Biol. Rev. (1974), **49**, *pp.* 51–84
BRCPAH 49–3

ON THE INHERITANCE OF DIFFERENTIATED TRAITS

By P. R. COOK

Sir William Dunn School of Pathology,
South Parks Road, Oxford

(*Received* 7 *June* 1973)

CONTENTS

I. INTRODUCTION

It is now generally assumed that nearly all the cells of one organism contain similar genetic information; their DNA contains identical base sequences. This assumption rests upon the demonstration that whole plants can be grown from one differentiated plant cell (Steward, 1970) or a whole animal generated from one differentiated nucleus transplanted into an enucleate frog's egg (Gurdon & Laskey, 1970). During differentiation, identical genes are expressed differently in the cells of one organism. In diploid cells the two copies of each gene are probably coordinately controlled and so differences in the expression of the same gene must be studied

in different cells. A more basic approach is to consider those situations where homologous DNA molecules, within one cell, behave differently. For example, genes on only one of the two *X*-chromosomes in female cells of eutherian mammals are expressed (Lyon, 1971, 1972). An analogous situation is that where only one of the two alleles that code for a flagellar protein is expressed in the bacterium *Salmonella* (Iino, 1969). In conjunction with these examples, position effects (Lewis, 1950; McClintock, 1950) and particularly, variegated position effects in the fruit-fly, *Drosophila* (Baker, 1968), will be discussed. Here identical genes, not within the same but within different cells of the same tissue, behave differently, and this behaviour is critically determined by linkage to neighbouring genes. An understanding of position effect variegation should prove fundamental to an understanding of differentiation, for this phenomenon reveals a mechanism whereby any gene may be switched on or off.

As a result of the success of Jacob and Monod's ideas (Jacob & Monod, 1961; Monod & Jacob, 1961) in explaining the regulation of bacterial gene expression, behavioural differences of identical base sequences in differentiated cells are usually explained in terms of an association of the base sequences with repressor or activator molecules. Alternative explanations of these phenomena will be discussed. For example, differences in the expression of identical base sequences might arise from differences in gene superstructure. The term 'superstructure' will be used to include the secondary and higher-order structures that might be superimposed on the primary base sequence of a nucleic acid. If gene superstructure is a major factor determining gene expression, it seems likely that superstructures of DNA – like primary base sequences – may be replicated and inherited. DNA may therefore contain two kinds of heritable information: one kind which is stored in the primary base sequence of a gene, and a second kind which is acquired during development and is contained in its superstructure. This argument has been summarized elsewhere (Cook, 1973).

A particular phenotype may be inherited by progeny cells arising as a result of mitosis or meiosis. The purpose of this paper is to discuss the assumptions inherent in various explanations of the way differentiated traits are inherited through mitotic events. The inheritance, through meiosis, of differentiated traits is discussed separately in Section VIII(3). The creation, *ab initio*, of differences in behaviour of hitherto identical base sequences will only be of peripheral concern.

Two terms will be introduced and defined. The term 'differential' is defined as that which constitutes the specific difference between nucleic acids that have identical coding properties and which causes their differing behaviour. The differential is recognized by its property of permitting a gene to be expressed. The creator of the differential is defined as the 'differentiator'. There are two kinds of differential, simple and complex. Identical base sequences may behave differently for one of two reasons: either because their superstructures are intrinsically different or because they are associated with regulatory molecules in the environment. A difference in gene superstructure would constitute a simple differential; a complex differential depends upon the continuing association of a differentiator with a gene. The

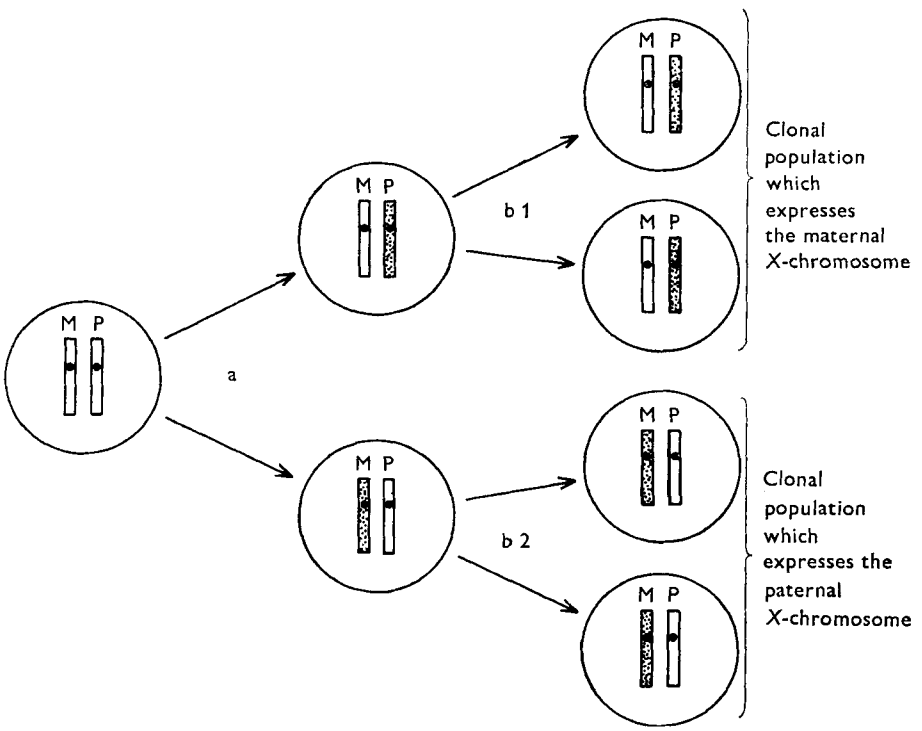

Fig. 1. *X*-chromosome inactivation. Two *X*-chromosomes – maternal (M) and paternal (P) – are schematically shown in the cells. Stippling indicates inactivity. Cell divisions are represented by arrows; each arrow may represent more than one cell division. At cell division a, the differential is acquired, it is then inherited in divisions b1 and b2. Cells arising from division b1 express their maternal *X*-chromosome, and those from b2, their paternal *X*-chromosome.

differentiators involved in creating a simple differential would affect gene superstructure. The repressor and activator molecules discussed by Jacob & Monod (1961) are examples of the differentiators involved in a complex differential.

II. MAMMALIAN *X*-CHROMOSOMES

Normal female cells from adult eutherian mammals possess two *X*-chromosomes, and there is convincing evidence that on the whole only one of these specifies the synthesis of proteins; the other is largely inert in this respect (for recent reviews see Lyon, 1970, 1971, 1972; Brown & Chandra, 1973). The inert *X*-chromosome replicates later than the other, and it is frequently condensed in interphase to form a heterochromatic Barr body (Barr, 1951). Usually two such homologous *X*-chromosomes differ in base sequence as one carries paternal and the other maternal genes. Presumably many homologous genes on the maternal and paternal *X*-chromosomes of highly inbred strains of mice share similar base sequences, but they nevertheless behave differently (Ohno & Hauschka, 1960). In any case, the mechanism that creates the

differential is insensitive to the parental origin of the normal *X*-chromosome. It acts randomly and so cannot be influenced by particular maternal or paternal base sequences. Differentiation of the two *X*-chromosomes occurs early in development and this differentiated state is heritable: a cell in which the maternal *X*-chromosome is inactive, gives rise to progeny cells with inactive maternal *X*-chromosomes. Adult females are mosaics consisting of two clonal populations of cells, one in which the maternal and the other in which the paternal *X*-chromosome is active (see Fig. 1). Occasionally autosomal genes may become translocated to the *X*-chromosome and as a result they too may become inactivated, producing a variegated phenotype (Eicher, 1971).

III. THE STABLE INHERITANCE OF THE DIFFERENTIAL

Any complete explanation of the differential should account for its stable inheritance when the cells divide. The inactivation of the *X*-chromosome, variegated position effects and the phase state of *Salmonellae* are all to a greater or lesser extent stably inherited in this way (Lyon, 1971; Baker, 1968; Iino, 1969). Variegated position effects are stable for many cell generations (Hadorn, Gsell & Schultz, 1970) and reactivation of inactive *X*-chromosomes has not been observed despite the use of powerful techniques (Sato, Slesinki & Littlefield, 1972; Migeon, 1972). In these cases, and in many others, acquired characteristics are inherited through mitotic events.

Mutations are inherited in the absence of the inducing mutagenic agent and this inheritance only requires the mechanisms needed for the inheritance of DNA. It was because of this automatic inheritance that the early embryologists favoured the notion that development proceeded by directed gene mutation. But if the differential depends upon the association of a gene with a differentiator then that differential can only be inherited if the differentiator concentration is maintained during the replication of the gene upon which it acts. If the differentiator is a protein then a special circuit involving the protein, the gene that codes for it, and the target gene must be involved. This is because proteins, unlike nucleic acids, cannot be templates for their own synthesis.

The inheritance of the lysogenic state of the prophage λ in the bacterium *Escherichia coli* requires the operation of such a self-maintaining circuit (Hayes, 1964). Lysogeny – which is characterized by a non-lytic replication of integrated virus in step with host DNA – is maintained by the action of a repressor, and at each cell division the repression must be reconstituted in the daughter cells by the action of some specific and heritable mechanism. While the repressor continues to be synthesized at a rate which maintains its concentration above a certain critical level, the prophage is unable to grow unrestrainedly and lyse the bacterium. If the repressor is continually produced then the repressed state is inherited. This self-generating circuit involves a protein repressor; other differentiator molecules might be nucleic acids (see Britten & Davidson, 1969) and their concentrations could be maintained by self-replication, but there seems to be no evidence for this idea.

IV. *CIS* AND *TRANS* EFFECTS

There are two general kinds of effects on gene expression – *cis* and *trans* effects – that any explanation of the differential must accommodate. These effects were originally studied in mutant bacteria, but the terms are now used to describe particular phenomena in normal eukaryotic cells. *Trans*-acting mutations affect both neighbouring genes and those on other chromosomes. *Trans* effects in bacteria are mediated by molecules which diffuse through the cytoplasm, and probably the underlying mechanism is similar in eukaryotes. It should be mentioned that there is evidence for some very short-range *trans* effects which act intranuclearly and not through the cytoplasm. For example, complementation between fungal genes may occur when those genes are in the same nucleus in a diploid, but not when in different nuclei in a heterokaryon (see Fincham & Day, 1971). Also some *trans* effects in *Drosophila* depend upon synapsis of the loci concerned (Ashburner, 1970).

Cis effects influence neighbouring genes on the same chromosome and not genes on other genetic elements in the same cell. In Fig. 2, gene *A* acts *cis* and controls the expression of gene *C1*, but not of the similar gene *C2* on a different genetic element. What entity connects *A* to *C1* and permits them to communicate with each other? This entity might be a migratory molecule. If so, this molecule must be restricted in its migration so that *C1* is affected but not *C2*. Molecules with this property have been invoked to explain some *cis* effects in bacteria (McFall, 1967). If this molecule migrates from *A* to *C1* by diffusion it must originate from *A*. It might be an RNA molecule since proteins are made in the cytoplasm of eukaryotes. Alternatively, information might be transferred from *A* to *C1* through a connecting structure. In bacteria, the structure may be a very long RNA molecule. An RNA polymerase binding at *A* might transcribe one RNA molecule complementary to *A*, *B* and *C1*. Genes *A* and *C1* might then be co-ordinately expressed. If transcription is unidirectional, information can be transmitted along the chromosome in one direction only. *Cis* mutations in bacteria affect only a few contiguous genes, whereas the *cis* repression by position effects in *Drosophila* can act over distances equivalent to 50 bands in the chromosomes of salivary glands (Demerec, 1941*a*). If these *cis* effects are mediated by one RNA molecule, it must be incredibly long. Perhaps transcription of one gene activates transcription in a co-operative manner in an adjacent gene, and in this way genes which were separated by considerable distances might be coordinately transcribed. A further possibility is that gene *A* is connected to gene *C1* by a nuclear membrane or a molecular bridge over which information flows. A bridge might be built from freely diffusible molecules by the action of co-operative effects: the binding of a repressor to *A* might enhance the binding affinity of *B* for repressor and so on. In this way *A* and *C1* might be co-ordinately repressed. All these models are compatible with a complex differential. However, as DNA connects *A* to *C1*, a simple interpretation of *cis* effects is that information is transmitted from *A* to *C1* through the superstructure of the DNA. Because simple salts and drug molecules can induce the nucleotides in DNA to undergo co-operative conformational changes in solution (Pohl, Jovin, Baehr &

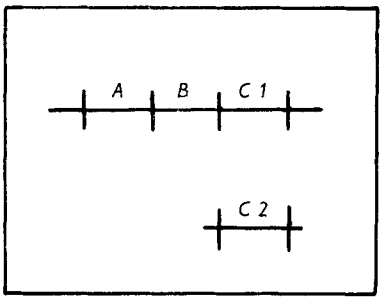

Fig. 2. *Cis* and *trans* effects. One cell containing two genetic elements is depicted. A mutation in gene *A* acts *trans* if it affects both *C1* and *C2*, and *cis* if only *C1* is affected.

