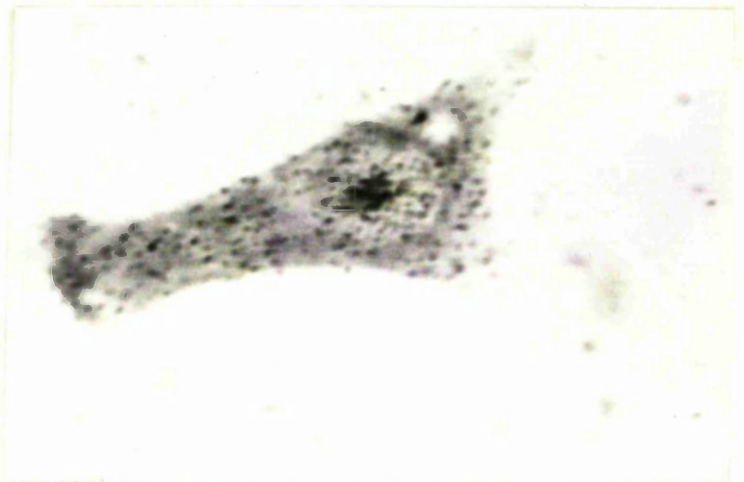
a). On the right is a dikaryon. Some debris lies over the upper pole of the cell. Below the hamster nucleus, which has a bilobed nucleolus, there is an erythrocyte nucleus with two primitive nucleoli. The grains produced by the dikaryon are on the left. The hamster nucleus and nucleolus are labelled more heavily than the cytoplasm. The grain distribution over them matches the morphology of the debris and the hamster nucleus and nucleolus. The erythrocyte nucleus is lightly labelled. The primitive nucleoli within it however are labelled much more heavily, having a grain density approaching that of the hamster nucleolus.



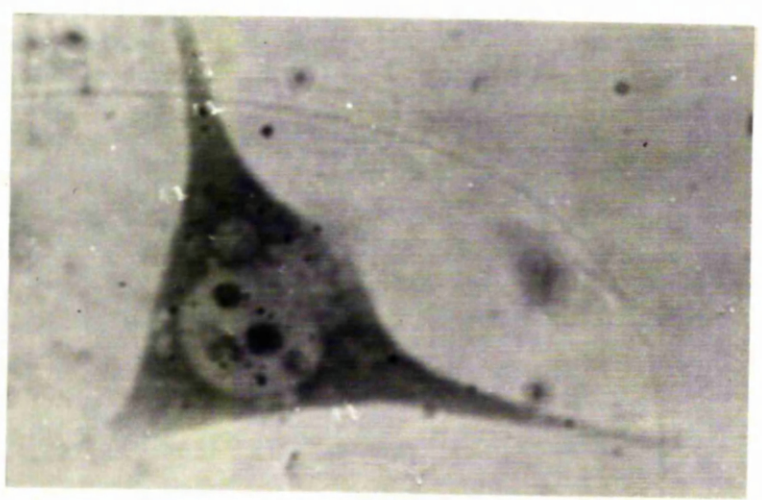
b). A second example from the same preparation. The heterokaryon contains two hamster nucleii and one erythrocyte nucleus which has two primitive nucleoli. In contact with the heterokaryon is an unfused hamster cell. The grains produced by the cells have shifted to the left. Again the distribution of the grains matches the morphology of the hamster nucleii and nucleoli. The erythrocyte nucleus is more heavily labelled than in a), but again there is heavier labelling over the primitive nucleoli. To emphasise the situation a more contrasty print of the grains is mounted as a flap over the heterokaryon.

FIGURE XXVII

Heterokaryons formed from unirradiated PyY TG/Car/BUdR cells and erythrocytes from 10 day old eggs. Fixed twentyone hours after fusion.



a) Incubated with tritiated adenine for four hours before fixation. The dikaryon is heavily labelled. The unfused cells in the preparation were unlabelled.



b) Incubated with tritiated hypoxanthine for four hours before fixation. The dikaryon is lightly labelled. The unfused cells in the preparation were unlabelled.

Though Sendai virus haemolysed adult hen erythrocytes, those from eggs were more resistant and not all were haemolysed. When using erythrocytes from eggs some erythrocyte cytoplasm containing enzyme or mRNA may enter with the avian nucleus at the time of fusion.

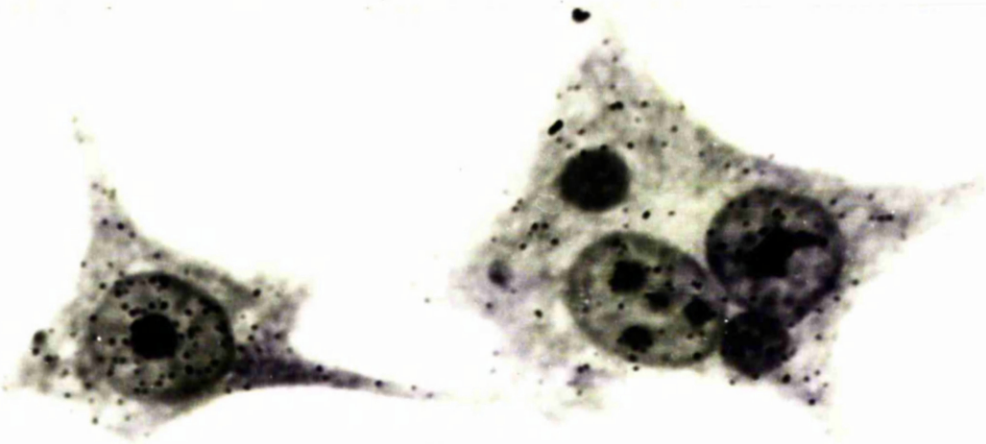
The half life of the APP activity in the heterokaryons was long enough for the activity to remain detectable for two or more days. The labelling by hypoxanthine was lighter and passively transferred activity was rarely detected after two days. The difference in the degree of labelling by adenine and hypoxanthine may be due either to differences in the quantity of the enzyme transferred, or to differences in the activities of the two enzymes. Intact erythrocytes also incorporated more adenine than hypoxanthine. Both APP and IPP of the chicken remained functional in the environment of the hamster cell.

Using either tritiated adenine or hypoxanthine metabolic co-operation was demonstrable between the heterokaryons and cells that had not undergone fusion, Figure XXVIII. Cells not actually in contact, though close to the heterokaryons were usually unable to incorporate the label and retained their variant phenotype. Some unfused hamster cells not in contact with heterokaryons were also capable of incorporating purines, these had presumably taken part in an abortive fusion and had retained some passively transferred enzyme activity. Metabolic co-operation could therefore take place in situations where the wild type donor cell was unable to synthesise the enzyme whose activity was lacking in the recipient cell. The presence of the passively transferred chicken specified enzymes not only allowed the variant to incorporate purines into nucleic acid, but also these heterokaryons were fully competent to take part in metabolic co-operation.

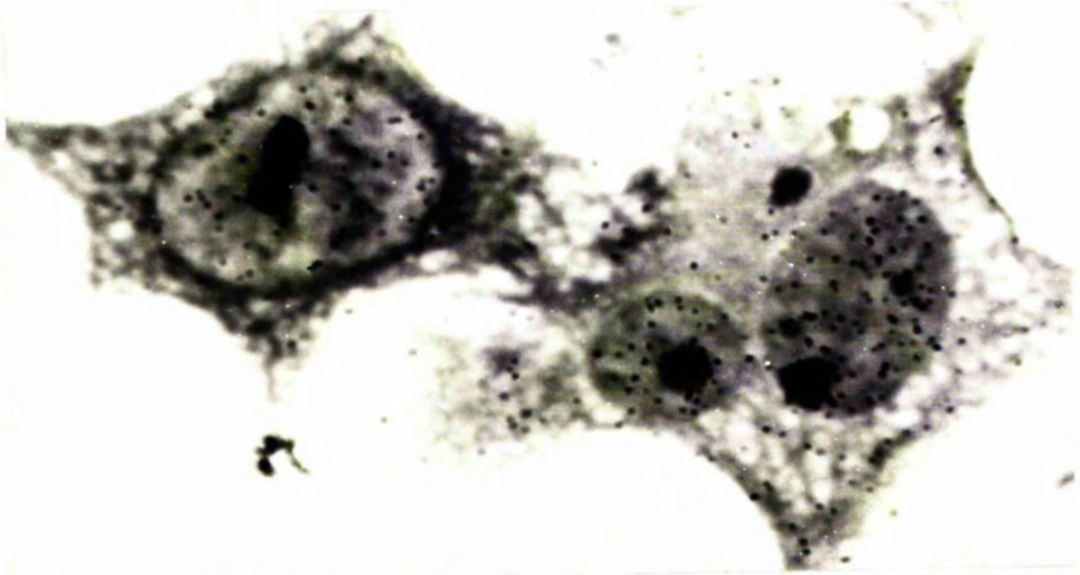
The presence of metabolic co-operation impeded the analysis of the reactivation of the chicken erythrocytes by grain counting. Heterokaryons containing reactivated erythrocytes were also capable of taking part in metabolic co-operation, Figure XXVIII. Only solitary cells could be utilised

FIGURE XXVIII.

Metabolic co-operation between unfused hamster cells and heterokaryons formed by fusion of erythrocytes from fifteen day old eggs with PyY AA/AAR/TG/TGR cells. In both cases the preparations were incubated with tritiated adenine for four hours before fixation.



a). Fixed at one day after fusion. The APP activity of the heterokaryon was derived from the erythrocyte by passive transfer at the time of fusion. The unfused hamster cell is labelled by virtue of metabolic co-operation with the heterokaryon. Unfused hamster cells in the same preparation not in contact with heterokaryons containing erythrocyte nucleii were very occasionally lightly labelled.



b). Fixed at four days after fusion. Note the enlargement of all the nucleii. The heterokaryon contains a reactivated erythrocyte nucleus with a large heavily labelled nucleolus. The APP activity derived by passive transfer has decayed and been replaced by enzyme synthesised within the heterokaryon. The unfused hamster cell in contact with the heterokaryon is labelled because of metabolic co-operation with the heterokaryon containing a reactivated erythrocyte nucleus.

for grain counts because the phenotype of cells in contact could have been altered by metabolic co-operation. Even then metabolic co-operation could not always be totally eliminated because the cells may move apart during the experiment or a bridging cell become detached during the processing. The effect this had on the counts was to transfer some of the heterokaryons from the unlabelled class to the labelled class and to increase the background labelling of some unfused cells. Of course metabolic co-operation did not work in the opposite direction and transfer the cells from the labelled to the unlabelled classes. Since the class of cell being searched for was labelled, heterokaryons containing reactivated erythrocytes, the result in qualitative terms will not have been affected by metabolic co-operation.