Holbrook, 1972), it would seem that the structure of DNA is well suited to signalling of this kind. The transmission of information through the superstructure could be bidirectional, gene *A* controlling the activity of gene *C1* and *vice versa.*

A consideration of *cis* and *trans* effects is particularly important in the present context because differentials which are based upon an association of the gene with freely diffusible molecules would probably result in *trans* effects. *Cis* effects can only be explained by a complex differential if additional mechanisms – which probably involve co-operative effects – are involved. On the other hand, a structural differential would lead directly to *cis* phenomena. A critical test of whether *cis* or *trans* effects are acting can only be made in cells which are at least diploid for the gene in question. The inactivation of the *X*-chromosome, position effect variegation and the variation of the phase state of *Salmonellae* are all characterized by *cis* effects.

V. EXPLANATIONS OF THE NATURE OF THE DIFFERENTIAL

(1) *Non-identity of base sequences*

Although the DNA content of cells in some organisms varies as a result of specific gene multiplication or by gene loss (for example, in Ascaroidea, Copepoda and Diptera) such variations are not widely thought to be the general cause of differentiation (Lewis & John, 1963).

Similarly, irreversible gene mutation is no longer thought to play a role in orderly development, but the notion that development does proceed by modification of the covalent bonds in the gene still has its supporters. This is largely because it is easy to see how changes in DNA – or its chemically modified counterpart – can be inherited. Changes in the covalent bonds of DNA would presumably be reversed when whole plants are grown from single differentiated cells, or when differentiated nuclei are transplanted into an enucleate egg. Two kinds of modifications have been suggested. First, development might involve recombination of cellular base sequences, perhaps with themselves (Lederberg & Stocker, 1970) or with some extrinsic DNA-containing element – an episome or protovirus (Temin, 1971). For example, during development an episome might become inserted at random into one, and only one, of the two *X*-chromosomes, and as a result this *X*-chromosome might become

inactivated (Grumbach, Morishama & Taylor, 1963; Cooper, 1971). It has been suggested that phase variation in *Salmonella* can be explained by an alteration of primary base sequence by episomal insertions (Iino, 1969) or by internal recombination events (Lederberg & Stocker, 1970). Secondly, chemical modification of DNA – for example, by methylation or glucosylation of specific bases (Chargaff, Crampton & Lipshitz, 1953; Sheid, Srinivasan & Borek, 1968; Koerner, 1970; Arber & Linn, 1969; Revel & Luria, 1970) or of the backbone deoxyribose-phosphate chain–might differentiate identical DNA molecules. There seems to be little evidence for or against these explanations, and they will not be given further consideration, although much of the discussion that follows is applicable to them.

(2) *Complex differentials*

The association of a gene with a differentiator might constitute the differential. A continuing association is necessary for the inheritance of complex differentials of this type. The differentiator might be a diffusible molecule which has a high affinity for an operator – a specific regulatory region of the nucleic acid. Most explanations of the inactivation of the *X*-chromosome (Lyon, 1971; Eicher, 1971), and for differentiation, involve binding of activators or repressors to operators (Britten & Davidson, 1969). These models stem from the work of Jacob and Monod (Jacob & Monod, 1961; Monod & Jacob, 1961) on the regulation of bacterial gene expression. Alternatively, the activity of genes might be controlled by varying concentrations of the same differentiator. For example, particular concentrations of sodium or potassium ions might cause specific parts of the genome to condense and so become inactive (Harris, 1970*a*). These differentiator molecules – being diffusible – should affect all target genes in a cell; therefore special mechanisms must be invoked to explain *cis* phenomena where the differentiator molecules act selectively. For example, when the differentiator concentration reaches a certain critical level all the genes on one, and only one, of the two *X*-chromosomes must become co-ordinately activated or repressed.

The inheritance of critical levels of differentiators must involve some kind of self-maintaining circuit. Where such circuits exist – for example, in the maintenance of repressor levels in lysogenized bacteria – they are not stably inherited. A pedigree analysis has shown that every bacterium in a lysogenic culture of *Bacillus megatherium* inherits the capacity to liberate phage (Lwoff & Gutman, 1950) at a probability ranging from about 10^{-2} to 10^{-5} per cell per generation (Hayes, 1964). The prophage remains integrated in the bacterium whilst the repressor concentration is maintained above a critical level: the phenomenon of prophage immunity indicates that this concentration is usually well above the critical level, as any superinfecting phage is unable to enter a lytic cycle (Hayes, 1964). Yet even this simple mechanism frequently breaks down. How can mechanisms like this explain the more complex requirements of the inactivation of *X*-chromosomes? Here the problem is not the simple one of inactivating all the *X*-chromosomes in a given cell, but only to inactivate some of them (Lyon, 1972). The number inactivated depends upon the chromosomal constitution of the cell, one chromosome usually remaining active. In diploid cells one

X-chromosome is active and the other is inactive; in cells with a normal autosomal set and four *X*-chromosomes, only one is active and three are inactive (Lyon, 1972). However, it is not always the case that there is only one active *X*-chromosome per cell. In human triploids two *X*-chromosomes are active (Schindler & Mikamo, 1970). When cell hybrids are made by fusing together two parental cell types, the *X*-chromosomes of the hybrid cell retain the properties they had before cell fusion: cells with varying numbers of active *X*-chromosomes may be constructed in this way (Siniscalco *et al.*, 1969; Migeon, 1972). These results suggest that an *X*-chromosome, once it has been inactivated, is not influenced by the presence of additional *X*-chromosomes in the cell. *X*-chromosome inactivation must be a *cis* phenomenon. Repressor (or activator) molecules would thus need to be maintained not only above, but also below a critical level, and this range would presumably be different in the various cases. The inactivation of the *X*-chromosome might therefore be expected to be less stable than the λ repressor-operator system. It is not (Sato, Slesinski & Littlefield, 1972), and not even in hybrid cells whose chromosome constitution is particularly labile (Migeon, 1972).

The stability of the inheritance of a differential that involves the binding of a differentiator might be improved in three ways. The first involves co-operative effects – the binding of one differentiator molecule enhancing the binding of further molecules. Such co-operation might lead to stable inheritance. Secondly, stability might be improved by an amplification of a small difference by a cascade of controlling devices, each perhaps involving the operation of diffusible molecules. Such cascades permit integration of controlling circuits (Britten & Davidson, 1969). It has, however, been pointed out that the control systems involved in differentiation are unlikely, on both theoretical and practical grounds, to involve complex cascades (for example, see Ohno, 1971). A third way of improving stability requires temporal fluctuations in the critical levels of differentiator molecules. For example, the differential might be created by the random binding of one repressor to one *X*-chromosome. This repressor-*X*-chromosome complex might then have two properties to distinguish it from the other *X*-chromosome; it might be late replicating and inactive in transcription. If the repressor concentration varies throughout the cell cycle as shown in Fig. 3, then any late-replicating chromosome could give rise to repressed progeny. So the differential would be inherited. A model of this type could be tested by fusion (mediated by Sendai virus) of cells in period *a* in the cell cycle with those in period *b* (see Fig. 3). In this way a hitherto active *X*-chromosome might become repressed.

Of course, some or all of these three ways of maintaining a complex differential might be combined. There might be a class of diffusible molecules, *i*, present early in the S phase in most cells. These molecules might only bind to DNA-*i* complexes and to DNA-differentiator complexes. If *i* molecules activate the genes to which they are bound, this combination of co-operative effects, a simple cascade and temporal fluctuations in *i* concentration might explain the *cis* inheritance of the activity of a range of genes. This range of gene activity which was originally selected by the specificity of differentiators, *d*, could be maintained subsequently

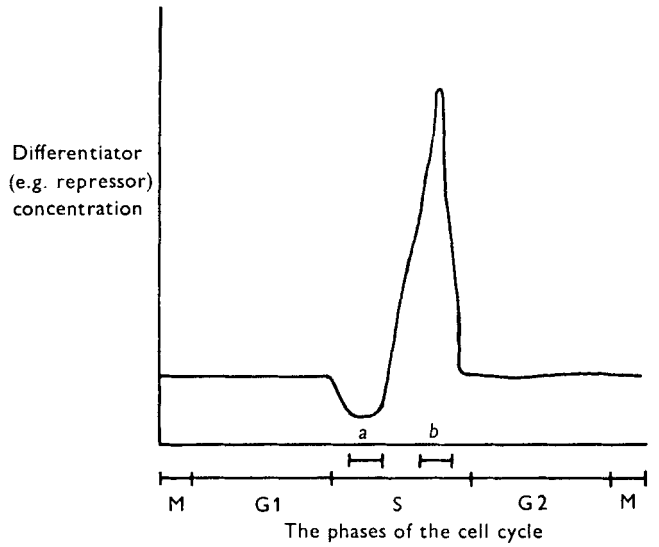

Fig. 3. Temporal fluctuations in differentiator concentration. DNA is replicated only during the S phase. Replication of the active and inactive *X*-chromosomes might take place during the periods *a* and *b* respectively. (M, mitosis; G1, growth phase 1; S, DNA synthetic phase; G2, growth phase 2.)

by the less-specific *i* molecules. A cascade allied to co-operative effects in this way has two important properties. First it permits integration of control systems (Britten & Davidson, 1969); *i* molecules might bind to a variety of DNA-differentiator complexes. Secondly, the synthesis of the regulator molecule *i* is unregulated, and yet in the absence of the differentiator molecules, *d*, the expression of their target genes is controlled.

If the differentiator is part of a structure that is much bigger than a gene, then gene expression could be said to be determined by the location of the gene relative to the associated differentiator. Differences in gene expression which appear to depend on differences in the spatial arrangements of cells are now being actively explored (for example, see Wolpert, 1969; Goodwin & Cohen, 1969). The inheritance of a differential of this type nevertheless depends upon the continuing association of the gene with the differentiator and so this differential is formally a complex differential. However, a differentiator that is part of a larger structure differs in one important respect from the differentiators that have been discussed previously; it does not diffuse so readily. *Cis* phenomena are therefore more easily explained.

If there was only one *X*-chromosome binding site at the nuclear membrane and if binding of an *X*-chromosome to the membrane led to its inactivity, then differences in behaviour of two *X*-chromosomes could be created (Comings, 1968). The inactive *X*-chromosome is indeed often found adjacent to the nuclear membrane (Barr, 1951). The data which are consistent with this hypothesis have been reviewed (Comings, 1968); they are mainly drawn from experiments which show that when chromosomes are spread at metaphase their distribution is non-random (Schneiderman & Smith,

1962; Miller, Mukherjee, Breg & Gamble, 1963*b*), for example the *X*-chromosomes are to be found at the edge of the spread (Morishama, Grumbach & Taylor, 1962; Miller *et al.*, 1963*a*). There is no precise way of determining the position of genes within most interphase nuclei, but the controlled induction of premature chromosome condensation in interphase nuclei may provide such a method of detecting characteristic differences in location of chromosomes (Johnson & Rao, 1970). However, models of this kind must explain how spatial arrangements of the genome might be stably inherited. There may be a precedent for this in the segregation of centrioles to particular positions during cell division.

(3) *The superstructural hypothesis*

(*a*) *Introduction*

In one cell, two homologous DNA duplexes may behave differently because they differ in superstructure and this difference in superstructure might cause differences in gene expression. It is just such a difference in the superstructure of two DNA duplexes which the proteins that regulate gene expression might recognize. Very little is known about the organization of DNA in chromosomes, so that ideas about superstructure are necessarily vague, but these differences may be in base stacking, in sense or degree of supercoiling (Pardon, Wilkins & Richards, 1967; Pardon & Wilkins, 1972), or in other forms of higher-order structures. Perhaps when DNA is in the B conformation, transcription may not occur because a nascent RNA molecule cannot adopt this conformation. A gene might then be expressed when its DNA exists in the A or C conformation while its homologous partner is not expressed, because it is in the B conformation. There is ample precedent for this sort of specificity in enzyme action: there are many pairs of enzymes with differing specificities for optical isomers [e.g. the two amino-transferases with differing specificities for D- and L-aspartate (E.C. 2.6.1.1 and E.C. 2.6.1.10)]. Indeed, it is the conformation of the DNA double helix that determines whether one or both DNA strands of simian virus 40 are transcribed *in vitro* by *E. coli* RNA polymerase (Westphal, 1970).

In the following discussions two assumptions are made unless stated otherwise. The first is that no covalent bonds are broken. The second is that there is no net rotation of bases about sugar-phosphate bonds in either of the backbone polynucleotide chains of the duplex. This is a plausible assumption since the forces maintaining the double helical structure restrict such free rotation.