6. Reactivation of the genetic information of the erythrocyte nucleus

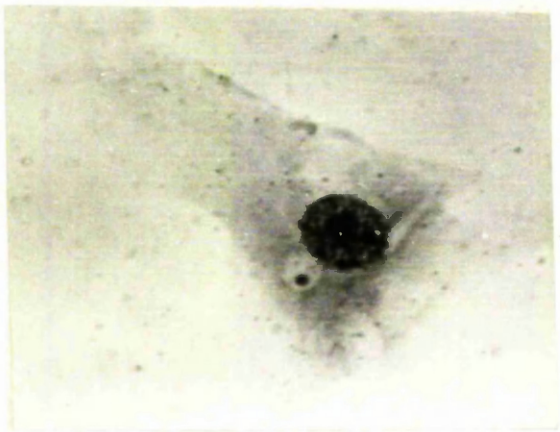
Figure XXIX demonstrates the ability of some heterokaryons formed from erythrocytes from ten day old eggs and unirradiated PyY TG/Car/BUdR cells to incorporate hypoxanthine, deoxycytidine and thymidine by twenty nine hours after fusion. Some heterokaryons were also labelled by these precursors at twenty hours after fusion. Since at this time all of the heterokaryons were making RNA, any having IPP activity were labelled. The TK and dCK activities however were only demonstrable in heterokaryons containing nucleii passing through S phase during the pulse.

On the basis of the biochemical and morphological changes that have taken place in some of the erythrocyte nucleii by twenty nine hours after fusion it is probable that some of the heterokaryons contained reactivated erythrocyte nucleii. Some erythrocyte nucleii had enlarged, were synthesising RNA and had one or more functional nucleolii. In some cases heterokaryons were labelled that contained erythrocyte nucleii that were not fully reactivated. In these cases the activity was due to passive transfer (see previous section).

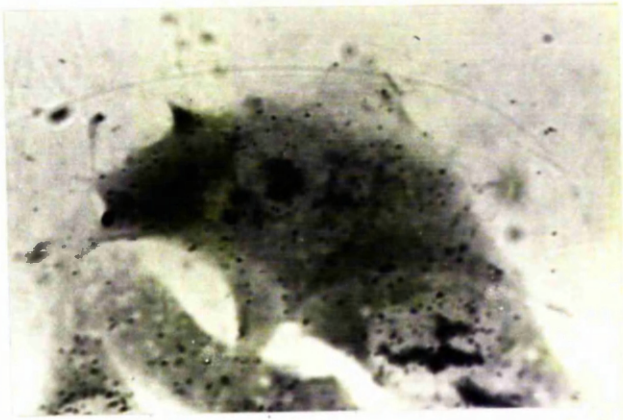
Heterokaryons formed by fusion of erythrocytes from ten day old eggs and unirradiated RyI TG/Car/BUdR cells. Incubated with tritiated nucleic acid precursors for four hours prior to fixation. Fixed at twenty nine hours after fusion.

a). Low power view. Pulsed with thymidine. The only cell labelled contains two erythrocyte nucleii and a hamster nucleus, all labelled.

b). Higher power view. Pulsed with tritiated thymidine. A heterokaryon containing one labelled hamster nucleus and two labelled erythrocyte nucleii. A second unlabelled hamster nucleus is present.



c). Pulsed with tritiated deoxycytidine. The hamster nucleus is labelled, the erythrocyte nucleus is unlabelled but contains a nucleolus.



d). Pulsed with hypoxanthine. The heterokaryon contains one hamster nucleus and three erythrocyte nucleii, two of which have labelled nucleoli. The heterokaryon is labelled, the adjacent unfused hamster cell is taking part in metabolic co-operation with the heterokaryon.

Erythrocytes from fifteen day old eggs were used in order to reduce the passive transfer of enzyme activity. The passive transfer was less both because the enzyme activities in the erythrocytes from older eggs were lower and also because there was greater haemolysis and therefore perhaps not so much cytoplasmic transfer at the time of fusion. The process of reactivation was slower in the erythrocyte nucleii from older eggs and so there was more time for decay of the passively transferred activity to occur.

The hamster cells were pre-irradiated prior to fusion to prevent nucleii within heterokaryons entering mitosis. PyY TG/Oar/BUdR cells underwent massive micronucleation after irradiation with doses of X-rays ranging from 1,000 to 10,000 rads and could not be used for long term experiments. The PyY AA/AAR/TG/TGR cells ceased to divide and in many cases retained a single nucleus for several days after 6,000 rads. There was however a reduction in the number of cells on the coverslips by up to 80% over the first four days after fusion.

The ability of the dikaryons formed between erythrocytes from fifteen day old eggs and pre-irradiated PyY AA/AAR/TG/TGR cells to incorporate adenine, adenosine, hypoxanthine and guanosine was followed at daily intervals after fusion by autoradiography. The number of grains over the hamster nucleus of forty dikaryons were counted at each point. Dikaryons were used to eliminate differences possibly present due to variations in the numbers of nucleii within the heterokaryons. Only solitary dikaryons were used to avoid the effects of metabolic co-operation. At one day after fusion there was heavy labelling of dikaryons after incubation for four hours with tritiated adenine, and some labelling after incubation with tritiated hypoxanthine. This labelling was due to passive transfer of APRT and IPP activity. There was no labelling of the heterokaryons at this time after incubation with adenosine or guanosine.

The results obtained by grain counting over the hamster nucleii of

dikaryons are shown in Figure XXX. The passively transferred activity allowing adenine incorporation declined over the first three days before initiating a rise that continued until the sixth day. The other activities showed a rise on the second day after fusion that continued throughout the experiment. The rise in the incorporation of adenosine and guanosine was just detectable in this experiment. Both behaved in a similar fashion and the rise was simultaneous with those of adenine and hypoxanthine incorporation.

The control cells counted were solitary hamster cells with single nucleii in the same preparations as the dikaryons. The rise in the grain counts over the controls during the experiment was due to two effects. Firstly metabolic co-operation could not be entirely eliminated for reasons that have already been mentioned. Secondly since the average area of the irradiated hamster nucleii doubled during the experiment, the counts due to autoradiographic background would also be expected to double. The initially raised level of adenine incorporation was probably due to passive transfer of enzyme activity at the time of an abortive fusion.

The ability of the heterokaryons containing reactivated erythrocyte nucleii to incorporate various purine precursors differed. The incorporation of adenine was the highest followed by hypoxanthine, lowest of all was that of the nucleosides adenosine and guanosine.

Figure XXII shows some of the dikaryons pulsed with hypoxanthine that were used for obtaining the grain counts plotted in Figure XXX. The dikaryons that have incorporated hypoxanthine contain erythrocyte nucleii with nucleoli. The size of the nucleoli in the reactivated erythrocyte nucleii varied. Occasionally a heterokaryon with an erythrocyte nucleus without a nucleolus was labelled. The label may have been incorporated either by virtue of metabolic co-operation with a heterokaryon containing a reactivated erythrocyte nucleus, or enzyme activity transferred at the time of fusion may remain. Some dikaryons containing erythrocyte nucleii with large nucleoli failed to incorporate any label. The erythrocyte nucleus

Grains/40 PyY nucleii.

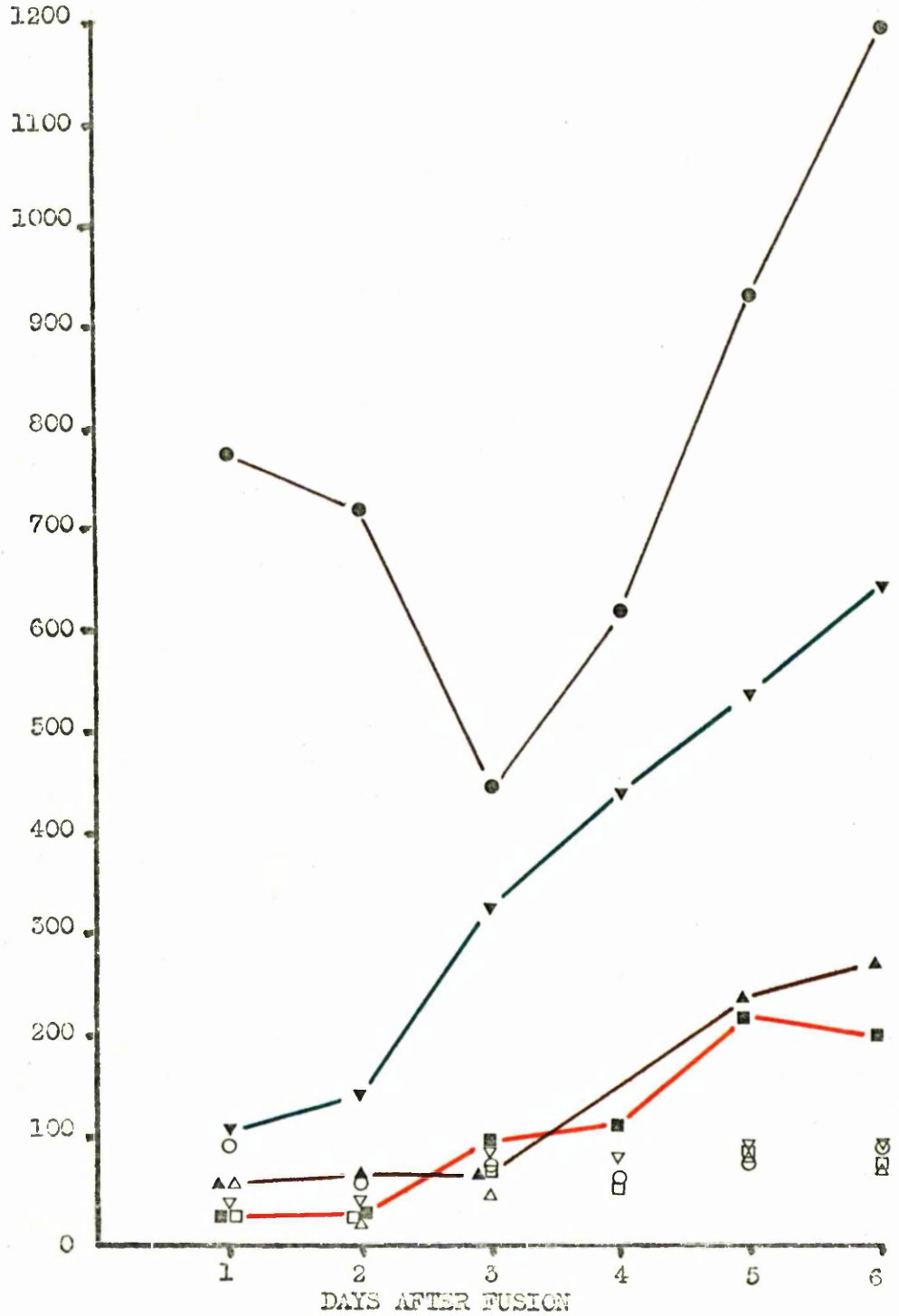


FIGURE XXX.GRAIN COUNTS OVER PyY NUCLEII OF DIKARYONS AND UNFUSED CELLS.

Dikaryons -●-Adenine -■-Adenosine -▼-Hypoxanthine -▲-Guanosine

Control ⊙ Adenine □ Adenosine ▽ Hypoxanthine △ Guanosine

may not have reactivated its ability to specify IPF or the heterokaryon may have been synthesising very little nucleic acid during the pulse.

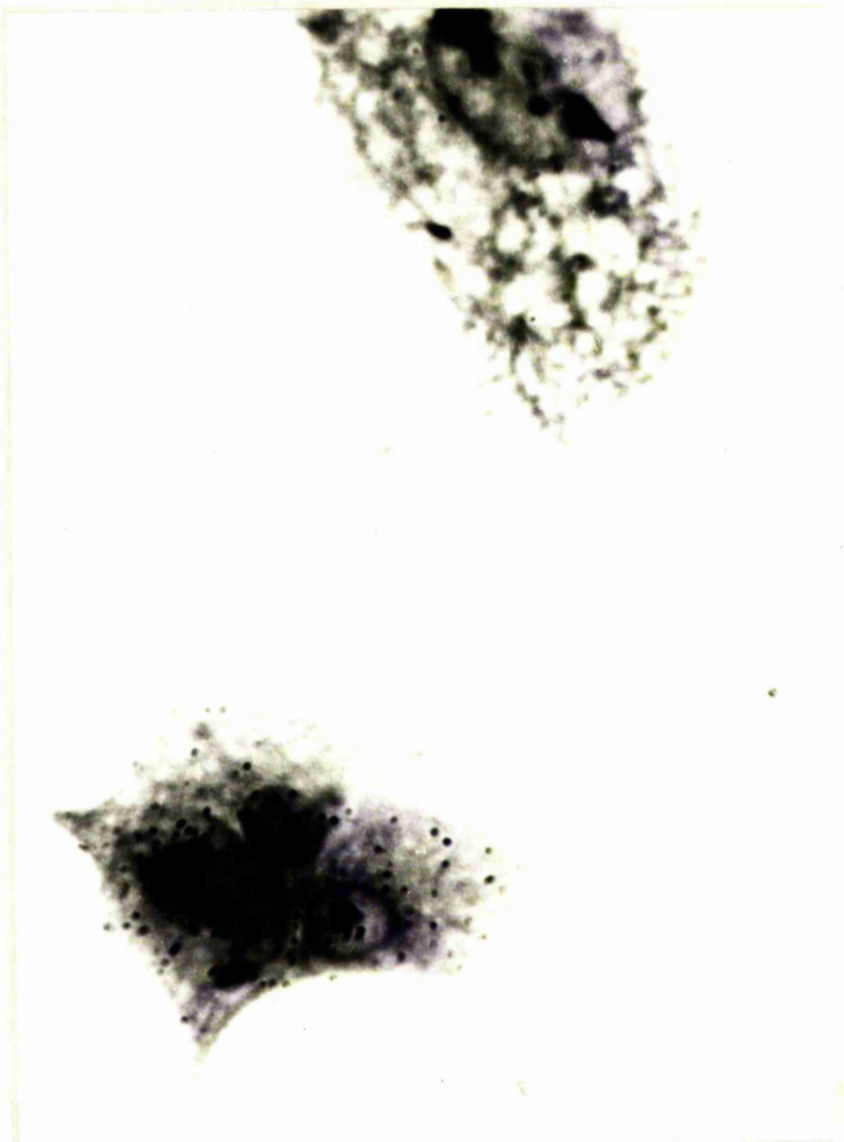
Figure XXXI shows a heterokaryon four days after fusion which contains two reactivated erythrocyte nuclei both of which have nucleoli. The unfused hamster cell partially in the photograph was one of those used as a control. The preparation had been pulsed with tritiated adenine for four hours before fixation. Only the heterokaryon is labelled and therefore capable of incorporating adenine. The unfused PyY AA/AAR/TG/TGR cells in the preparation remain incapable of incorporating adenine.

The estimations of the incorporation of preformed purines by the heterokaryons was open to subjective errors of judgement since it was impossible to perform them blind in this system. The reactivation was also followed by scintillation counting in an attempt to eliminate observer error. The coverslips, instead of being mounted and autoradiographed, were counted in a scintillation counter after pulsing as usual with the labelled precursors.

Figure XXXII shows the results obtained after fusing erythrocytes from sixteen day old eggs with X-irradiated PyY AA/AAR/TG/TGR cells. Each point is the average of the counts incorporated by two coverslips. There was a decay in the incorporation of each purine precursor during the first three days after fusion before it rose again to nearly return to the initial levels by the sixth day. The similarity in the behaviour of all the activities is striking. The incorporation of uridine was determined in parallel and is also plotted as a percentage of the initial value on Figure XXXII. It dropped rapidly until the fourth day after fusion after which it settled at 10-15% of the initial value. The decay in the incorporation was due to the loss of cells from the coverslips because of their X-irradiation before fusion. The uridine incorporation by the fused preparation and control unfused X-irradiated PyY AA/AAR/TG/TGR cells were identical throughout the experiment. Treatment with Sendai virus and the process of cell fusion therefore did not grossly alter the ability of the variant cells to

FIGURE XXXI

A heterokaryon four days after fusion with fifteen day old egg erythrocytes. Two reactivated erythrocyte nucleii, both of which have nucleoli, are present in the heterokaryon. An unfused hamster cell is partially in the photograph. The preparation was incubated with tririated adenine for four hours before fixation. Only the heterokaryon is labelled.



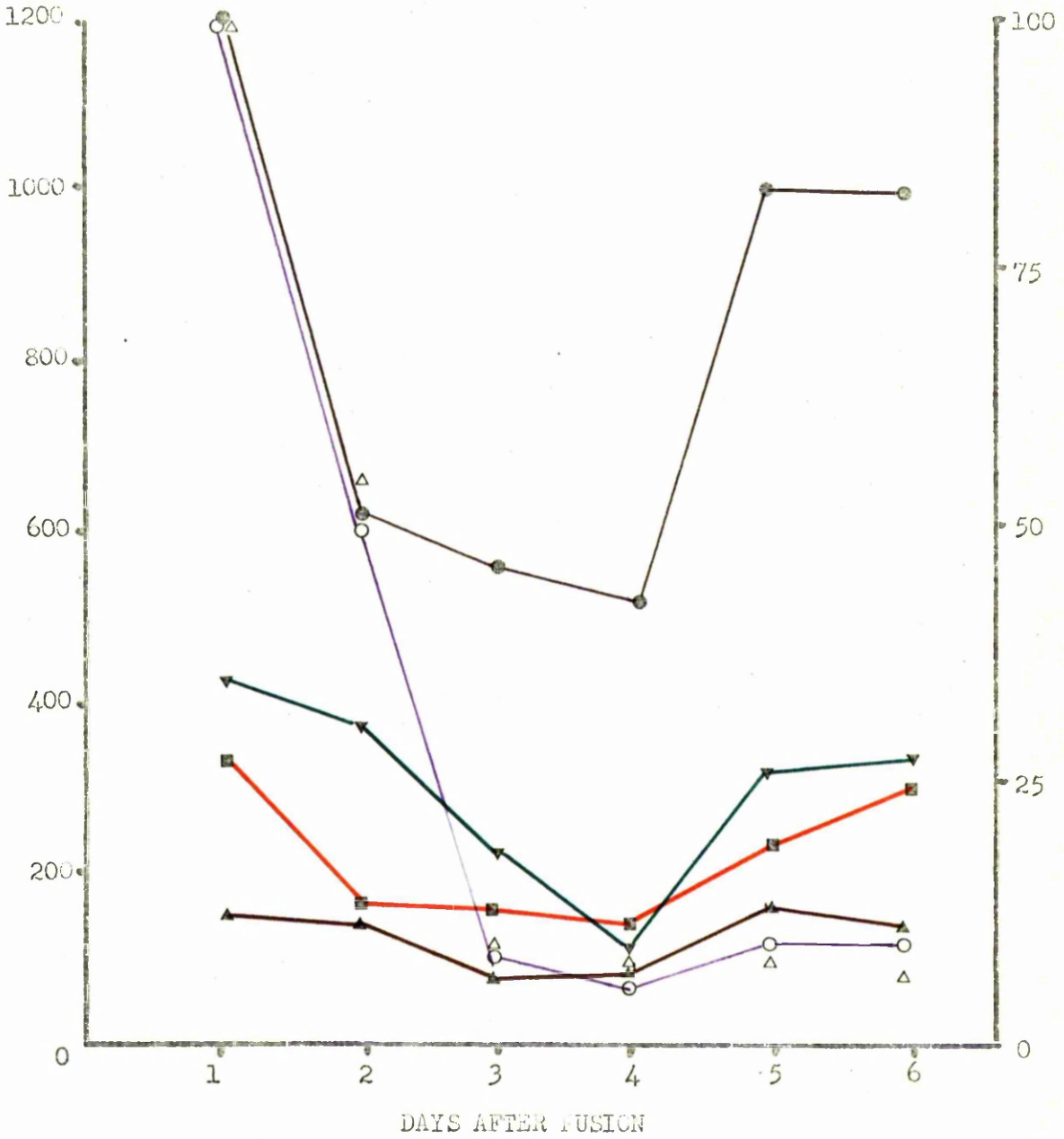


FIGURE XXXII. INCORPORATION OF HETEROKARYONS AS DETERMINED BY SCINTILLATION COUNTING.