(*b*) *Maintenance of a superstructural differential*

A simple form of superstructure is a coiled coil in which tertiary turns are imposed upon the helical turns of the DNA duplex. Such extra supercoils can be maintained in DNA if net rotation about the helical axis is restricted. Rotation might be restricted as a result of the binding of histones (Phillips, 1971) or intercalating agents, by methylation of bases or by attachment of the ends of the duplex to a rigid structure – for example, the nuclear membrane. Extrinsic factors restrict rotation in these cases: rotation would be intrinsically restricted in a circle formed by joining the ends of the duplex. (Circles are formally equivalent to linear duplexes in which free

rotation about the ends is forbidden.) Extra supercoils in the covalently continuous and circular DNA of viruses are maintained in this way (see Section V. 3*e*). Additional supercoils in a linear molecule could be maintained intrinsically if the DNA were organized into loops – for example, of the type seen in the lampbrush chromosomes of amphibian oocytes (Gall, 1955). A specific pairing between base sequences at different points along one duplex could give rise to circle formation. When linear duplexes of DNA are sheared they give rise to fragments which are circular (Thomas, Hamkalo, Misra & Lee, 1970). These circles may be closed at the pairing sites by hydrogen bonds between different base sequences on the one duplex. DNA with highly reiterated sequences (Britten & Kohne, 1968; Walker, 1971; Yunis & Yasmineh, 1971) has many of the properties to be expected of such a pairing site. Alternatively, pairing might involve a protein or RNA bridge between the two sites. Whatever their nature, these sites would be involved in maintaining supercoils within one linear DNA molecule. Pairing between sites on different duplexes might be involved in chromosome pairing and crossing over during meiosis.

More complex coiling of DNA (Crick, 1971*a*; Paul, 1972) may also be involved in maintaining a defined superstructure in a gene. A globular region perhaps maintains the conformation of an associated gene. This idea is attractive for many reasons, but especially as it provides an explanation for the banding patterns seen in the polytene chromosomes of some Diptera (Beerman, 1966). One band and interband are now thought to constitute one complementation group (Judd, Shen & Kaufman, 1972), although there is clearly much more DNA per duplex in this region than would be required to code for a protein of average molecular weight (Rudkin, 1965; Daneholt & Edström, 1967). This 'extra' DNA might not specify the amino-acid sequence of a protein; instead it might be involved in maintaining the conformation of an associated gene that did code for a protein, thus regulating its expression. Therefore some point mutations in much of the band and interband may not be deleterious and so the mutational load on the organism might not be as great as has sometimes been predicted from its DNA content (Haldane, 1957; Muller, 1950). The 'extra' DNA that regulates the expression of an adjacent structural gene might include many of the non-identical, but closely related DNA sequences that are characteristic of higher organisms (Britten & Kohne, 1968).

In the foregoing discussion it has been suggested that superstructures might be maintained either intrinsically in DNA or by the action of extrinsic molecules – for example, by histones, intercalating agents or molecules that bridge pairing sites. These extrinsic molecules might be non-specific in the sense that they are produced in nearly all cells. They are not differentiators and they are quite unlike the activators or repressors discussed by Jacob & Monod (1961). Nevertheless, where these extrinsic molecules are missing, this type of superstructural differential would be lost. On the other hand, the maintenance of superstructures in circles or loops need not require extrinsic factors.

(*c*) *Creation of a superstructural differential*

How might differences in superstructure be created in DNA molecules with similar base sequences? *Trans*-acting differentiators are probably involved. Superstructures might be introduced after synthesis by coiling the duplex or by cutting one strand of the duplex, introducing super-helical turns into it and then mending the cut. Enzymes that change the superhelical properties of DNA have recently been isolated from both animal and bacterial cells infected with viruses (Alberts & Frey, 1970; Wang, 1971; Champoux & Dulbecco, 1972). Alternatively, superstructures might be introduced during the replication of DNA. (This notion is attractive as it is consistent with the idea that 'transdetermination' in imaginal disks of *Drosophila* is dependent upon DNA replication (Gehring, 1972).) Since the two strands of a DNA duplex are antiparallel, DNA replication might well be modified to produce daughter helices with different superstructures. For example, if DNA synthesis on one strand occurs in the A configuration and on the other in the C configuration, on subsequent relaxation to the stable B form, progeny supercoils of opposite sense would be formed.

(*d*) *Inheritance of a superstructural differential*

How might superstructures be replicated and inherited? This might be achieved in two ways. First, defined superstructures could be reintroduced into the DNA during each cell cycle by the action of diffusible differentiator molecules. A differential of this type is formally a complex differential; the superstructure – although it controls gene expression – is maintained by a continuing association with differentiator molecules. It may therefore have the properties discussed previously. Secondly, the replicating machinery of cells might be such that, by itself, not only the primary base sequence but also the superstructure of DNA is duplicated.

Consider the semi-conservative replication of a circular duplex of DNA. This might proceed by strand separation without breakage of covalent bonds followed by the progressive synthesis of daughter strands around the circle. Because of the two conditions which forbid covalent bond breakage and net rotation of bases about sugar-phosphate bonds in either of the polynucleotide backbone chains of the duplex, two interlocking circles result. Each is topologically identical to the parent. This is so, whether or not the parental molecule has superhelical turns. (The number of times one circle interlocks with the other is a function of duplex and superhelical turns.) It is not surprising that superstructure is conserved on replication in this way since, from the point of view of one strand of the parental double helix, all that has been done is to remove a complementary strand and replace it with a newly synthesized strand.

The progeny circles, even if they lack superhelical turns, can only be freed from one another by cutting covalent bonds to open the duplex. (This constitutes part of the 'unwinding' problem (Cairns, 1963; Watson, 1970).) The cut ends must then be mended after separation of the progeny to reform two isolated circles. If rotation about the axis of the helix is forbidden during opening of the circle, the

progeny duplexes remain topologically identical to the parent. (Of course, circle opening and closing would probably occur many times as synthesis proceeds around the circle.) Even in the absence of superhelical turns, it seems likely that during circle opening the cut ends of the duplex would be held in some way, perhaps by the enzymes responsible for making the cut or by some supramolecular organization of the duplex. If the movement of the cut ends was not restricted, it seems unlikely that the ends could reunite again after separation of the progeny. This holding of the cut ends implies a restriction of rotation about the axis of the helix. Whether the parental duplex was supercoiled or not, these plausible restrictions ensure that the superstructure of a circular DNA molecule is automatically replicated together with the primary base sequence. When the ends of a linear duplex are held so that rotation is restricted, the duplex may formally be considered as a circle; therefore, when such a duplex is replicated in the manner described, its superstructure is also replicated automatically with its primary base sequence. It may be that newly replicated DNA is not covalently continuous, but superstructures would nevertheless be automatically replicated if this restriction concerning rotation were obeyed. If supercoils can be maintained in DNA molecules, no particular problems are posed by their replication and inheritance.

One chromosome probably carries many sequences of DNA which differ in superstructure, so that the chromosome might be divided by barriers (for example pairing sites) which contain particular superstructures within a section. Replication of eukaryotic chromosomes is discontinuous (Pelling, 1966; Huberman & Riggs, 1968; Callan, 1972); replication could start and stop at these barriers, otherwise superstructures in neighbouring sections might interact if replication progressed across the barrier.

Differences in superstructure at the level of the gene might be reflected in the gross organization of chromosomes. Indeed, there is a remarkable structural difference between the two *X*-chromosomes in adult female cells. During interphase, one *X*-chromosome – the inactive one – is frequently condensed to form a densely staining heterochromatic body (Barr, 1951). Heterochromatin has many characteristics but the most striking is the correlation between its appearance and transcriptional inactivity (Brown, 1966; Yunis & Yasmineh, 1971). In *Drosophila*, the inactivation of genes by position effects is almost invariably associated with their heterochromatization (see Section VI; Baker, 1968; Schultz, 1947; Prokofyeva-Belgovskaya, 1948). In each of these cases gene inactivity is correlated with gross condensation of the chromatin: this can be simply interpreted if superstructures at the level of the gene affect directly both gene expression and gross structure.

If superstructure is the basis of the differential then a number of restrictions are imposed on the organization and replication of the DNA within a chromosome. Certain models proposed for the replication of DNA are incompatible in their simplest form with these restrictions (Watson, 1970). However, many of the experimental data lead one to suspect the existence of these restrictions.

It has been argued that an intrinsic differential can be automatically replicated and inherited. Once established, a superstructural differential – unlike other differen-

ials – does not require the continued operation of the mechanisms that created it. The idea that development proceeded by a process of directed gene mutation appealed to the early embryologists for the same reason. Specific mutations can be inherited without the operation of specific self-generating circuits; so, too, might specific superstructures of DNA.

(*e*) *Polymorphism of DNA*

The superstructural hypothesis requires that DNA molecules which have identical base sequences may be polymorphic. This notion runs counter to the assumption that a primary sequence necessarily defines a unique secondary and tertiary structure. It is commonplace for proteins and even RNA (Adams, Lindahl & Fresco, 1967; Sanger, 1971; Lodish, 1970, 1971; Fukami & Imahori, 1971; Min Jou, Haegeman, Ysebaert & Fiers, 1972) to be polymorphic and for the polymorphs to differ in function. We now know that crystalline DNA is able to adopt a wider variety of conformations than the three types, A, B and C, that were initially observed (Bram & Tougard, 1972). Furthermore, a comparison of the circular dichroism spectra of the DNA component of chromatin and of purified DNA indicates that the B and C forms might coexist in chromatin (Hanlon, Johnson, Wolf & Chan, 1972). But this is polymorphism amongst DNA molecules of different sequences. Is there evidence that polymorphism exists amongst DNA molecules which have identical base sequences? The answer to this question comes from studies on the small circular DNA molecules of viruses.

These molecules contain superhelical turns in addition to the helical turns of the DNA duplex. When one strand of the DNA duplex is cut the majority of these superhelical turns disappears; the duplex turns remain. The superhelical density is related to the number of superhelical turns per unit length of the molecule (Eason & Vinograd, 1971; Bauer & Vinograd, 1968, 1971). It can be measured in the presence of intercalating dyes such as ethidium bromide or propidium diiodide. One of the strands of a closed circular DNA molecule of simian virus 40 (SV_{40}) can be cut; these cut molecules which have lost superhelical turns can bind more dye than native molecules. DNA-dye complexes have a lower buoyant density than pure DNA and the pure and complexed forms can be resolved in caesium chloride density gradients. In this way differences in superhelical densities can be visualized as differences in buoyant density (Bauer & Vinograd, 1970; Hudson, Upholt, Devinny & Vinograd, 1969; Eason & Vinograd, 1971; Crawford & Waring, 1967).

Intact and closed circular DNA of SV_{40} can be purified from isolated virions or from cells infected with virus. Although these DNA molecules have identical base sequences they differ in superhelical density (Eason & Vinograd, 1971). A similar result has been found with the bacteriophage PM2 (Espejo, Espejo-Canelo & Sinsheimer, 1971). This is another situation where DNA molecules which have identical base sequences are probably behaving differently; those with one configuration are being replicated while those with another configuration are being encapsulated.

One strand of the double-stranded DNA from these viruses can be cut *in vitro*,

and this is followed by a dissipation of superhelical turns; the cut can be subsequently mended by polynucleotide ligase. When the superhelical density of the treated or 'relaxed' DNA is compared to the original sample it is found to have changed. (This is so when all native circular DNA's are compared with their cut and mended counterparts (Bauer & Vinograd, 1971).) Intact and circular DNA molecules from SV_{40} may therefore have one of three structures superimposed upon the same base sequence. These forms can be obtained from the virions or from cells infected with virus or by making the 'relaxed' form *in vitro*. Parental and progeny virion DNA molecules have the same superhelical density which differs from that of 'relaxed' molecules; a defined configuration is faithfully replicated and inherited during the normal growth of the virus.

Evidence that even the DNA of *E. coli* is folded in a complex manner has recently been reported (Worcel & Burgi, 1972). Intact DNA is supercoiled when released by careful lysis from this bacterium. A single cut in one strand of the DNA duplex does not eliminate all supercoiling as one might expect if the duplex were a single circle; it only reduces it slightly. Additional cuts reduce it further. This is interpreted as showing that the circular DNA molecule is folded into many loops, in such a way that a single cut reduces supercoiling only within the affected loop and not in the whole chromosome. It appears that differences in superhelical density do occur in DNA and can exist side by side along a single DNA duplex.