Tritiated precursor supplied:

—●— Adenine —■— Adenosine —▼— Hypoxanthine —▲— Guanosine

—○— Uridine fused △ Uridine control (unfused).

incorporate uridine.

The drop in the incorporation of uridine by the coverslip preparations necessitated the normalization of the incorporation of the labelled purine precursors before the incorporation at different times could be compared. Figure XXXIII shows the counts obtained due to the incorporation of the four different purine precursors normalized to the incorporation of uridine. The initial plot on each curve is the incorporation expected by unfused PyY AA/AAR/TG/TGR cells. There was increased APP activity in the fused preparation at one day after fusion, this was due to passive transfer. Little if any increase in activity occurred before the second day. Between the second and the fourth days after fusion there was an increase in the incorporation of all four purine precursors. The increase in the activities of the purine salvage enzymes was closely similar to that obtained by grain counting (Figure XXX) both in the timing of the reactivation process and the relative incorporation of the different precursors. In both cases APP was by far the most active. The two determinations were carried out using material obtained in different experiments.

The loss of cells makes interpretation of this experiment difficult. Preliminary results indicate that it will be possible to reduce the loss by lowering the dose of X-rays given to the PyY AA/AAR/TG/TGR cells to 1000 rads.

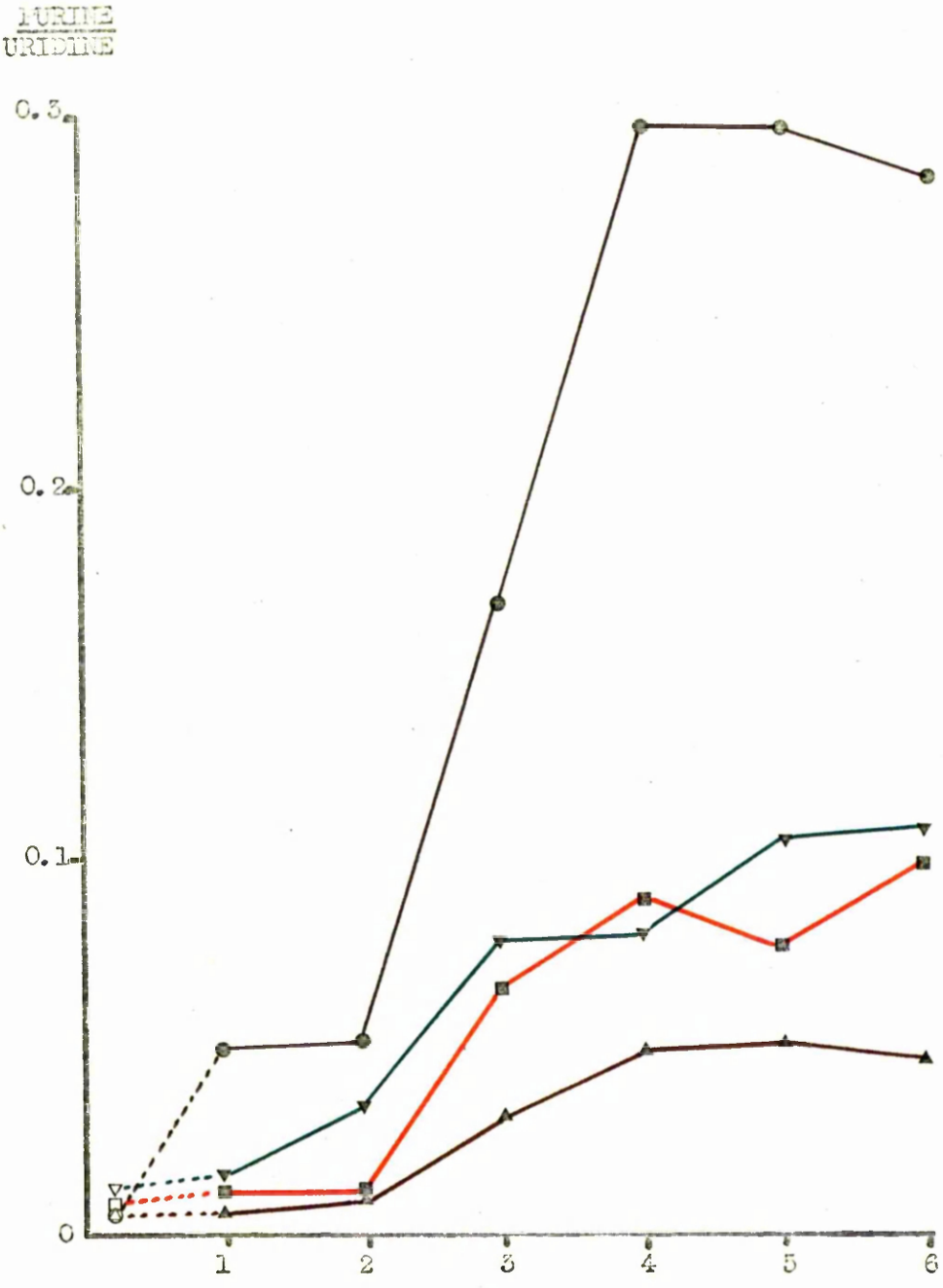


FIGURE XXXIII. INCORPORATION OF HETEROKARYONS EXPRESSED AS THE RATIO OF THE COUNTS AFTER INCUBATING WITH VARIOUS PURINES AND URIDINE.

Tritiated precursor supplied to the heterokaryons:
—●—Adenine —■—Adenosine —▼—Hypoxanthine —▲—Guanosine
Control ratios of unfused FyI AA/AAR/TG/TGR cells. (Figure XII.)
○Adenine □Adenosine ▽Hypoxanthine △Guanosine

10. DISCUSSION

1. Characterisation of cell lines

The growth curves for the PyY and PyY AA/AAR/TG/TGR cells were identical. Single point determinations of the number of PyY AA/AAR and PyY TG/TGR cells at 7 days after plating indicated that these too had similar growth rates. The similarity was confirmed by results shown in Chapter 7. After inoculating 1 x 10^5 cells to a baby bottle, there was about a fifty-fold increase in the number of both PyY, PyY AA/AAR and PyY TG/TGR cells during the week after plating. The rate of growth was not constant, there being a slowing after 3-4 days, until by 6-7 days the number of cells in the monolayer ceased to increase. Changing the medium at 6 days stimulated growth and higher cell densities were achieved. Otsuka (1972), also using polyoma transformed BHK 21/C13 cells, observed the same phenomenon, and showed that stimulation could be achieved without changing the medium by adding serine, glycine or pyruvate. Limitation of these substances may in some way cause the cessation of growth.

The process of biochemical selection had thus not affected the rates of growth of the variants in liquid medium and all continued to be capable of being cloned in agar suspension.

When exposed to purine and pyrimidine analogues for which resistance had not been selected, the variant cells showed the same sensitivity as the wild type cells. The development of resistance was not a blanket one to all purine or pyrimidine analogues, but specific to each analogue. An exception was the PyY TG/Car/BUdR variant being resistant to TGR, which will be discussed later, and perhaps another exception was in the case of the PyY TG/TGR cells surviving slightly better than the PyY cells at low concentrations of AA. Slight alterations in sensitivity such as this could be achieved by an increase in the de novo synthesis of purines, diluting

out the analogue and competing with it inside the cell. This alteration might be selected for by growth in the other inhibitors.

The phenotypes of the variants were also confirmed by growth in aminopterin with either adenine or hypoxanthine as a purine source. PyY AA/AAR cells grew in HAT but not AAT and PyY TG/TGR cells grew in AAT but not in HAT. The PyY AA/AAR cells could not utilize preformed adenine as a purine source and PyY TG/TGR could not utilize hypoxanthine.

The incorporation of preformed purine and pyrimidine nucleic acid precursors was directly tested by supplying the cells with tritiated material. The incorporation of the tritiated purines and pyrimidines into macromolecules was assayed both by scintillation counting and by autoradiography. The patterns of incorporation by the variants were as expected. Those resistant to an analogue of a purine or a pyrimidine were, in general, incapable of incorporating significant quantities of that purine or pyrimidine.

In the case of one variant there was unexpected incorporation. PyY AA/AAR cells continued to incorporate small quantities of adenine and had only a 50% reduction in their capacity to incorporate adenosine. Some adenine may have become deaminated, either intracellularly or extracellularly, to produce hypoxanthine which the PyY AA/AAR cells were capable of incorporating.

In the case of adenosine deamination by adenosine deaminase to produce inosine is well established. Tubercidin does not undergo deamination (Roy-Burman, 1970) and so since the enzyme adenosine deaminase does not take part in a lethal synthetic step, there is no selection for its absence. Cells resistant to tubercidin therefore might be expected to continue to be capable of incorporating adenosine via the deamination pathway. They would be expected to have a reduced ability to incorporate adenosine via adenosine kinase since tubercidin is capable of phosphorylation to an inhibitory derivative, tubercidin monophosphate, by this enzyme. Normal levels of adenosine kinase activity have however been found in the PyY AA/AAR cell variants (Edwards, 1970). The biochemical basis of resistance to

tubercidin is not fully understood, but there is evidence to suggest that there may have been a modification in the permeability of the cells to tubercidin.

Conversely, there are examples of variants being unable to incorporate purines and pyrimidines to whose analogues they had not previously been exposed. PyY TG/TGR cells have a reduced capability to incorporate uridine. PyY TG/TGR and PyY TG/Car/BUdR cells have a reduced capacity to incorporate adenosine. PyY TG/Car/BUdR cells incorporate very little guanosine.

The PyY TG/TGR variant had not been selected for resistance to any analogues of uridine and there is no reason to consider there to have been any selective pressure in favour of a low uridine incorporation. The PyY AA/AAR/TG/TGR cells, which had been exposed to the analogues TG and TGR in exactly the same manner as the PyY TG/TGR cells did not have such a drastic reduction in their uridine incorporation. Impurities were not present in the preparations of the inhibitors used that selected for the inability to incorporate uridine. It is of interest to note, however, that Syzbalski et al. (1959) found that D 98/AG cells resistant to 8-azaguanine were three-fold more resistant to the pyrimidine analogue 5-fluorouracil than the wild type D 98. No testing of the resistance of the PyY variants has yet been carried out using 5-fluorouracil.

The reduced incorporation of adenosine by the PyY TG/TGR and PyY TG/Car/BUdR cells could be explained by suggesting that the majority of the adenosine was incorporated as inosine after deamination. Since the PyY TG/TGR and PyY TG/Car/BUdR cells were unable to incorporate inosine, label presented to them as adenosine was poorly incorporated. The deamination may take place extracellularly (Hakala, 1969) and so is not incompatible with the suggestion that resistance of these variants to nucleosides lies in an alteration in their permeability (Edwards, 1970).

PyY TG/Car/BUdR cells were found to be cross-resistant to TGR and unable to incorporate labelled guanosine, though not selected for resistance

to TGR. These observations suggest that the routes of incorporation of guanosine and guanine have one or more steps in common. Since the resistance to TG is a single step process and the PyY TG/TGR cells have been shown to lack detectable IPP activity, guanosine may normally be acted on by nucleoside phosphorylase and incorporated as the base by the enzyme IPP. However other pathways may also be used because there was no cross-resistance between TG and TGR during the initial selection of the PyY TG/TGR and PyY AA/AAR/TG/TGR variants (Subak-Sharpe 1969).

The PyY AA/AAR/TG/TGR variant was shown to be capable of reversion in three activities after long periods of growth in the absence of the analogues, becoming capable of incorporating adenosine, hypoxanthine or guanosine. No reversion with respect to APP activity was ever seen. These findings confirm those obtained by Lockhart (1972). In those cases where reversion was seen the resistance to the analogues could not have been due to a deletion.

2. Metabolic co-operation

The presence of metabolic co-operation was demonstrated using double label autoradiography to distinguish the two variants after mixing. The results obtained by Blrk et al. (1968), Subak-Sharpe et al. (1969) and Subak-Sharpe (1969) were confirmed using double label autoradiography. Metabolic co-operation occurred between cells in contact at light microscopic level and caused an alteration in the phenotype of the co-operating variant cells to that of the cell with which it was in contact. Metabolic co-operation requires a donor-recipient relationship between two cells, with the cell exhibiting the enzyme activity being the donor. Depending on the precursor provided a particular variant may act either as the donor or the recipient. When a mixture of PyY AA/AAR and PyY TG/TGR cells was pulsed with adenine PyY AA/AAR cells were the recipient, but when hypoxanthine was used they acted as the donor. Conversely the PyY TG/TGR cells acted as the donor

during pulsing with adenine and the recipient during pulsing with hypoxanthine.

Under selective conditions, the phenotypic change caused by metabolic co-operation may result in either of two effects depending on the type of selective system being used (Fujimoto et al., 1971). When the cells were made auxotrophic for a purine source an intact salvage path allowing the incorporation of preformed purine from the medium was essential for their survival. Co-operating cells, though normally incapable of incorporating the purine supplied, survived and grew under these conditions because of their phenotypic change. In this situation metabolic co-operation has been termed "the kiss of life". In the presence of a purine analogue an intact purine salvage pathway was lethal for the cell. Co-operating cells were killed under these conditions because of their phenotypic change. Metabolic co-operation in these circumstances has been termed "the kiss of death". The kiss of life and the kiss of death were first demonstrated using mixtures of IPP negative and wild type human fibroblasts.

AA, TG, HAT and AAT provided four selective systems to test for the presence of "the kiss of death" and "the kiss of life" using PyY AA/AAR and PyY TG/TGR cells both as donors and as recipients. "The kiss of life" was demonstrated using either variant as the recipient. Conditions allowing "the kiss of death" to affect the selective process were not achieved. It was probable that an overkill situation was present. The sensitive cells were killed rapidly and perhaps also some of the co-operating cells, but enough resistant cells survived to grow to confluence. "The kiss of death" might be demonstrable in this system by using different initial proportions of the two variants. If the sensitive cells were present in excess, the resistant cells would have less chance of escaping the effects of metabolic co-operation.

The half lifes of the phenotypic change in the incorporation of adenine by the PyY AA/AAR cells and that of hypoxanthine by PyY TG/TGR cells brought about by metabolic co-operation were investigated. They were found to be

too short to detect by resuspending a confluent culture containing a mixture of both variants and pulsing immediately in suspension. The incorporation of adenine and hypoxanthine due to metabolic co-operation was almost certainly in both cases dependent on continued cell contact. Metabolic co-operation in these systems was therefore due to the transfer of labelled nucleotide or nucleic acid between the cells. Cox et al. (1970) using Lesch-Nyhan cells, and Pitts (1971) using PyY TG/TGR cells, showed that the half life of metabolic co-operation was negligible in the case of hypoxanthine incorporation.

A second line of evidence pointing to the molecular basis of metabolic co-operation was obtained. After fusing PyY AA/AAR/TG/TGR cells with chicken erythrocytes there was in some cases passive transfer of APP and IPP activities to the heterokaryons. Heterokaryons containing non-reactivated erythrocyte nuclei could take part in metabolic co-operation by acting as donors of the ability to incorporate either adenine or hypoxanthine to unfused PyY AA/AAR/TG/TGR cells in contact with them. Metabolic co-operation in this situation could not have been due to the transfer of informational macromolecules because they were not being synthesised, (assuming that the passively transferred activity from the chicken erythrocyte was due to the transfer of protein and not mRNA). The transfer of regulating molecules can be discarded for the same reason. The remaining possibilities are the transfer of labelled material, either nucleotides or labelled nucleic acid, or the transfer of preformed enzyme. The former possibility is probably the case. A limited number of molecules of the chicken enzyme enter the heterokaryon at the time of cell fusion and it is unlikely that these would be transferred to the unfused co-operating cells.

Some evidence suggested that metabolic co-operation could take place in the absence of labelled precursor in the medium. After mixing prelabelled cells with unlabelled cells, some of the cells in contact with heavily labelled cells became lightly labelled. If the light labelling can be shown

to be due to metabolic co-operation, the molecular basis could only have been by the exchange of labelled material between the cells. The labelled cells were the sole source of labelled material during the time the cells were in contact.

More than one mechanism of metabolic co-operation may act in any one cell type, and in the case of different cells and different enzyme activities involve the use of several mechanisms. However all the above evidence points to there being a transfer of nucleotides or labelled nucleic acid between co-operating BHK 21/C13/PyY cell variants.

Autoradiography is a highly sensitive technique for detecting enzyme activity. The presence of the protein is not detected directly, and very few molecules of an active enzyme need be present in a cell in order to produce detectable labelling. The position of the labelled material in the cell at the time of fixation may bear no relationship to the location of the enzyme causing the incorporation. In the case of the purine and pyrimidine salvage pathways, once the precursor has been phosphorylated it is free to travel anywhere inside the cell either as a nucleotide or a macromolecule. The cell membrane is normally impermeable to nucleotides and macromolecules and the labelled purine precursors are retained within the cell after phosphorylation. However, during metabolic co-operation either nucleotides or labelled nucleic acid are capable of passing between cells in contact at the level of the light microscope. Because of the degree of labelling of co-operating cells and the passage of significant quantities of labelled material over distances of several cells within a few hours the small rapidly diffusing nucleotides are most probably transferred between co-operating cells.

Excitable animal cells are in ionic contact by means of low resistance pathways (Lowenstein, 1966). Ionic coupling also can exist between both normal and transformed cells in culture (Furschpan and Potter, 1968). Studies on the cell contacts between coupled cells have suggested that the gap junction serves as the low resistance pathway (Peyton, Bennett and

Pappas, 1969). Gilula, Reeves and Steinbach (1972), have correlated the presence of gap junctions, ionic coupling, and metabolic co-operation using two cells unable to incorporate hypoxanthine when grown alone, DA and A9, and cells capable of incorporating hypoxanthine, Don cells. In mixed culture metabolic co-operation took place between Don:DA cells, but not between the Don:A9 cells. Don cells were ionically coupled and also made gap junctions with DA cells. Neither ionic coupling nor gap junctions were seen between A9 cells and Don cells. These results are strong circumstantial evidence that the presence of gap junctions is needed between cells to allow them to be in ionic contact and to take part in metabolic co-operation. They are also compatible with the molecular basis of metabolic co-operation being the transfer of nucleotides between cells via gap junctions. It is not known why L cells and their derivatives should be incapable of forming gap junctions and participating in metabolic co-operation.

Metabolic co-operation has been demonstrated to occur over long distances and to involve several cells (Subak-Sharpe et al., 1969). Metabolic co-operation has been shown to take place between human cells in vitro (Freedman et al., 1968), and in vivo. Tumours in hamsters formed from PyY TG/TGR cells were capable of incorporating tritiated hypoxanthine (Pitts, 1972). Within the body, metabolic co-operation may be an important form of intracellular communication over quite large distances. It may explain why heterozygotes for the Lesch-Nyhan syndrome are phenotypically normal. Crick (1970) lent weight to the theories based on the formation of gradients that have been advanced in attempts to understand morphogenesis.

There appears to be no physical reason why molecules about the size of cyclin AMP should not be able to set up gradients over distances of 30 cells or more and influence the behaviour of the cells. Crick termed such molecules morphogens. The setting up of a gradient over a line of cells will be considered as a model. At one end of the line there is a source of the morphogen which produces the chemical and maintains a constant

concentration. At the other end of the line there is a sink, destroying the morphogen and keeping the concentration at a fixed low level. The morphogen diffuses from one cell to the next down the line and after a time approaches a dynamic equilibrium. If the diffusion constant is everywhere the same, the concentration gradient of the morphogen will be linear. At any one point the concentration of the morphogen is unique and allows the cell at that place to relate its position to those of the source and the sink.

It would be inefficient to have the cell membranes easily permeable to the morphogen and there may be a special mechanism allowing the rapid passage of morphogen between the cells within which the gradient has been set up. Crick suggested that tight junctions may be involved. In practice gradients may be set up in three dimensions, and a single cell may be simultaneously affected by several gradients. If, as seems likely, metabolic co-operation is due to the transfer of nucleotides between cells, it provides a model system for the study of concentration gradients within cells.