VI. VARIEGATED POSITION EFFECTS IN *DROSOPHILA*

Both *cis* and *trans* effects characterize the remarkable phenomenon of position effect variegation in the fruit-fly *Drosophila* (Baker, 1968). Gene expression may be suppressed as a result of a chromosomal rearrangement which occurs adjacent (or *cis*) to the affected gene. This suppression, which occurs early in development, does not necessarily affect all the cells carrying the rearrangement. Once initiated, the suppression is inherited by progeny cells within the fly, so that gene expression in different clones of cells within one tissue may produce a variegated phenotype in that fly (see Fig. 4; Becker, 1969; Baker, 1967; Hadorn *et al.*, 1970). Baker has listed some criteria that can be used to determine whether variegation of gene expression is caused by position effect (Baker, 1968). The first is its *cis* nature; the phenotype of progeny flies reverts from variegated to wild-type when crossing over restores the original genotype. A second criterion concerns the involvement of heterochromatin. Heterochromatin is generally characterized by its peculiar staining properties and by its inactivity in RNA synthesis. In almost all cases of position effect variegation a determining rearrangement involves a break in the neighbourhood of the locus which shows variegation and another break in a heterochromatic region. When active euchromatic genes are relocated close to a break in heterochromatin they assume the genetic inactivity which generally characterizes heterochromatin. Thirdly, a variegation due to a position effect can be suppressed by extra heterochromatin: the extra heterochromatin – which can be carried by *Y*-chromosomes – acts *trans*.

There is another remarkable situation where *Y*-chromosomes have a suppressive

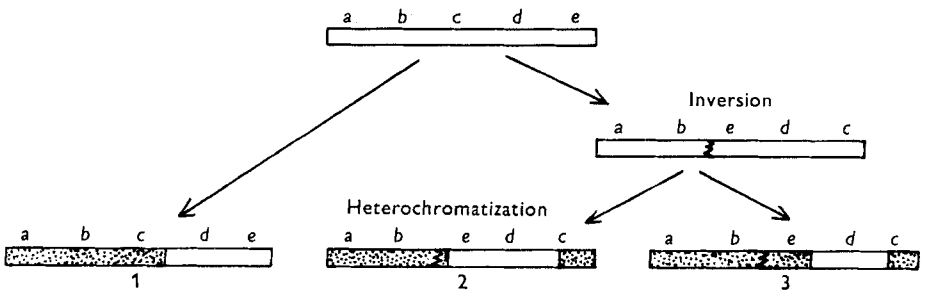

Fig. 4. Position effect variegation. One chromosome carrying heterochromatic loci *a*, *b* and *c*, and euchromatic genes *d*, *e* is depicted (1). Heterochromatin is stippled. In (2) and (3) an inversion leads to the relocation of the euchromatic gene *e* close to a break in the heterochromatic region. During development, the euchromatic gene *e* may (3) or may not (2) become heterochromatic and thereby inactive. Stable inheritance by the clonal derivatives of (2) and (3) leads to a variegated phenotype.

effect. X-rays can be used to induce rearrangements involving sex-linked recessive loci which are not lethal in *XY* males, but lethal in *XO*. These lethal mutations are accompanied by a chromosomal rearrangement with one break in a euchromatic region of the *X*-chromosome and another break in a heterochromatic region. Although the phenotype of these mutants cannot be variegated – they are lethal mutations – these are genes whose actions are being suppressed by a process analogous to that responsible for the suppression characterizing position effect variegation. When the X-rays induce the rearrangement, a hitherto euchromatic gene becomes relocated close to heterochromatin. The embryo dies if this gene, which is *X*-linked and therefore present in only one dose in the *XO*, becomes repressed by the action of the neighbouring heterochromatin. On the other hand, this repression can be prevented by the presence of a *Y*-chromosome: flies with the *XY* constitution are viable. In one study, 20 per cent of all sex-linked and recessive mutations induced by X-rays showed this type of position effect (Lindsley, Edington & von Halle, 1960). As Baker says, 'there is no evidence against the assumption that every gene is subject to this type of suppression' (Baker, 1968). An understanding of this situation should prove fundamental to an understanding of differentiation for here is a mechanism whereby any gene may be switched on or off. Studies of this type provide the strongest evidence that some gene switches act *cis*.

A further characteristic of variegated position effects is the polarity of the inactivation which spreads outwards from the heterochromatic breakpoint. A gene close to the breakpoint is likely to be inactivated; if its activity is suppressed, then the next most distal gene may also be inactivated (Demerec & Slizynska, 1937; Schultz, 1941). There is a correlation between this spreading of inactivation of gene expression and heterochromatization (Schultz, 1947; Prokofyeva-Belgovskaya, 1948). A similar kind of inactivation which spreads along a chromosome gives rise to mosaicism in mice bearing *X*–autosome translocations (Eicher, 1971; Baker, 1968).

The variegated phenotype may be influenced by parental effects, and an extreme form of such *trans* effects which reaches through both time and space will now be

considered. Parental genes, though not inherited by progeny flies, may nevertheless have an effect on variegation in those flies (Baker, 1968). It is easy to see how a maternal effect may be transferred through the cytoplasm of the egg; the most likely medium for the transmission of a paternal effect is the chromatin of the sperm. Whatever this *trans*-acting differentiator proves to be – and sperm would be an excellent place to search for it – it is unlikely that a sufficient amount of it could be transferred through the germ cells to have a direct and later effect on the variegated phenotype in many of the cells of the resulting adult fly. In some way the information carried by this parental differentiator must be replicated and inherited.

If the differential depends upon the continued association of a gene with a differentiator then the parental differentiator must be replicated, or its information transferred to a secondary differentiator that can replicate. A self-maintaining circuit might result, which, persisting in the nucleus or cytoplasm could influence the creation of the differential in the adult fly. Rather unlikely models of this type can explain the *trans* effects that do occur, but they must also account for what is fundamentally a *cis* phenomenon.

The *cis* nature, the invariable association with heterochromatin and the characteristic spread of repression from heterochromatic regions point to a structural basis for the phenomenon of position effect variegation. There is one major argument against this simple structural interpretation: *trans* effects, which imply the action of diffusible differentiators, may emanate from extra heterochromatin within the affected cell or even from parental chromosomes through egg and sperm. These *trans* effects may suppress variegation. It is possible that the genes that become relocated close to heterochromatin express a variegation in phenotype as a reflexion of a 'variegation' of DNA superstructure. As *trans* effects are known to occur in this system, one interpretation is that such effects only influence the creation of the differential, and do not act subsequently. This notion could be tested by removal (for example, by somatic crossing over) of a *Y*-chromosome that is suppressing the lethal effects of a rearrangement involving both heterochromatin and a sex-linked recessive locus in an *XY* male (see Lindsley *et al.*, 1960). The inactivation of genes by position effects occurs very early in development (Baker, 1971; Nöthiger, 1972; García-Bellido, 1972) so that these parental differentiators can probably act directly at the creation of the differential. Only superstructural differentials can be maintained and replicated in the absence of the differentiators; differentials of other types cannot.

How might variegated position effects arise from the operation of a superstructural differential? A defined superstructure created for example by histones or intercalating agents might characterize heterochromatin. If a gene adopts the superstructural characteristics of neighbouring DNA, a euchromatic gene translocated to a heterochromatic region would become heterochromatic. This is sometimes the case (Schultz, 1947; Prokofyeva-Belgovskaya, 1948). The closer the relocated gene was to a break in heterochromatin the more likely it is that it would be affected. When extra heterochromatin is present during heterochromatization, the suppression of euchromatic genes that have been translocated adjacent to heterochromatin becomes less likely. This might be due to competition by the extra heterochromatin for diffusible

and therefore *trans*-acting differentiator molecules. In some cells the translocated euchromatic gene would become heterochromatic, in others it would not. This would ultimately give rise to the variegated phenotype. The relocated euchromatic gene would now have adopted one of two superstructures: one would resemble that of the adjacent heterochromatin while the other would retain that of other euchromatic genes. The former would render it incapable of acting as a template for RNA transcription, the latter would not. Once created, the differential would be replicated automatically and the factors that helped create the differential might have no further effect. A superstructural differential can therefore account simply for variegation produced by position effects.

VII. PHASE VARIATION IN *SALMONELLA*

It is arguable whether the mechanisms responsible for the inactivation of the mammalian *X*-chromosome have any counterpart in bacteria. Clearly, the gene repression which characterizes the classical examples of lysogeny and the *lac* operon cannot be simply compared to the repression of the *X*-chromosome; all similar *lac* operons within a normal bacterium are co-ordinately repressed, whereas only one of two *X*-chromosomes is normally inactive. Where *cis* effects are seen in the *lac* operon, they usually result from the effects of mutations. A closer counterpart to the different behaviour of two *X*-chromosomes – and perhaps also to the problem of eukaryotic differentiation in general – is the phenomenon of phase variation in the bacterium, *Salmonella*. At any time, only one of two loci that code for a flagellar protein is expressed and this difference in behaviour of the two loci is heritable. This phenomenon cannot be explained in terms of a complex differential.

Strains of the bacterium *Salmonella* are characterized by their somatic (O) and flagellar (H) antigens. When a mass culture of *Salmonella* is plated out, two types of colony are obtained. All bacteria within a colony usually express the same flagellar (H) antigen and this antigen defines the phase; or in other words, the ability to express one or other of the two flagellar antigens is heritable. Occasionally, a bacterium will spontaneously change phase; it will then breed true, until one of its offspring reverts to the original phase (see Fig. 5). This variation of phase resembles forward and back mutation in some respects, but differs from it by its oscillation between two fixed alternatives (see Iino, 1969, for a review).

Genes may be transferred between different diphasic strains by transduction and such studies have demonstrated two series of multiple alleles at unlinked loci, *H1* and *H2*. These are the structural loci for the flagellar (H) antigens which characterize the two phases. Closely linked to *H2* is a locus, *vh2*, which governs the rate of alternation between the two phases (Iino, 1961). When a bacterium is in phase 1, *H1* is active and *H2* inactive; and when in phase 2, *H2* is active and *H1* inactive. Transduction between single-phase cultures of diphasic strains showed that a donor *H1* allele can only be expressed when transferred into phase 1 bacteria; this is so regardless of the phase of the donor. On the other hand, a donor *H2* can be expressed in any phase of the recipient, but only when the donor is in phase 2 (Lederberg & Iino,

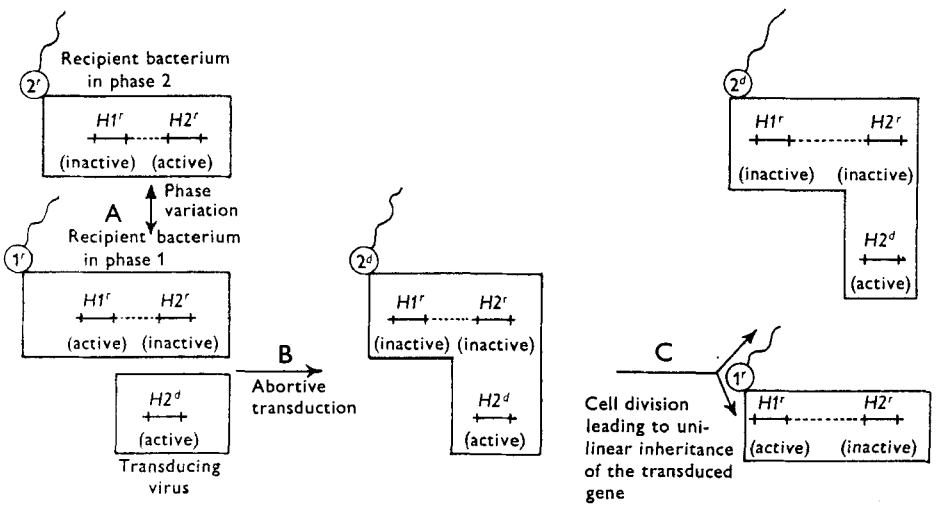

Fig. 5. Phase variation in *Salmonella*. (A) Phase variation between phase 1 and phase 2 in a bacterium^r. Only the genes *H1* and *H2* are shown. (B) A virus carrying the *H2* allele of the donor (*H2*^{d}) is introduced by abortive transduction into the recipient bacterium^r in phase 1. The transducing virus is derived by growing virus on a phase 2 donor strain of *Salmonella*. (C) The partial diploid bacterium formed as a result of the abortive transduction can replicate its recipient chromosome but not the donor chromosomal fragment, and so the unreplicated donor fragment can only be inherited by one of the two progeny cells.

Superscripts *r* and *d* refer to donor and recipient genes respectively. The activity of the *H* alleles is indicated in the brackets and the type of flagellar antigen which characterizes each cell is shown in a circle (simplified from Iino, 1969; Pearce & Stocker, 1967).

1956). For example, when an active *H2* allele from a phase 2 bacterium is introduced by abortive transduction into a phase 1 cell (i.e. *H1* active, *H2* inactive) the partial diploid bacterium ceases to express its *H1* allele (see Fig. 5). A bacterium in phase 2 is therefore thought to produce a diffusible repressor which inactivates all *H1* alleles in the cell. But the expression of the *H2* alleles cannot be explained in the same way by a simple model which involves diffusible repressors as these would inactivate all *H2* alleles in the cell. The partial diploid expresses its donor *H2* allele but not the recipient *H2* allele (Pearce & Stocker, 1967). The expression of an *H2* gene depends therefore on the 'state' of a phase determinant on the same chromosome; it is unaffected by the 'state' of another phase determinant on a different genetic element in the same bacterium (Pearce & Stocker, 1967). This phase determinant therefore acts *cis*. Here again is a situation where two alleles in the same cell behave differently; one *H2* allele is expressed, the other is not. As the transducing virus conveys very little, if any, donor cytoplasm to the partial diploid cell, the differential is probably transferred from donor to recipient with a gene. The 'state' of the phase determinants of the two *H2* alleles cannot be re-determined after gene transfer by a *trans*-acting system, otherwise both phase determinants would adopt the same state.

The difference in behaviour of the two *H2* alleles in the partial diploid cell has

some similarities with the difference in behaviour of the two *X*-chromosomes in female cells of mammals. Perhaps the recipient *H2* allele became inactive at a particular time in the cell cycle when a repressor concentration was high. If the repressor concentration had fallen by the time the donor *H2* was introduced into the cell, then the donor *H2* might remain active (Fig. 3). However, the difference in behaviour of the two *H2* alleles in the partial diploid cell cannot be explained in this way. This cell arose by abortive transduction: the chromosome of the recipient can replicate, the donor fragment cannot. This results in the unilinear inheritance of the transduced gene by progeny cells (see Fig. 5). The partial diploid cell repeatedly passes through a whole cell cycle, at some time during which the hypothetical repressor concentration should rise. If so, both *H2* alleles should be inactivated. They are not; donor *H2* continues to be expressed.

Where a *cis* phenomenon is found to occur naturally in bacteria, it cannot be explained by a complex differential. Perhaps the phase determinant should be sought in the superstructure of the *H2* allele; in one form this might lead to the synthesis of *H2* gene product and also of a repressor of *H1*, and in another form, to *H2* gene repression. Phase variation would then be an expression of a spontaneously occurring oscillation between two gene superstructures.

VIII. SOME SPECULATIONS

(1) *The action of bromodeoxyuridine*

This discussion of the nature of the differential has deliberately been restricted to those situations which are clearly characterized by *cis* effects. One of the problems posed by differentiation is the inheritance by DNA molecules of an acquired behaviour and it may be that differentiation is a *cis* phenomenon. If so, the control of gene expression by specific superstructures of DNA may be a very general mechanism. It has been stressed that a variety of mechanisms probably gives rise to the differentials operative during development (Harris, 1970*a*; Hadorn *et al.*, 1970). Those situations where the differentiation of a cell can be maintained and inherited in the presumed absence of specific external stimuli will now be considered (Gehring, 1972; Konigsberg, 1961; Stevens, 1960; Moore, 1964; Yasumura, Tashjian & Sato, 1966; Coon, 1966; Cahn & Cahn, 1966; Yaffe, 1968; Richardson, Tashjian & Levine, 1969).

What specific agents affect the synthesis of proteins characteristic of the differentiated state? It is perhaps surprising to find that classical mutagens (e.g. X-rays) have little effect. The best-known example of such an agent is 5-bromodeoxyuridine - an analogue of thymidine – whose actions are both striking and varied. It specifically suppresses a variety of differentiated functions with only marginal effects on cell growth and RNA and protein synthesis (Stellwagen & Tomkins, 1971*a*, *b*; Weintraub, Campbell & Holtzer, 1972). The effects are reversed by removal of the drug (Stellwagen & Tomkins, 1971*b*; Weintraub *et al.*, 1972). For example, haemoglobin production in cells of the erythropoietic series (Weintraub *et al.*, 1972), myosin synthesis in developing myoblasts (Stockdale, Okazaki, Nameroff & Holtzer, 1964; Bischoff & Holtzer, 1970), synthesis of inducible tyrosine amino-transferase in

hepatoma cells (Stellwagen & Tomkins, 1971*a*, *b*), the specific synthesis of proteins in chondrocytes (Abbott & Holtzer, 1968; Marzullo, 1972), in the pancreas (Wessells, 1964; Rutter *et al.*, 1968) and of mucopolysaccharides in amnion cells (Mayne, Sanger & Holtzer, 1971) and melanin production and tumorigenicity of a mouse melanoma cell-line (Silagi, Beju, Wrathall & Deharven, 1972) are all suppressed. Furthermore it and a related compound induce growth of latent DNA and RNA viruses (Watkins, 1970; Rowe, Lowy, Teich & Hartley, 1972).

There seems to be no satisfactory explanation for these effects of bromodeoxyuridine. Since they occur in most of the cells treated with the drug, and as they are readily reversed when the drug is removed, it seems unlikely that bromodeoxyuridine is acting as a classical mutagen (Brockman & Anderson, 1963). Incorporation of the drug into DNA seems to be necessary for its action; indeed there is a positive correlation between the percentage substitution of thymidine by bromodeoxyuridine and the suppressive effect, maximum suppression occurring at very high levels of substitution (20–50%) (Stellwagen & Tomkins, 1971*b*; Weintraub *et al.*, 1972). This is another reason why the analogue is unlikely to be acting as a mutagen or to be affecting the coding properties of DNA. Perhaps the binding of differentiator molecules to DNA is affected by bromodeoxyuridine substitution. Alternatively, changes in gene expression might result from modifications of the superstructure of DNA due to replacement of thymidine by its analogue (Iball, Morgan & Wilson, 1966). At a gross level bromodeoxyuridine is known to affect the spiralization of chromosomes (Zakharov & Egolina, 1972).

When the substituted DNA is replicated after removal of excess analogue the induced superstructure should revert to its original state with restoration of its initial function. However, in some cases the superstructure induced by bromodeoxyuridine might be stably inherited after replacement of the bromodeoxyuridine by thymidine. This might partially explain the surprising finding that bromodeoxyuridine induces nutritionally deficient and drug-resistant 'mutations' in a Chinese hamster cell-line at a frequency which is orders of magnitude higher than those induced by several chemical mutagens and by X-rays (Chu, Sun & Chang, 1972). For example, 0·1–10% of the cells surviving treatment with the drug turned out to be auxotrophic 'mutants'.

One further study of great interest concerns the association of purified *lac* repressor with *lac* operator DNA from *E. coli*, in which 90% of the thymidine has been replaced by bromodeoxyuridine (Lin & Riggs, 1972). The rate of dissociation of repressor from the drug-substituted DNA was ten times slower than from the unsubstituted DNA: in other words, the repressor is bound tenfold more strongly to the substituted DNA. So the remarkably tight binding of repressor to operator DNA is enhanced by bromodeoxyuridine substitution. Are the various effects of bromodeoxyuridine due to enhanced binding of repressors or is the superstructure of even the *lac* operator of crucial importance to the control of the gene expression of the *lac* operon?

If superstructures of DNA can be inherited by descendant organisms there may be a class of 'mutants' in which gene expression is changed by virtue of a heritable

change in gene superstructure. This class might include the bromodeoxyuridine induced 'mutations' described above and the unstable mutants of fungi (Barnett & De Serres, 1963; Nasim, 1967) and *Drosophila* (Demerec, 1941*b*) that oscillate between two fixed phenotypes in a manner reminiscent of phase variation. There is evidence to indicate that some polar mutations exert an effect through the superstructure of genes. For example, it seems that it is the position of an amber mutation within the superstructure of a gene that determines whether or not an adjacent gene is to be affected (see Min Jou *et al.*, 1972; Fukami & Imahori, 1971). Furthermore there is a class of insertions in the *lac* operon of *E. coli* that exert a complete polar effect which does not entirely result from changes in reading frame or the effects of nonsense mutations (Malamy, 1970; Malamy, Fiandt & Szybalski, 1972). Perhaps the insertion changes the superstructure of adjacent genes. A 'mutagen' which affects superstructure might change the superstructure of both strands of a DNA duplex so that all progeny might be mutant.

(2) *'Determination' and 'differentiation'*

The imaginal disk cells of the larvae of *Drosophila* are predestined to give rise to particular adult tissues – for example, antennae or thorax (Nöthiger, 1972; García-Bellido, 1972). The establishment of this predestined or 'determined' state occurs quite early in the development of the larvae and it is inherited for many cell generations before metamorphosis leads to the overt expression of the consequences of a 'determined' state. The proteins that are characteristic of the adult tissue are not made until this overt expression or 'differentiation' occurs (see Ephrussi (1972) for a recent discussion). Not everybody is convinced that the molecular mechanisms involved in 'determination' are different from those concerned with 'differentiation' (Gross, 1968). This view results, in part, because both events have been described in terms of the same kind of complex differential. Perhaps 'determination' involves the creation of a simple differential and 'differentiation' the addition of a complex one.

The 'determined' state of genes in different cells might be characterized by the same superstructure. This superstructure would be imposed upon the appropriate genes in the different cells, by operation during 'determination' of some *specific* mechanism. But a common effector, such as ecdysone or cyclic adenosine monophosphate might induce overt 'differentiation' of the various types of 'determined' cells. In some way ecdysone might recognize all superstructures characteristic of the 'determined' state, and cause their expression. Cells from different imaginal disks might contain superstructures characteristic of 'determination' imposed on different genes. When these cells are exposed to ecdysone those different genes would be expressed. So although specific mechanisms might be involved in 'determination', 'differentiation' would involve the operation of a common effector.

The differences between a 'determining' event and the subsequent expression of that event have been stressed. Whatever the nature of the differential proves to be, some specific mechanism must be invoked to explain specific 'determinations'. Why then do 'determination' and 'differentiation' not take place simultaneously?

What are the possible advantages of a temporal dissociation of the two events? Theoretical arguments have been advanced to suggest that spatial differences in the critical levels of differentiator molecules are responsible for the creation of differentials (Wolpert, 1969). These differences, perhaps arising by diffusion, could only extend through several hundreds of cells (Crick, 1971*b*). Differentiator molecules in the egg, if not themselves replicated, must become diluted by cell division and so they must also act on small numbers of cells. The 'determination' of imaginal disk cells in *Drosophila* does seem to involve small numbers of cells (Nöthiger, 1972; García-Bellido, 1972). If the differentials created in these few cells can be replicated and inherited, then cell division will amplify the numbers of such 'determined' cells. During this amplification, there would be no requirement for the expression of the differential. When cell division has generated cell numbers sufficient to constitute a tissue, then a non-specific and perhaps humoral signal could induce overt 'differentiation' in the tissue. In this way, a superstructure which can only be acquired originally by a few cells, is inherited by many; and it is only in the many cells that the differentiated phenotype must be expressed.

(3) *Some examples of non-Mendelian inheritance*

It has been argued that heritable information in DNA exists in two forms – in the primary base sequence and in the superstructure, the second regulating the expression of the first. The superstructure may be acquired during development and inherited through mitotic cell generations. A swimming tadpole can grow from an enucleate egg into which a nucleus from a differentiated frog cell has been transplanted (Gurdon & Laskey, 1970). In the egg, the acquired superstructures of the transplanted nucleus may be lost, preventing their inheritance through the germ cells. But this may not always be the case; it may be that some differentials can be inherited through the sperm and the egg. The inheritance, through meiosis, of acquired superstructures might cause paternal effects in *Drosophila*, and the specific inactivation of the paternal *X*-chromosomes of marsupials (Cooper, Vande-Berg, Sharman & Poole, 1971; Sharman, 1971; Brown & Chandra, 1973), or of the whole paternal chromosome set in certain *Coccids* (Chandra, 1971).

Paramutation at the *R* locus in the maize plant is an example of the inheritance of an acquired characteristic which extends through several sexual generations. At the *R* locus there are a number of alleles which control the production of the pigment anthocyanin. One class of *R* alleles is never, or rarely, recoverable in standard form from particular heterozygotes and in all their gametes the potential to form pigment is reduced (see Brink, 1964, for a review). Maize kernels are triploid, containing one paternal and two maternal alleles at any locus. Consider the crosses illustrated in Table 1 in which only progeny with the genotype $R^{r}r^{g}r^{g}$ in the kernel and $R^{r}r^{g}$ in the plant are considered. Kernels obtained from cross 2a are less pigmented than genotypically identical kernels derived from cross 1a. R^{st} is said to be 'paramutagenic', and its action gives rise to a 'paramutant' R^{r} allele (or $R^{r'}$). The 'paramutant' $R^{r'}$ allele is less effective in pigment production than the standard R^{r} allele. It is gametically transmitted and may be inherited by subsequent generations. This is demon-

Table 1. *Paramutation in maize*

Four types of pollen are crossed with females with the genotype $r^g r^g$. Only progeny with kernels of the genotype $R^r r^g r^g$ are considered. Usually the phenotype of the alleles in the seeds is as follows: R^r, darkly mottled in 1 dose; r^g, colourless; R^{st}, stippled. The phenotypes of the seeds resulting from the four crosses are given in the brackets. The R^r allele in the $R^r r^g r^g$ kernels resulting from cross 2*a* and 2*b* gives the paramutant $R^{r'}$ phenotype (lightly mottled). (From Brink, 1964.)