3. Reactivation of chicken erythrocyte nuclei within BHK 21/C13/PyY cell variants

The reactivation of the chicken erythrocyte took place within the PyY variants. It followed a similar pattern to that described in mouse A9 cells, (Harris et al., 1969; Harris and Cook 1969; Cook, 1970; Deák et al., 1972 and Dendy, 1972). The avian erythrocyte is a metabolically inactive and highly specialized cell, but after fusion with a metabolically active cell the pattern of differentiation of its nucleus can be altered. Other examples of nuclear reactivation occurring are sperm pronuclei following fertilization and nuclei from Xenopus brain cells after injection into activated eggs (Gurdon and Woodland, 1968). In both cases the sequence of events was the same as within the reactivating chick erythrocyte nuclei. The mechanisms by which chromatin is packed in these condensed nuclei are

unknown but the chick erythrocyte nucleii and sperm nucleii have distinctive histones which may in some way be related to the packaging.

The reactivation process of chicken erythrocytes from eggs of the same age within the BHK 21/C13/PyY hamster cell variants took place more rapidly than in the A9 mouse cell. However the appearance of all the chick specified functions followed to date (IPP activity, chick surface antigen and sensitivity to diphtheria toxin using L cells, nucleolar material in HeLa cells, and IPP, APP, the two purine nucleoside permeases and perhaps also dCK and TK using BHK 21/C13/PyY variants) could be correlated with the presence of a functional nucleolus within the erythrocyte nucleus of the heterokaryon.

Ringertz et al. (1971) followed the process of reactivation in HeLa cells using fluorescent labelled antibody against various nuclear components. They demonstrated the entry of human proteins into the erythrocyte nucleus during reactivation. The appearance of nucleoli was followed using antisera to both human and chick nucleolar material. Material reacting with an antiserum to human nucleoli was present in some fifteen day old erythrocyte nucleii less than ten hours after fusion. The pattern of fluorescence altered from a few weakly staining spots to two or three distinct nucleolar bodies as the nucleus enlarged and reactivation progressed. Positive results using anti-chick nucleolar antiserum were initially obtained in 20% of the erythrocyte nucleii by twenty hours after fusion. The reaction with the anti-human antiserum was stronger than with the anti-chick. At later times the nucleoli of the HeLa nucleii of the heterokaryons became positive when treated with anti-chick nucleolar antiserum. Reactivated erythrocyte nucleii also reacted with anti-human nucleoplasm antiserum.

Human nucleolar antigens accumulated in a specific manner within the reactivating erythrocyte nucleii perhaps because of an affinity for the nucleolar organizing regions. Whether the affinity was sufficient to allow nucleoli which were composed of human material to function within erythrocyte

nucleii has yet to be established. The appearance of chicken nucleolar material within the HeLa nucleoli of the heterokaryons, which there was no reason to suppose were non-functional, supports the notion that nucleolar material can function within the nucleolus of another species. The nucleolus is closely involved in the expression of the genetic information within its nucleus. The reactivating erythrocyte nucleus within a HeLa cell may require the formation of a functional primary nucleolus from human material to allow synthesis of material needed for the production of a chicken specified nucleolus.

The small nucleoli seen in the PyY variants may initially be formed of hamster material. The timing of their appearance and their morphology are very similar to those described by Ringertz et al. (1971) being present in 50% of erythrocyte nucleii from twelve day eggs at twenty-four hours after fusion. X-irradiation of the PyY variants before fusion had no effect on the course of reactivation or the appearance of nucleoli. These small nucleoli were shown in some cases to be heavily labelled by RNA precursors. Nucleoli were present in 10% of the dikaryons formed between eleven to fourteen day old egg erythrocytes and X-irradiated mouse A9 cells on the fourth day after fusion (Harris and Cook, 1969). The difference in timing of the appearance of nucleoli in erythrocyte nucleii fused with different cells may be due to the use of different criteria for the appearance of nucleoli (which in the PyY cells was a gradual process) or to a difference in the process of reactivation.

The entry of foreign proteins into the erythrocyte nucleus is an integral part of the reactivation process. The ability of the foreign material to function within the erythrocyte nucleus may be critical in determining the rate of reactivation, and may differ between host cells. The formation of a functional primary nucleolus from foreign material may be one such critical step. Perhaps nucleolar material from HeLa and PyY cells acts more effectively than that of the A9 cells and allows the production of detectable

chick-specified functions after a shorter time.

The biochemical defects present in the PyY AA/AAR/TG/TGR cells have been precisely determined in two of the four systems. The enzyme activities APP and IPP were lacking from these cells by in vitro assays. However, normal levels of AK and I/GK were present despite the inability of the cells to incorporate tritiated nucleosides (Edwards, 1970). There was no cross-resistance between any of the analogues so all four resistances were genetically distinct. It is thought most likely that resistance to tubercidin and 6-thioguanosine was due to the loss of a specific permease in each case. Whatever the precise nature of the defects, heterokaryons formed between these cells and reactivated erythrocyte nuclei were capable of incorporating every purine precursor tested. Probably at least four different chicken proteins needed to be specified in order to repair the genetic defects of the hamster cell variant.

It might be suggested that enzymes involved in the purine salvage pathway of the chicken have a different specificity to those of the hamster. For example, there may be only one phosphorylase for all the purine bases, so simultaneous appearance of APP and IPP activities would be expected. It is doubtful if there would be any permease activity for nucleosides resulting from the production of a pyrophosphorylase, so differing substrate specificities between the enzymes of the chick and the hamster do not explain the repair on the basis of a single chick enzyme. Chicken APP and IPP activity can be separated because fifteen day old erythrocytes incorporate significant quantities of adenine, but very little hypoxanthine. Therefore a minimum of three chick proteins need to be specified in order to restore the purine salvage pathway of the PyY AA/AAR/TG/TGR cells, APP, IPP and one (or two) nucleoside permeases. The appearance of all of these in heterokaryons followed the same time course.

Another explanation could be that the enzyme activities were hamster specified and induced by the presence of the reactivated erythrocyte nucleus.

There was significant reversion in three of the systems, allowing the cells to incorporate adenosine, hypoxanthine and guanosine. The structural genes for these activities were still present in the genome of the PyY AA/AAR/TG/TGR cells and thus potentially available for transcription. In the case of APP activity reversion was never detected, so the structural gene could have been deleted. To prove the production of chick specified purine salvage enzymes, it would be necessary to extract them from heterokaryons containing reactivated erythrocyte nucleii and show a difference in properties from the enzymes of the PyY cells and a similarity to those of chicken cells. Cook (1970) showed the IPP activity in heterokaryons containing A9 cells and reactivated erythrocyte nucleii differed in its properties from that of the mouse enzyme and was indistinguishable from chick IPP.

The striking similarity in the kinetics of the appearance of all four activities in heterokaryons formed from PyY AA/AAR/TG/TGR cells and reactivating erythrocyte nucleii, their biochemical diversity, genetic independence and association with a reactivated erythrocyte nucleus containing a nucleolus all point to their being due to chicken specified proteins.

It has been suggested that the nucleolus is in some way involved in the transfer from the nucleus to the cytoplasm of RNA carrying information for the synthesis of specific proteins (Harris et al., 1969). Confirmation that some function at or near the nucleolus was essential for the full expression of genetic information was obtained after inactivating the nucleoli of reactivated erythrocyte nucleii in A9 cells by irradiating with a U-V microbeam and following the ability of the heterokaryon to incorporate hypoxanthine (Deák et al., 1972). It was found that there was decay in the incorporation after irradiating the nucleolus if only one was present in the erythrocyte nucleus. Both nucleoli had to be irradiated if two were present to achieve a reduction in the incorporation. Irradiation of only one of two nucleoli or an extranucleolar area did not achieve a reduction in the incorporation of hypoxanthine.

The manner in which the nucleolus or para-nucleolar structures are involved in the expression of genetic information is unknown. The production of processed mRNA and rRNA may be intimately linked, nucleolar participation may in some way be essential for the export of mRNA to the cytoplasm, the nucleolus may be involved in the control of translation or the relationship may be more distant through mechanisms at present unknown. Results obtained using the PyY variants confirm that some relationship exists but throw no further light on the mechanism.

4. Therapeutic applications

Genetically marked cells were used in this study for two avenues of investigation, metabolic co-operation and the reactivation of the avian erythrocyte nucleus. Both involve the alteration of the phenotype of the variant cells and have implications for the treatment of human genetic disease.

The way in which metabolic co-operation affects the diagnosis of carriers of the Lesch-Nyhan syndrome and the use of selective procedures to overcome the effect have been discussed.

At present, inborn errors of metabolism are treated symptomatically in one of two ways. Firstly, by restricting the intake of compounds unable to be metabolized. For example, a diet low in phenylalanine reduces the incidence of mental retardation in phenylketonurics. Secondly, drug therapy can prevent the accumulation of harmful metabolites by de novo synthesis. The enzyme xanthine oxidase can be inhibited by allopurinol, reducing the excess production of uric acid in the Lesch-Nyhan syndrome and gout. The majority of these diseases have no treatment however. With the ability to transfer genetic information between cells, there arises the possibility of removing the symptoms by repairing the genetic defect in the somatic cells of the patient.

The presence of a minute fraction of the chicken genome is sufficient to correct the A9 cell defect (Schwartz, et al., 1971). Cells from a Lesch-Nyhan patient might be able to be manipulated in a similar fashion and reimplanted in the patient. The presence of metabolic co-operation implies not all the cells of a defective individual need be treated in this way, but the presence of a few cells of normal genotype may be able to modify the phenotype of a whole organ. Though theoretically possible at present, and perhaps practicable in the near future, such techniques may be of value in only a few situations. The Lesch-Nyhan syndrome produces brain damage that is irreversible, probably at an early stage, and any treatment after this has occurred could never produce a full return to normal. Nor is it known whether the syndrome is to some extent due to a function of IPP not connected with the phosphorylation of hypoxanthine or guanine but necessary in certain target cells.

Metabolic co-operation has implications in the field of cancer chemotherapy, the success of which depends on exploiting any differences that may exist between normal and malignant cell to selectively kill the malignant cells. Metabolic co-operation obscures differences in the behaviour of cells in contact and reduces the discrimination that can be achieved by a selective system. It may remove the possibility of achieving selective conditions discriminating efficiently between some malignant and normal cells in vivo unless metabolic co-operation can be temporarily prevented. However, the cells of some tumours are not electrically coupled (Lowenstein, 1972) and therefore may be incapable of taking part in metabolic co-operation and more amenable to chemotherapy.

BIBLIOGRAPHY

Albertini, R. J., DeMars, R., 1970. Science, N.Y. 169, 482.