	Cross				Progeny genotype	
	Female genotype		Male genotype		Kernels	Plants
1a	$r^g r^g$ ♀♀	×	$R^r R^r$ ♂	→	$R^r r^g r^g$ (darkly mottled)	$R^r r^g$
2a	$r^g r^g$ ♀♀	×	$R^r R^{st}$ ♂	→	$R^r r^g r^g$ (lightly mottled)	$R^r r^g$
1b	$r^g r^g$ ♀♀	×	$R^r r^g$ ♂ (pollen from progeny of cross 1a)	→	$R^r r^g r^g$ (darkly mottled)	$R^r r^g$
2b	$r^g r^g$ ♀♀	×	$R^r r^g$ ♂ (pollen from progeny of cross 2a)	→	$R^r r^g r^g$ (lightly mottled)	$R^r r^g$

strated by back-crossing the pollen collected from crosses 1a and 2a with $r^g r^g$ females (see crosses 1b, 2b in Table 1). The $R^r r^g r^g$ kernels resulting from cross 2b are again less pigmented than those from cross 2a.

There are many intriguing characteristics of paramutation, but only a few will be considered here. Mutation is not thought to cause the phenomenon as the repressive action of R^{st} is both directed and ubiquitous; the effect is exerted on almost all R^r gametes produced by $R^r R^{st}$ heterozygotes. The $R^{r'}$ allele is metastable; it tends to revert towards R^r. The extent of repression of R^r varies from plant to plant, so the paramutagenic effect of R^{st} is variable. Furthermore it has been shown that the change from R^r to $R^{r'}$ is unlikely to be mediated by a cytoplasmic particle that is independent of chromosomal genes.

This behaviour is explicable if R^{st} induces a heritable superstructural change in the R^r allele, and if this rearrangement leads to a repression of R^r gene activity. Like position effect variegation in *Drosophila*, heterochromatin exerts a suppressive effect; the action of R^{st} on R^r is somewhat suppressed if that R^r allele is adjacent to a heterochromatic region. The repression of *R* gene activity is unlike the other repressions discussed previously in two main respects. First, this repression is variable rather than absolute. This variety of gene expression might reflect a variety of gene superstructures each differing slightly in superhelical density. Secondly, this differential is inherited through both cell and sexual generations; an acquired characteristic is inherited. Presumably it is immune from those influences active in gametogenesis which remove acquired differentials.

Paramutation is not the only example of non-Mendelian inheritance of this type. Brink (1964) has reviewed other examples to be found in the plant kingdom and

examples from the animal world might include the differential imprinting of chromosomes in *Sciara* (Rieffel & Crouse, 1966; Crouse, Brown & Mumford, 1971), the effects of the segregation-distorter locus in *Drosophila* (Zimmering, Sandler & Nicoletti, 1970) and the changes in mating type of certain ciliates (Nanney, 1964). Does an inheritance of superstructure underlie the inheritance of these acquired characteristics? If so, then a genetic analysis of such situations should define the factors which affect the differential.

(4) *Segregation of chromosomes*

Every time a cell divides chromatids with identical base sequences behave differently; they move to opposite poles of the dividing cell (see Luykx, 1970, for a recent review). The specificity of segregation is usually explained in terms of the bilateral symmetry of duplicated or paired chromosomes and an association of the centromeres with micro-tubules of the spindle apparatus. Bilateral symmetry cannot provide a complete explanation for the specificity of segregation since during the first anaphase of meiosis, unpaired sex chromosomes move to opposite poles of the dividing cell.

There is an alternative explanation based upon superstructure for the specificity of segregation. The movement of a chromosome to a particular pole of the cell might be determined by the superstructure of its centromere. Centromeres might be characterized by one of two superstructures, and the micro-tubules joined to one pole might recognize one superstructure and micro-tubules joined to the other pole, the second. The segregation of many chromosomes in accordance with Mendelian laws can be explained if duplication of a centromeric base sequence leads to the creation of two progeny, the first with one kind of superstructure, and the second with the other kind of superstructure. One further assumption must be made if the segregation of chromosomes during meiosis is to be explained. It seems likely that centromeric base sequences of the paired chromosomes have been duplicated by the time when the first of the two segregations occurs. During the first segregation (at anaphase I) duplicated centromeres move together, only to separate at the second segregation (at anaphase II). Perhaps during the first segregation, the activity of one or other of the two centromeres of dyads is suppressed randomly.

Genetic loss or aberrations in the superstructure of the centromere might lead to segregation of unlinked genes in a manner which disobeyed Mendelian laws. Aneuploidy or the phenomenon of meiotic drive (Zimmering *et al.*, 1970) could result.

(5) In vitro *transformation by viruses*

Some viruses, for example SV_{40} and polyoma, can induce a morphological transformation of mammalian cells *in vitro*: some of these transformed cells may be malignant (Dulbecco, 1969). This phenomenon has been explained either by a gain of viral genes or by a loss of cellular genes as a result of the integration of viral DNA into cellular genes. The superstructure of both viral and cellular base sequences may be changed by the integration of superhelical molecules of viral DNA. If so, only some of the integrated viral genes might be expressed and this seems to be the

case. Alternatively host genes might adopt a new superstructure which changes their expression. The virus might also affect centromeric superstructure and so alter the segregation of chromosomes. This might cause the aneuploidy which is characteristic of the transformed phenotype. Such mechanisms are attractive if one believes that malignancy may be caused by a combination of genetic or epigenetic loss and a generation of genetic variation, followed by selection of the malignant variant (Harris, 1971).

IX. EXPERIMENTAL APPROACH

How might one distinguish between the various explanations of the differential? Where a complex differential exists, it should always be possible to isolate the differentiator (for example, a repressor), since this differential is conditional upon the presence of a differentiator-gene complex. However, if the differential is merely an intrinsic difference in the superstructures of identical base sequences, no such differentiator need be present once the differential has been created. Superstructural differentials may be destroyed without a concomitant loss of material: the destruction of complex differentials probably involves the loss of the differentiator.

It has been discussed how the superstructure of a gene may be determined by that of neighbouring DNA and so gene translocation might affect gene function. The superstructural differential can therefore be distinguished from complex differentials since if the latter are involved, translocated genes should still be subject to regulation by diffusible differentiators and so translocation need not affect gene function.

Cell fusion mediated by Sendai virus can be used to construct binucleate cells from parental cells with genomes that contain identical base sequences but which have different phenotypes (Harris, 1970*b*). Such studies should provide insight into whether or not a particular differentiated phenotype results from *cis* or *trans* phenomena. But the interpretation of these experiments is difficult as it is not always clear whether the mechanisms governing 'determination' or 'differentiation' are being studied. Where dividing and mononucleate hybrid cells are studied there is an additional complication of the organizational instability of the genome (Harris, 1970*b*; Ephrussi, 1972).

If superstructures of DNA can be changed – for example, by incorporation of bromodeoxyuridine – gene expression might be modified. Intercalating agents, which are widely used to measure the superhelical properties of DNA (Bauer & Vinograd, 1968, 1970; Hudson *et al.*, 1969) have also been used to change the superhelical conformation of the closed circular DNA of polyoma virus and so affect its velocity of sedimentation (Crawford & Waring, 1967; Waring, 1970). Addition of small amounts of ethidium bromide to DNA decreased the velocity of sedimentation of the DNA; larger amounts increased it again. This was interpreted as a dissipation of supercoiling by small amounts of the intercalating agent until eventually none remained. Further intercalation of ethidium bromide induced turns of an opposite sense with a concomitant increase in sedimentation velocity. This technique provides a means of varying both the sense and degree of supercoiling: can a gene's activity

be modified when its supercoiling is changed in this way? In fact, ethidium bromide and acridine orange inhibit the replication of cytoplasmic superhelical DNA molecules in bacteria (Hayes, 1964), yeast (Whittaker, Hammond & Luha, 1972) mammalian cells (Nass, 1970), *Chlamydomonas* (Flechtner & Sager, 1973) and trypanosomes (Riou & Delain, 1969).

Of course, a proof or disproof of a role for superstructure in the control of gene expression must depend on a comparison of the superstructures of two DNA molecules with identical base sequence and differing behaviour. It will still remain to show that these superstructures might be inherited.

X. CONCLUSIONS

Any complete explanation of the differential should include a description of its inheritance. If the differential depends on the continuing association of a gene with a differentiator, then that differential can only be inherited if the differentiator concentration is maintained during replication of its target gene. Where the differentiator is a protein, a special self-maintaining circuit involving the protein, the gene that codes for it, and the target gene must be involved. This is because proteins, unlike nucleic acids, cannot be templates for their own synthesis. Where such circuits exist – for example, in the maintenance of repressor levels in lysogenized bacteria – they are not stably inherited. Ways in which the stability of complex differentials might be improved have been discussed. In organisms which show complex patterns of differentiation, each pattern must be associated with a specific self-maintaining circuit unless these circuits are integrated. This might involve the operation of cascades and co-operative effects. The operation of a complex differential would not result directly in *cis* phenomena unless the differentiator was part of a large and so non-diffusing structure. Where the differentiator is freely diffusible, *cis* phenomena could result from complex differentials if co-operative effects were involved.

It may be that development proceeds by a modification of the covalent bonds in genes. It is easy to imagine how changes in the covalent bonds of DNA can be inherited and how they might give rise to *cis* effects. Alternatively, it has been argued that the superstructure of any gene might control its expression, and that superstructures can be replicated and inherited. If so, a description of the differential based on gene superstructure provides a simple explanation for the inheritance of differentials and for *cis* effects. Only a superstructural differential or changes in the covalent structure of DNA can be maintained and replicated in the absence of differentiators.

It would seem likely that differentials that are heritable have a superstructural basis: those that are not might be of other types. The superstructure of a gene would be one factor in the hierarchy of mechanisms which govern the expression of the gene. Critical levels of hormones or other diffusible agents might regulate gene expression within the limits imposed by that superstructure. Critical levels of proteins would almost certainly be involved at the creation of the superstructural differential. Throughout this discussion it has been assumed that gene expression

is controlled at the level of transcription; it is not intended that this should preclude the possibility of controls at other levels or even of the transmission of superstructures from DNA to RNA. Information might be stored in the superstructure of RNA molecules. Transcription of the different superstructures of a gene might give rise to RNA molecules with differing superstructures. The superstructure of the RNA molecule might determine whether or not it was transferred to the cytoplasm or translated into protein (Lodish, 1970, 1971; Fukami & Imahori, 1971).

If superstructure proves to be a basis for the differential, then DNA may contain two kinds of heritable information: one kind being stored in the primary base sequence of a gene and the second kind, which is acquired during development, being contained in its superstructure. The superstructure would be a major factor which determined whether a gene was expressed or not. The development of an organism would then proceed by orderly and directed changes in gene superstructures.

XI. SUMMARY

1. During differentiation identical genes are expressed differently in the cells of one organism. This difference in behaviour can be inherited. The following examples are described: the inactivity of only one of two *X*-chromosomes in female cells of eutherian mammals, the phenomenon of variegated position effect in the fruit-fly *Drosophila* and the variation in expression of the two alleles that code for a flagellar protein in the bacterium *Salmonella*.

2. The assumptions inherent in various explanations of the way differentiated traits are inherited are discussed. The possible bases of the *cis* and *trans* effects seen in eukaryotes are also described.

3. It is now generally believed that behavioural differences of identical base sequences arise from an association of the base sequences with diffusible molecules. An alternative explanation based upon gene superstructure is proposed. Perhaps gene superstructure controls the expression of any gene. Superstructures of DNA-like primary base sequences – might be replicated and inherited. If so, DNA may contain two kinds of heritable information: one kind being stored in the primary base sequence of a gene, and the second kind, which is acquired during development. being contained in its superstructure.

4. Some speculations are made concerning (1) the mode of action of bromodeoxyuridine, (2) the nature of 'determination' and 'differentiation', (3) non-Mendelian inheritance with particular reference to paramutation in maize, (4) the segregation of chromosomes, and (5) *in vitro* transformation by viruses.

XII. ACKNOWLEDGEMENTS

This work was carried out during the tenure of a Stothert Research Fellowship of The Royal Society and Research Fellowships at Brasenose College, Oxford. I thank Professor H. Harris for his support and encouragement and also my many colleagues in Oxford who have helped in the preparation of this manuscript. Special

thanks are due to Mr C. J. Marshall whose continuing help has been invaluable in formulating these ideas, and to Dr P. R. Dendy for his help with the writing of the manuscript.

XIII. REFERENCES

ABBOTT, J. & HOLTZER, H. (1968). The loss of phenotypic traits by differentiated cells. V. The effect of 5-bromodeoxyuridine on cloned chondrocytes. *Proc. natn. Acad. Sci., U.S.A.* **59**, 1144–51.

ADAMS, A., LINDAHL, T. & FRESCO, R. (1967). Conformation differences between the biologically active and inactive forms of a transfer ribonucleic acid. *Proc. natn. Acad. Sci., U.S.A.* **57**, 1684–91.

ALBERTS, B. M. & FREY, L. (1970). T4 bacteriophage gene 32: a structural protein in the replication and recombination of DNA. *Nature, Lond.* **227**, 1313–18.

ARBER, W. & LINN, S. (1969). DNA modification and restriction. *A. Rev. Biochem.* **38**, 467–500.

ASHBURNER, M. (1970). A prodromus to the genetic analysis of puffing in *Drosophila*. *Cold Spring Harb. Symp. quant. Biol.* **35**, 533–8.

BAKER, W. K. (1967). A clonal system of differential gene activity in *Drosophila*. *Devl Biol.* **16**, 1–17.

BAKER, W. K. (1968). Position-effect variegation. *Adv. Genet.* **14**, 133–69.

BAKER, W. K. (1971). Evidence for position-effect suppression of the ribosomal RNA cistrons in *Drosophila melanogaster*. *Proc. natn. Acad. Sci., U.S.A.* **68**, 2472–76.

BARNETT, W. E. & DE SERRES, F. J. (1963). Fixed genetic instability in *Neurospora crassa*. *Genetics, Princeton* **48**, 717–23.

BARR, M. L. (1951). The morphology of neurological nuclei in the cat according to sex. *Expl Cell Res.* **2**, 288–90.

BAUER, W. & VINOGRAD, J. (1968). The interaction of closed circular DNA with intercalative dyes. I. The superhelix density of SV_{40} in the presence and absence of dye. *J. molec. Biol.* **33**, 141–71.

BAUER, W. & VINOGRAD, J. (1970). The interaction of closed circular DNA with intercalative dyes. III. Dependence of the buoyant density upon superhelix density and base composition. *J. molec. Biol.* **54**, 281–98.

BAUER, W. & VINOGRAD, J. (1971). The use of intercalative dyes in the study of closed circular DNA. *Progress in molecular and subcellular biology* **2**, 181–215.

BECKER, H. J. (1969). Genetic and variegation mosaics in the eye of *Drosophila*. *Current Topics Devl. Biol.* **1**, 155–71.

BEERMANN, W. (1966). Differentiation at the level of the chromosomes. In *Cell Differentiation and Morphogenesis* (ed. W. Beermann and others), pp. 24–51. Amsterdam: North-Holland.

BISCHOFF, R. & HOLTZER, H. (1970). Inhibition of myoblast fusion after one round of DNA synthesis in 5-bromodeoxyuridine. *J. Cell Biol.* **44**, 134–50.

BRAM, S. & TOUGARD, P. (1972). Polymorphism of natural DNA. *Nature New Biol., Lond.* **239**, 128–31.

BRINK, R. A. (1964). Genetic repression of *R* action in maize. In *The Role of Chromosomes in Development* (ed. M. Locke), pp. 183–230. New York: Academic Press.

BRITTEN, R. J. & DAVIDSON, E. H. (1969). Gene regulation for higher cells: a theory. *Science, N.Y.* **165**, 349–57.

BRITTEN, R. J. & KOHNE, D. E. (1968). Repeated sequences in DNA. *Science, N.Y.* **161**, 529–40.

BROCKMAN, R. W. & ANDERSON, E. P. (1963). Biochemistry of cancer (metabolic aspects). *A. Rev. Biochem.* **32**, 463–512.

BROWN, S. W. (1966). Heterochromatin. *Science, N.Y.* **151**, 417–25.

BROWN, S. W. & CHANDRA, H. S. (1973). Inactivation system of the mammalian *X*-chromosome. *Proc. natn. Acad. Sci., U.S.A.* **70**, 195–9.

CAHN, R. D. & CAHN, M. B. (1966). Heritability of cellular differentiation: clonal growth and expression of differentiation of retinal pigment cells *in vitro*. *Proc. natn. Acad. Sci., U.S.A.*, **55**, 106–14.

CAIRNS, J. (1963). The chromosomes of *Escherichia coli*. *Cold Spring Harb. Symp. quant. Biol.* **28**, 43–5.

CALLAN, H. G. (1972). Replication of DNA in the chromosomes of eukaryotes. *Proc. R. Soc. Lond.* B **181**, 19–41.

CHAMPOUX, J. J. & DULBECCO, R. (1972). An activity from mammalian cells that untwists superhelical DNA – a possible swivel for DNA replication. *Proc. natn. Acad. Sci., U.S.A.* **69**, 143–6.

CHANDRA, H. S. (1971). Inactivation of whole chromosomes in mammals and coccids: some comparisons. *Genet. Res.* **18**, 265–76.

CHARGAFF, E., CRAMPTON, C. F. & LIPSHITZ, R. (1953). Separation of calf thymus deoxyribonucleic acid into fractions of different composition. *Nature, Lond.* **172**, 289–92.

CHU, E. H. Y., SUN, N. C. & CHANG, C. C. (1972). Induction of auxotrophic mutations by treatment of Chinese hamster cells with 5-bromodeoxyuridine and black light. *Proc. natn. Acad. Sci., U.S.A.* **69**, 3459–63.

COMINGS, D. E. (1968). The rationale for an ordered arrangement of chromatin in the interphase nucleus. *Am. J. hum. Genet.* **20**, 440–60.

COOK, P. R. (1973). Hypothesis on differentiation and the inheritance of gene superstructure. *Nature, Lond.* **245**, 23–5.

COON, H. G. (1966). Clonal stability and phenotypic expression of chick cartilage cells *in vitro*. *Proc. natn. Acad. Sci., U.S.A.* **55**, 66–73.

COOPER, D. W. (1971). Directed genetic change model for *X*-chromosome inactivation in eutherian mammals. *Nature, Lond.* **230**, 292–4.

COOPER, D. W., VANDEBERG, J. L., SHARMAN, G. B. & POOLE, W. E. (1971). Phosphoglycerate kinase polymorphism in kangaroos provides further evidence for paternal *X*-inactivation. *Nature, New Biol., Lond.* **230**, 155–7.

CRAWFORD, L. V. & WARING, M. J. (1967). Supercoiling of polyoma virus DNA measured by its interaction with ethidium bromide. *J. molec. Biol.* **25**, 23–30.

CRICK, F. H. C. (1971*a*). General model for the chromosomes of higher organisms. *Nature, Lond.* **234**, 25–7.

CRICK, F. H. C. (1971*b*). The scale of pattern formation. *Symp. Soc. exp. Biol.* **25**, 429–38.

CROUSE, H. V., BROWN, A. & MUMFORD, B. C. (1971). L-chromosome inheritance and the problem of chromosome 'imprinting' in *Sciara* (Sciaridae, Diptera). *Chromosoma* **34**, 324–39.

DANEHOLT, B. & EDSTRÖM, J.-E. (1967). The content of deoxyribunucleic acid in individual polytene chromosomes of *Chironomus tentans*. *Cytogenetics* **6**, 350–6.

DEMEREC, M. (1941*a*). The nature of the changes in the white-Notch region of the *X*-chromosome of *Drosophila melanogaster*. *Proc. 7th Int. Congr. Genet. Edinburgh* 1939, pp. 99–103.

DEMEREC, M. (1941*b*). Unstable genes in *Drosophila*. *Cold Spring Harb. Symp. quant. Biol.* **9**, 145–9.

DEMEREC, M. & SLIZYNSKA, H. (1937). Mottled white 258–18 of *Drosophila melanogaster*. *Genetics, Princeton* **22**, 641–9.

DULBECCO, R. (1969). Cell transformation by viruses. *Science, N.Y.* **166**, 962–8.

EASON, R. & VINOGRAD, J. (1971). Superhelix density heterogeneity of intracellular simian virus 40 deoxyribonucleic acid. *J. Virol.* **7**, 1–7.

EICHER, E. M. (1971). *X*-autosome translocations in the mouse: total inactivation versus partial inactivation of the *X*-chromosome. *Adv. Genet.* **15**, 175–259.

EPHRUSSI, B. (1972). *Hybridization of Somatic Cells*. Princeton: Princeton University Press.

ESPEJO, R., ESPEJO-CANELO, E. & SINSHEIMER, R. L. (1971). A difference between intracellular and viral supercoiled PM2 DNA. *J. molec. Biol.* **56**, 623–6.

FINCHAM, J. R. S. & DAY, P. R. (1971). *Fungal Genetics*, 3rd ed. Oxford: Blackwell.

FLECHTNER, V. R. & SAGER, R. (1973). Ethidium bromide induced selective and reversible loss of chloroplast DNA. *Nature, New Biol., Lond.* **241**, 277–9.

FUKAMI, H. & IMAHORI, K. (1971). Control of translation by the conformation of messenger RNA. *Proc. natn. Acad. Sci., U.S.A.* **68**, 570–3.

GALL, J. G. (1955). On the submicroscopic structure of chromosomes. *Brookhaven Symp. Biol.* **8**, 17–32.

GARCÍA-BELLIDO, A. (1972). Pattern formation in imaginal disks. In *The Biology of Imaginal Disks* (ed. H. Ursprung and R. Nöthiger), pp. 59–91. Berlin: Springer-Verlag.

GEHRING, W. (1972). The stability of the determined state in cultures of imaginal disks in *Drosophila*. In *The Biology of Imaginal Disks* (ed. H. Ursprung & R. Nöthiger), pp. 35–58. Berlin: Springer-Verlag.

GOODWIN, B. C. & COHEN, M. H. (1969). A phase-shift model for the spatial and temporal organisation of developing systems. *J. theoret. Biol.* **25**, 49–107.

GROSS, P. R. (1968). Biochemistry of differentiation. *A. Rev. Biochem.* **37**, 631–60.

GRUMBACH, M. M., MORISHAMA, A. & TAYLOR, J. H. (1963). Human sex chromosome abnormalities in relation to DNA replication and heterochromatinization. *Proc. natn. Acad. Sci., U.S.A.* **49**, 581–9.

GURDON, J. B. & LASKEY, R. A. (1970). The transplantation of nuclei from single cultured cells into enucleate frogs' eggs. *J. Embryol. exp. Morphol.* **24**, 227–48.

HADORN, E., GSELL, R. & SCHULTZ, J. (1970). Stability of a position-effect variegation in normal and transdetermined larval blastemas from *Drosophila melanogaster*. *Proc. natn. Acad. Sci., U.S.A.* **65**, 633–7.

HALDANE, J. B. S. (1957). The cost of natural selection. *J. Genet.* **55**, 511–24.

HANLON, S., JOHNSON, R. S., WOLF, B. & CHAN, A. (1972). Mixed conformations of deoxyribonucleic acid in chromatin: a preliminary report. *Proc. natn. Acad. Sci., U.S.A.* **69**, 3263–7.