Arlett, C. F., Potter, J., 1971. Mutation Res. 13, 59.

Arnow, L. 1959. J. Pharm. Exptl. Therap. 127, 116.

Ayad, S. R., Fox, M., 1968. Nature, London, 220, 35.

Barban, S. 1962. J. biol. Chem., 237, 291.

Barski, G., Sorieul, S., Cornfert, F. 1960. C.r. hebal. Seanc. Acad. Sci., Paris, 251, 1825.

Barski, G., Sorieul, S., Cornfert, F., 1961. J. natn. Cancer Inst. 26,1269.

Barski, G., 1968. Int. J. Cancer, 3, 320.

Baserga, R. 1962. J. Cell Biol. 12 633.

Benzer, S. 1971 Nature, N.B., London 234 129. Report of 3rd Ciba Found. Symp.

Boeveri, M. 1890. Quoted in Wilson, E.B., 1925. The cell in development and heredity 3rd Edition.

Bolund, L., Darzynkiewicz, Z., and Ringertz, N.R. 1969. Expl. Cell Res., 56, 406.

Boyle, J. A., Raivio, K. O., Astrin, H. H., Schulman, J. D., Graf, M. L., Seegmiller, J. E., Jacobson, C. B. 1970. Science, N.Y. 169, 688.

Bradley, T. R., Roosa, R. A., Law, L. W. 1962. J. Cell Comp. Physiol. 60, 127.

Brenner, S., Milstein, C., 1966. Nature, London, 211, 242.

Brockman, R. W., Kelley, G. G., Stutts, P., Copeland, V. 1961 Nature, London, 191, 469.

Brockman, R. W., 1963. Mechanism of resistance to Anticancer Agents. In Advances in Cancer Research. 7, 129. Eds. Haddow, A., Weinhouse, S.

Bürk, R. R., Pitts, J. D., Subak-Sharpe, J. H., 1968. Expl. Cell Res. 53, 297.

Burchenal, J. H., Robinson, E., Johnson, S. F., Kushida, M. M. 1950. Science, N.Y. 111, 116.

Cameron, I. L., Prescott, D. 1963. Expl. Cell Res. 30, 609.

Chang, R. S. 1957. Proc. Soc. exp. Biol. Med. 96, 818.

Chu, E. H. Y., Malling, H. V., 1968. Proc. natn. Acad. Sci. U.S.A. 61, 1306

Chu, E. H. Y., Brimer, P., Jacobson, K. B., Merriam, E. V. 1969 Genetics, 62, 359.

Cook, P. R. 1970. J. Cell Sci. 7, 1.

Cox, R. P., Krauss, M. J., Earl Balliss, M., Dancis, J., 1970. Proc. natn. Acad. Sci., U.S.A. 67, 1573.

Crick, F., 1970. Nature, London, 225, 420.

Darwin, C., 1859. The Origin of Species.

Davidson, R. L., 1970. Proc. natn. Acad. Sci., U.S.A., 67, 1870.

Davidson, R. L., 1972. Proc. natn. Acad. Sci., U.S.A., 69, 951.

Davidson, J. D., Bradley, T. R., Roosa, R. A., Law, L. W., 1962. J. natn. Canc. Inst., 29, 739.

Davies, H. G., 1961. J. biophys, biochem. cytol. 9, 671.

Deák, I., Sidebottom, E., Harris, H. In press.

DeMars, R. G., Hooper, J. L., 1960. J. exp. Med., 111, 559.

DeMars, R. G., Sarto, J. S., Felix, J. S., Benke, P., 1969. Science, N.Y. 164, 1303.

Dendy, P. R., 1972. D. Phil. Thesis, University of Oxford.

Dubbs, D. R., Kit, S., Detorres, R. A., Anken, M., 1967. J. Virol. 1, 968.

Edwards, R. L., 1970. Personal communication.

Elkind, M. M., Sutton, H., 1960. Radiation Res., 13, 556.

Enders, J. F., Peebles, T. C., 1954. Proc. Soc. exp. Biol. Med. 86, 277.

Engel, E., McGee, B. J., Harris, H., 1969. J. Cell Sci. 5, 93.

Ephrussi, B., Scaletta, L. J., Stenchever, M. A., Yoshida, M. C., 1964. In Cytogenetics of cells in culture. Ed. R. J. C. Harris.

Ephrussi, B., Sorieul, S., 1962. Approaches to the genetic analysis of mammalian cells. 81. In Michigan Conference on Genetics. Eds. Merchant, D. J., Neel, J. V.

Fell, H. B., Hughes, A. F., 1949. Q. Jl. Microsc. Sci. 90, 355.

Fischer, G. A., 1959. Cancer Res., 19, 372.

Flacks, J. G., Erwin, M. J., Buchanan, J. M., 1957. J. biol. Chem. 228, 201.

Flemming, W., 1879. Quoted in Wilson, E. B., 1925. The cell in development and heredity 3rd Edition.

Fox, M., Fox, B. W., Ayad, S. R., 1969. Nature, London, 222, 1086.

Fratantoni, J. L., 1968. Science, N.Y., 162, 570.

Frazer, R. C., 1964. Expl. Cell Res. 33, 473.

Freed, J. J., Mezger-Freed, L., 1970. Proc. natn. Acad. Sci., U.S.A. 65, 337.

Friedman, T., Seegmiller, J., Subak-Sharpe, J. H., 1968. Nature, London, 220, 272.

Fujimoto, W. Y., Seegmiller, J. E., 1970. Proc. natn. Acad. Sci., U.S.A., 65, 577.

Fujimoto, W. Y., Seegmiller, J. E., Uhlendorf, B. W., Jacobson, C. B., 1968. Lancet 11, 511.

Fujimoto, W. Y., Subak-Sharpe, J. H., Seegmiller, J. E., 1971. Proc. natn. Acad. Sci., U.S.A., 68, 1516.

Furshpan, E. J. and Potter, D. D., 1968. Curr. Top. Develop. Biol. 3, 95.

Gadd, R. E. A., Henderson, J. F., 1970. Can. J. biochem., 48, 295.

Gangel, S. G., Merchant, D. J., Shreffler, D. C., 1966. J. natn. Cancer Inst., 36, 1151.

Garrod, A. E. 1909. Inborn Errors of Metabolism.

Grobstein, C. 1961. Expl. Cell Res. Suppl., 8, 234.

Gurdon, J. B., 1962. J. Embryol. exp. Morph. 10, 622.

Gurdon, J. B., Uehlinger, V., 1966. Nature, London, 210, 1240.

Gurdon, J. B., Woodland, H. R., 1968. Biol. Rev. 43, 233.

Hakala, M. T., 1957. Science, N.Y., 126, 255.

Hakala, M. T., 1969. In Axenic cell reactions. Ed. G. L. Trisch.

Hakala, M. T., Ishihara, T., 1962. Cancer Res., 22, 987.

Hakala, M. T., Zakrzewski, S. F., Nichol, C. A. 1961. J. biol. Chem. 236, 952.

Handmaker, S., 1971, Nature 233, 416.

Harris, H., 1965. Nature, London, 206, 583.

Harris, H., 1966, Proc. Roy. Soc. B. 166, 358.

Harris, H., 1967. J. Cell Sci. 2, 23.

Harris, H., 1970. Cell Fusion, The Dunham Lectures.

Harris, H., Cook, P. R., 1969. J. Cell Sci. 5, 121.

Harris, H., Sidebottom, E., Grace, D. M., Bramwell, M. E., 1969. J. Cell Sci., 4, 499.

Harris, H., Watkins, J. F., Ford, C. E., Schoefl, G. I. (1966). J. Cell Sci., 1, 1.

Harris, H., Watkins, J. F., 1965. Nature, London, 205, 640.

Harris, M., 1960. Expl. Cell Res., 21, 439.

Harris, M., 1967. J. natn. Cancer Inst., 38, 185.

Harris, M., Ruddle, F. H., 1960. Cell Physiology of Neoplasia.

Hayflick, L., Moorhead, P. S., 1961. Expl. Cell Res., 25, 585.

Herzenberg, L. A., 1962. J. Cell Comp. Physiol., 60 (Suppl. 1) 145.

Hill, M., Hillova, J., 1972. Nature, N.B. London, 237, 35.

Hosaka, Y., 1962. Biken, J., 5, 121.

Johnson, R. T., Harris, H., 1969. J. Cell Sci. 5, 625.

Johnson, R. T., Rao, A. N., 1970. Nature, 226, 717.

Kao, F., Puck, T. T., 1968. Proc. natn. Acad. Sci., N.Y., 60, 1275.

Kao, F., Puck, T. T., 1969. J. Cell Phys., 74, 245.

Kao, F., Puck, T. T., 1970. Nature, London, 228, 329.

Kelley, G. G., Vail, M. H., Adamson, D. J., Palmer, E. H., 1961.
Am. J. Hyg., 73, 231.

Kidder, G. W., Dewey, V. C., 1949. J. biol. Chem., 179, 181.

Kit, S., Dubbs, D. R., Frearson, P. M., 1966. Int. J. Cancer, 1, 19.

Kit, S., Dubbs, D. R., Piekarski, L. J., Hsu, T. C., 1963. Expl. Cell
Res., 31, 297.

Klebe, R. J., Chen, T. R., Ruddle, F. H., 1970. Proc. natn. Acad. Sci.
N.Y., 66, 1220.

Klein, G., 1959. Can. Cancer Conf. 3, 215.

Kohn, A., 1965. Virology, 26, 228.

Kohn, A., Fuchs, P., 1970. Current Topics in Microbiol. Immunity, 52, 94.

Korn, E. D., Remy, C. N., Wasilejko, H. C., Buchanan, J. M., 1955.
J. biol. Chem., 217, 875.

Kornberg, A., 1957, in The Chemical Basis of Heredity 579.
Eds. McElroy, W. D., Glass, B.

Kornberg, A., Lieberman, I., Simms, E. S., 1955. J. biol. Chem., 215, 417.

Krenitsky, T. A., Elion, G. B., Henderson, A. M., Hitchings, G. H., 1968.
J. biol. Chem., 243, 2876.

Krooth, R. S., Sell, E. K., 1970. J. Cell Phys., 76, 311.

La Du, B. N., Zannoni, V. G., Laster, L., Seegmiller, J. E., 1958. J. biol. Chem., 230, 251.

Lamarck, J. B. P. A., 1815. Quoted in Bell, P. R., (Ed.) 1959. Darwin's biological work.

Lambert, R. A., 1912. J. exp. Med., 15, 510.

Law, L. W., 1951. Proc. Soc. exp. Biol. Med., 78, 499.

Lemann, H., Carrell, R. W., 1969. Brit. Med. Bulletin, 25, 14.

Lesch, M., Nyhan, W. L., 1954. Am. J. Med., 36, 561.

Lieberman, I., Ove, P., 1959a. Proc. natn. Acad. Sci., U.S.A., 45, 867.

Lieberman, I., Ove, P., 1959b. Proc. natn. Acad. Sci. U.S.A., 45, 872.

Liebman, K. C., Heidelberger, C., 1955. J. biol. Chem., 216, 823.

Littlefield, J. W., 1963. Proc. natn. Acad. Sci., U.S.A., 50, 568.

Littlefield, J. W., 1964. Cold Spring Harb. Symp. Quant. Biol., 29, 161.

Lockhart, N., 1972. Personal communication.

Lowenstein, W. R., 1966. Ann. N. Y. Acad. Sci., 137, 441.

Lowenstein, W. R., 1972. Cell interactions Third Lepetit colloquium, 296, Ed. L. G. Silvestri.

Lucy, J. A., 1970. Nature, London, 227, 815.

Lukens, L. N., Herrington, K. A., 1957. Biochim. biophys. Acta., 24, 432.

Lyon, M. F., 1961. Nature, London, 190, 372.

Mathias, A. P., Fischer, G. A., 1962. Biochem. Pharmacol., 11, 69.

Matsumoto, T., Maeno, V., 1962. Virology, 17, 563.

Matsuya, Y., Green, H., Basilico, C., 1968. Nature, London, 220, 1199.

Mendel, G., 1865. Trans. 1966. The Origin of Genetics, a Mendel Source Book, Eds. Stern, C., Sherwood, E. R.

Merril, C., Geir, M. R., Petricciani, J. C., 1971. Nature, London, 233, 398.

Metzgar, D. P., Moskowitz, M., 1963a. Expl. Cell Res., 30, 379.

Metzgar, D. P., Moskowitz, M., 1963b. Expl. Cell Res., 30, 388.

Mezgar-Freed, L., 1972. Nature, N. B., London., 235, 245.

Migeon, B. R., der Kaloustain, V. M., Nyhan, W. L., Young, W. L., Childs, B., 1968. Science, N.Y., 160, 425.

Migeon, B. R., Miller, C. R., 1968. Science, N.Y., 162, 1005.

Miller, O. J., Cook, P. R., Meera Khan, P., Shin, S., Siniscalco, M., 1971. Proc. natl. Acad. Sci., U.S.A., 68, 116.

Minna, J., Nelson, P., Peacock, J., Glazer, D., Nirenberg, M., 1971. Proc. natl. Acad. Sci., U.S.A., 68, 234.

Mohit, B., Fan, K., 1971. Science, N.Y., 171, 75.

Moore, G., 1964. Expl. Cell Res., 36, 422.

Morris, N. R., Fischer, G. A., 1960. Biochim biophys. Acta, 42, 183.

Naha, P. M., 1969. Nature, London, 223, 1380.

Naha, P. M., 1970. Nature, London, 228, 166.

Neff, J. M., Enders, J. F., 1967. Proc. Soc. exp. Biol. Med., 127, 260.

O'Callaghan, C., Stevens, G. W., Wood, J. F., 1969. Brit. J. Radiol. 42, 862.

Okada, Y., 1958. Biken J., 1, 103.

Okada, Y., 1962. Exptl. Cell Res., 26, 98.

Okada, Y., 1970. Current Topics in Microbiology and Immunity, 48, 102.

Okada, Y., Tadokoro, J., 1962. Exptl. Cell Res., 26, 108.

Otsuka, H., 1972. J. Cell. Sci., 16, 137.

Pasternak, C. A., Fischer, G. A., Handschumacher, R. E., 1961. Cancer Res., 21, 110.

Payton, B. W., Bennett, M. V. L., Pappas, G. D., 1969. Science, N.Y., 166, 1641.

Peters, R. A., 1952. Proc. Roy. Soc. Ser. B, 139, 143.

Peterson, J. A., Weiss, M. C., 1972. Proc. natl. Acad. Sci., U.S.A., 69, 571.

Pitts, J., 1971. Ciba Found. Symp. Growth Control in Culture 89. Eds. Wolstenholme, G. E. W. and Knight, J.

Pitts, J., 1972. Cell interactions, Third Lepetit Colloquium, 272. Ed. L. G. Silvestri.

Pontecorvo, G., 1959. Trends in Genetic Analysis.

Pontecorvo, G., 1971. Nature, London, 230, 367.

Poole, A. R., Howell, J. I., Lucy, J. A., 1970. Nature, London, 227, 810.

Poste, G., 1970. Adv. Virus Res., 16, 303.

Potter, M., Law, L. W., 1957. J. natn. Cancer Inst., 18, 413.

Puck, T. T., Kao, F-T., 1967. Proc. natl. Acad. Sci., U.S.A., 58, 1227.

Puck, K. K., Marcus, P. I., 1955. Proc. natl. Acad. Sci., U.S.A., 41, 432.

Quasba, P. K., Aposhian, H. V., 1971. Proc. natl. Acad. Sci., U.S.A., 68, 2345.

Remy, C. N., Smith, M. S., 1957. J. biol. Chem., 228, 325.

Ringertz, N. R., Bolund, L., 1969. Expl. Cell Res., 55, 205.

Ringertz, N. R., Carlsson, S-M., Ege, T., Bolund, L., 1971. Proc. natl. Acad. Sci., U.S.A., 68, 3228.

Roosa, R. A., Bradley, T. R., Law, L. W., Herzenberg, L. A., 1962. J. cell. comp. Physiol., 60, 109.

Rosenbloom, F. M., Kelley, W. N., Henderson, J. F., Seegmiller, J. E., 1967. Lancet, 11, 305.

Roy-Burman, P., 1970. Analogues of nucleic acid components. Recent Adv. Cancer Research, 25.

Ruddle, F. H., Chapman, V. M., Chen, T. R., Klebe, R. J., 1970. Nature, 227, 251.

Ruddle, F. H., Chapman, V. M., Ricciuti, F., Murnane, M., Klebe, R., Meera-nahn, P., Nature, N.B. London, 232, 69.

Salzmann, J. n., DeMars, R., Benke, P., 1968. Proc. natl. Acad. Sci. U.S.A. 60, 545.

Sanford, K. K., Earle, W. R., Likely, G. D., 1948. J. natn. Cancer. Inst., 9, 773.

Santichiara, A. S., Nabholz, M., Miggiano, V., Darlington, A. J., Bodmer, W., 1970. Nature, 227, 248.

Scaletta, L. J., Ephrussi, B., 1965. Nature, London, 205, 1169.

Schneeberger, E. E., and Harris, H., 1966. J. Cell Sci., 1, 401.

Schneider, J. A., Weiss, M. C., 1971. Proc. natl. Acad. Sci., U.S.A., 68, 127.

Schwartz, A. G., Cook, P. R., Harris, H., 1971. Nature, N.B., London, 230, 5.

Seegmiller, J. E., Rosenbloom, F. M., Kelley, W. N., 1967. Science, N.Y. 115, 1682.

Sidebottom, E., Harris, H., 1969. J. Cell Sci., 5, 351.

Sinclair, W. K., Morton, R. A., 1965. Biophys. J., 5, 1.

Stoker, M., 1967. J. Cell Sci., 2, 293.

Stoker, M., Macpherson, I., 1964. Nature, London, 203, 1355.

Subak-Sharpe, J. H., 1965. Expl. Cell Res., 38, 1066.

Subak-Sharpe, J. H., 1969. In Ciba Found. Symp. on Homeostatic Regulators. 276. Eds. Wolstenholme, G. E. W., Knight, J.

Subak-Sharpe, J. H., Bürk, R. R., and Pitts, J. D., 1966. Heredity, 21, 342.

Subak-Sharpe, J. H., Bürk, R. R., Pitts, J. D., 1969. J. Cell Sci., 4, 353.

Subak-Sharpe, J. H., Gentry, G. A. and Jamieson, A., 1972. Proc. 2nd Int. Congress of Virology. 103, Ed. J. L. Melnick.

Szybalska, E. M., Szybalski, W., 1962. Proc. natl. Acad. Sci., U.S.A., 48, 2026.

Szybalski, W., 1959. Expl. Cell Res., 18, 867.

Szybalski, W., Smith, M. J., 1959a. Proc. Soc. exp. Biol. Med., 101, 662.

Szybalski, W., Smith, M. J., 1959b. Fed. Proc. 18, 336.

Szybalski, W., Szybalska, E. M., Ragni, G., 1962. Nat. Canc. Inst. Monograph, 7, 75.

Tomkins, G. M., Thompson, E. B., Hayashi, S., Gelehrter, T., Spanner, D., Petekovsky, A., 1966. Cold Spring Harb. Symp. Quant. Biol., 31, 349.

Thompson, L. H., Mankovitz, R., Baker, R. A., Till, J. E., Siminovitch, L., Whitmore, G. F., 1970. Proc. natl. Acad. Sci., U.S.A., 66, 377.

Thompson, L. H., Mankovitz, R., Baker, R. M., Wright, J. A., Till, J. E., Siminovitch, L., Whitmore, G. F., 1971. J. Cell Phys., 78, 431.

Tomizawa, S., Arnow, L., 1960. J. Pharm. Explt. Therap. 128, 107.

Van Beneden, E., 1884. Quoted in Wilson, E. B., 1925. The cell in development and heredity 3rd Ed.

Vogt, M., 1959. Genetics, 44,1257.

de Vries, 1900. Quoted in Sturtevent, A. H., Beadle, G. W., 1939. An introduction to genetics.

Way, J. L., Parkes, R. E., 1958. J. biol. Chem. 231, 467.

Weiler, E., 1959. Explt. Cell Res. Suppl., 7, 244.

Weismann, A., 1885. Quoted in Wilson, E. B., 1925. The cell in development and heredity 3rd Edition.

Weiss, M. C., Ephrussi, B., 1966. Genetics, 54, 1095.

Weiss, P., 1939. Principles of development.

Williams, W. J., Buchanan, J. M., 1953. J. biol. Chem., 203, 583.