HARRIS, H. (1970*a*). *Nucleus and Cytoplasm*, 2nd ed. Oxford: Clarendon Press.
HARRIS, H. (1970*b*). *Cell Fusion*. Oxford: Clarendon Press.
HARRIS, H. (1971). Cell fusion and the analysis of malignancy. *Proc. R. Soc. Lond.* B **179**, 1–20.
HAYES, W. (1964). *The Genetics of Bacteria and Their Viruses*. Oxford: Blackwell.
HUBERMAN, J. A. & RIGGS, A. D. (1968). On the mechanism of DNA replication in mammalian chromosomes. *J. molec. Biol.* **32**, 327–41.
HUDSON, B., UPHOLT, W. B., DEVINNY, J. & VINOGRAD, J. (1969). The use of an ethidium analogue in the dye-buoyant density procedure for the isolation of closed circular DNA: the variation of the superhelix density of mitochondrial DNA. *Proc. natn. Acad. Sci., U.S.A.* **62**, 813–20.
IBALL, J., MORGAN, C. H. & WILSON, H. R. (1966). Structures of 5-bromodeoxyuridine and 5-bromouridine. *Nature, Lond.* **209**, 1230–2.
IINO, T. (1961). A stabiliser of antigenic phases in *Salmonella abortus-equi*. *Genetics, Princeton* **46**, 1465–9.
IINO, T. (1969). Genetics and chemistry of bacterial flagella. *Bact. Rev.* **33**, 454–75.
JACOB, F. & MONOD, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *J. molec. Biol.* **3**, 318–56.
JOHNSON, R. T. & RAO, P. N. (1970). Mammalian cell fusion: induction of premature chromosome condensation in interphase nuclei. *Nature, Lond.* **226**, 717–22.
JUDD, B. H., SHEN, M. W. & KAUFMAN, T. C. (1972). The anatomy and function of a segment of the *X*-chromosome of *Drosophila melanogaster*. *Genetics, Princeton* **71**, 139–56.
KOERNER, J. F. (1970). Enzymes of nucleic acid metabolism. *A. Rev. Biochem.* **39**, 291–322.
KONIGSBERG, I. R. (1961). Some aspects of myogenesis *in vitro*. *Circulation* **24**, 447–57.
LEDERBERG, E. M. & STOCKER, B. A. D. (1970). Phase variation in *rec*⁻ mutants of *Salmonella typhimurium*. *Bact. Proc.* p. 35.
LEDERBERG, J. & IINO, T. (1956). Phase variation in *Salmonella*. *Genetics, Princeton* **41**, 743–57.
LEWIS, E. B. (1950). The phenomenon of position effect. *Adv. Genet.* **3**, 73–115.
LEWIS, K. R. & JOHN, B. (1963). *Chromosome Marker*. London: J. & A. Churchill.
LIN, S.-Y. & RIGGS, A. D. (1972). *Lac* operator analogues: bromodeoxyuridine substitution in the *lac* operator affects the rate of dissociation of the *lac* repressor. *Proc. natn. Acad. Sci., U.S.A.* **69**, 2574–6.
LINDSLEY, D. L., EDINGTON, C. W. & VON HALLE, E. S. (1960). Sex-linked recessive lethals in *Drosophila* whose expression is suppressed by the *Y*-chromosome. *Genetics, Princeton* **45**, 1649–70.
LODISH, H. (1970). Secondary structure of bacteriophage f2 ribonucleic acid and the initiation of *in vitro* protein biosynthesis. *J. molec. Biol.* **50**, 689–702.
LODISH, H. (1971). Thermal melting of bacteriophage f2 RNA and initiation of synthesis of the maturation protein. *J. molec. Biol.* **56**, 627–32.
LUYKX, P. (1970). Cellular mechanisms of chromosome distribution. *Int. Rev. Cytol.* Suppl. 2.
LWOFF, A. & GUTMAN, A. (1950). Recherches sur un *Bacillus megatherium* lysogène. *Ann. Inst. Pasteur* **78**, 711–39.
LYON, M. F. (1970). Genetic activity of sex chromosomes in somatic cells of mammals. *Phil. Trans. R. Soc. Lond.* B **259**, 41–52.
LYON, M. F. (1971). Possible mechanisms of *X*-chromosome inactivation. *Nature, New Biol., Lond.* **232**, 229–32.
LYON, M. F. (1972). *X*-chromosome inactivation and developmental patterns in mammals. *Biol. Rev.* **47**, 1–35.
MCCLINTOCK, B. (1950). The origin and behaviour of mutable loci in maize. *Proc. natn. Acad. Sci., U.S.A.* **36**, 344–55.
MCFALL, E. (1967). Dominance studies with stable merodiploids in the D-serine deaminase system of *Escherichia coli* K-12. *J. Bact.* **94**, 1982–8.
MALAMY, M. H. (1970). Some properties of insertion mutations in the *lac* operon. In *The Lactose Operon* (ed. J. R. Beckwith and D. Zipser), pp. 359–73. Cold Spring Harbor: Cold Spring Harbor Laboratory.
MALAMY, M. H., FIANDT, M. & SZYBALSKI, W. (1972). Electron microscopy of polar insertions in the *lac* operon of *Escherichia coli*. *Molec. gen. Genet.* **119**, 207–22.
MARZULLO, G. (1972). Regulation of cartilage enzymes in cultured chondrocytes and the effect of 5-bromodeoxyuridine. *Devl Biol.* **27**, 20–6.
MAYNE, R., SANGER, J. W. & HOLTZER, H. (1971). Inhibition of mucopolysaccharide synthesis by 5-bromodeoxyuridine in cultures of chick amnion cells. *Devl Biol.* **25**, 547–67.
MIGEON, B. R. (1972). Stability of *X*-chromosomal inactivation in human somatic cells. *Nature, Lond.* **239**, 87–9.

MILLER, O. J., BREG, W. R., MUKHERJEE, B. B., GAMBLE, A. VAN N. & CHRISTAKOS, A. C. (1963*a*) Non-random distribution of chromosomes in metaphase figures from cultured human leukocytes II. The peripheral location of chromosomes 13, 17–18 and 21. *Cytogenetics* **2**, 152–67.

MILLER, O. J., MUKHERJEE, B. B., BREG, W. R. & GAMBLE, A. VAN N. (1963*b*). Non-random distribution of chromosomes in metaphase figures from cultured human leukocytes. I. The peripheral location of the Y chromosome. *Cytogenetics* **2**, 1–15.

MIN JOU, W., HAEGEMAN, G., YSEBAERT, M. & FIERS, W. (1972). Nucleotide sequence of the gene coding for the bacteriophage MS2 coat protein. *Nature, Lond.* **237**, 82–8.

MONOD, J. & JACOB, F. (1961). General conclusions: teleonomic mechanisms in cellular metabolism, growth and differentiation. *Cold Spring Harb. Symp. quant. Biol.* **26**, 389–401.

MOORE, G. E. (1964). *In vitro* cultures of a pigmented hamster melanoma cell line. *Expl Cell Res.* **36**, 422–3.

MORISHAMA, A., GRUMBACH, M. M. & TAYLOR, J. H. (1962). A synchronous duplication of human chromosomes and the origin of sex chromatin. *Proc. natn. Acad. Sci., U.S.A.* **48**, 756–63.

MULLER, H. J (1950). Our load of mutations. *Am. J. hum. Genet.* **2**, 111–76.

NANNEY, D. L. (1964). Macronuclear differentiation and subnuclear assortment in ciliates. In *The Role of Chromosomes in Development* (ed. M. Locke), pp. 253–73. New York: Academic Press.

NASIM, A. (1967). The induction of replicating instabilities by mutagens in *Schizosaccharomyces pombe*. *Mutation Res.* **4**, 753–63.

NASS, M. M. K. (1970). Abnormal DNA patterns in animal mitochondria: ethidium bromide-induced breakdown of closed circular DNA and conditions leading to oligomer accumulation. *Proc. natn. Acad. Sci., U.S.A.* **67**, 1926–33.

NÖTHIGER, R. (1972). The larval development of imaginal disks. In *The Biology of Imaginal Disks* (ed. H. Ursprung and R. Nöthiger), pp. 1–34. Berlin: Springer-Verlag.

OHNO, S. (1971). Simplicity of mammalian regulatory systems inferred by single gene determination of sex phenotypes. *Nature, Lond.* **234**, 134–7.

OHNO, S. & HAUSCHKA, T. S. (1960). Allocycly of the *X*-chromosome in tumours and normal tissues. *Cancer Res.* **20**, 541–5.

PARDON, J. F. & WILKINS, M. H. F. (1972). A super-coil model for nucleohistone. *J. molec. Biol.* **68**, 115–24.

PARDON, J. F., WILKINS, M. H. F. & RICHARDS, B. M. (1967). Superhelical model for nucleohistone. *Nature, Lond.* **215**, 508–9.

PAUL, J. (1972). General theory of chromosome structure and gene activation in eukaryotes. *Nature, Lond.* **238**, 444–6.

PEARCE, U. B. & STOCKER, B. A. D. (1967). Phase variation of flagellar antigens in *Salmonella*: abortive transduction studies. *J. gen. Microbiol.* **49**, 335–49.

PELLING, C. (1966). A replicative and synthetic chromosomal unit – the modern concept of the chromomere. *Proc. R. Soc. Lond.* B **164**, 279–89.

PHILLIPS, D. M. P. (1971). *Histones and Nucleohistones*. New York: Plenum.

POHL, F. M., JOVIN, T. M., BAEHR, W. & HOLBROOK, J. J. (1972). Ethidium bromide as a cooperative effector of a DNA structure. *Proc. natn. Acad. Sci., U.S.A.* **69**, 3805–9.

PROKOFYEVA-BELGOVSKAYA, A. A. (1948). Heterochromatization as a change of the chromosome cycle. *J. Genetics* **48**, 80–98.

REVEL, H. R. & LURIA, S. E. (1970). DNA-glucosylation in T-even phage: genetic determination and role in phage-host interaction. *A. Rev. Genetics* **4**, 177–92.

RICHARDSON, U. I., TASHJIAN, A. H. & LEVINE, L. (1969). Establishment of a clonal strain of hepatoma cells which secrete albumin. *J. Cell Biol.* **40**, 236–47.

RIEFFEL, S. M. & CROUSE, H. V. (1966). The elimination and differentiation of chromosomes in the germ line of *Sciara*. *Chromosoma* **19**, 231–76.

RIOU, G. & DELAIN, E. (1969). Abnormal circular DNA molecules induced by ethidium bromide in the kinetoplast of *Trypanosoma cruzi*. *Proc. natn. Acad. Sci., U.S.A.* **64**, 618–25.

ROWE, W. P., LOWY, D. R., TEICH, N. & HARTLEY, J. W. (1972) .Some implications of the activation of murine leukemia virus by halogenated pyrimidines. *Proc. natn. Acad. Sci., U.S.A.* **69**, 1033–5.

RUDKIN, G. T. (1965). The relative mutabilities of DNA in regions of the *X*-chromosome of *Drosophila melanogaster*. *Genetics, Princeton* **52**, 665–81.

RUTTER, W. J., KEMP, J. D., BRADSHAW, W. S., CLARK, W. R., RONZIO, R. A. & SANDERS, T. G. (1968). Regulation of specific protein synthesis in cytodifferentiation. *J. Cell Physiol.* **72**, Suppl. 1, 1–18.

SANGER, F. (1971). Nucleotide sequences in bacteriophage ribonucleic acid. *Biochem. J.* **124**, 833–43.

SATO, K., SLESINSKI, R. S. & LITTLEFIELD, J. W. (1972). Chemical mutagenesis at the phosphoribosyltransferase locus in cultured human lymphoblasts. *Proc. natn. Acad. Sci., U.S.A.* **69**, 1244–8.

SCHINDLER, A.-M. & MIKAMO, K. (1970). Triploidy in man: Report of a case and a discussion in etiology. *Cytogenetics* **9**, 116–30.

SCHNEIDERMAN, L. J. & SMITH, C. A. B. (1962). Non-random distribution of certain homologous pairs of normal human chromosomes in metaphase. *Nature, Lond.* **195**, 1229–30.

SCHULTZ, J. (1941). The function of heterochromatin. *Proc. 7th Int. Cong. Genet.*, Edinburgh, 1939, pp. 257–62.

SCHULTZ, J. (1947). The nature of heterochromatin. *Cold Spring Harb. Symp. quant. Biol.* **12**, 179–91.

SHARMAN, G. B. (1971). Late DNA replication in the paternally derived *X*-chromosome of female kangaroos. *Nature, Lond.* **230**, 231–2.

SHEID, B., SRINIVASAN, P. R. & BOREK, E. (1968). Deoxyribonucleic acid methylase of mammalian tissues. *Biochemistry, N.Y.* **7**, 280–5.

SILAGI, S., BEJU, D., WRATHALL, J. & DEHARVEN, E. (1972). Tumorigenicity, immunogenicity and virus production in mouse melanoma cells treated with 5-bromodeoxyuridine. *Proc. natn. Acad. Sci., U.S.A.* **69**, 3443–7.

SINISCALCO, M., KLINGER, H. P., EAGLE, H., KOPROWSKI, H., FUJIMOTO, W. Y. & SEEGMILLER, J. E. (1969). Evidence for intergenic complementation in hybrid cells derived from two human diploid strains each carrying an *X*-linked mutation. *Proc. natn. Acad. Sci., U.S.A.* **62**, 793–9.

STELLWAGEN, R. H. & TOMKINS, G. M. (1971*a*). Differential effect of 5-bromodeoxyuridine on the concentrations of specific enzymes in hepatoma cells in culture. *Proc. natn. Acad. Sci., U.S.A.* **68**, 1147–50.

STELLWAGEN, R. H. & TOMKINS, G. M. (1971*b*). Preferential inhibition by 5-bromodeoxyuridine of the synthesis of tyrosine aminotransferase in hepatoma cell cultures. *J. molec. Biol.* **56**, 167–82.

STEVENS, L. C. (1960). Embryonic potency of embryoid bodies derived from a transplantable testicular teratoma of the mouse. *Devl Biol.* **2**, 285–97.

STEWARD, F. C. (1970). From cultured cells to whole plants: the induction and control of their growth and morphogenesis. *Proc. R. Soc. Lond.* B **175**, 1–30.

STOCKDALE, F., OKAZAKI, K., NAMEROFF, M. & HOLTZER, H. (1964). 5-bromodeoxyuridine: effect on myogenesis *in vitro*. *Science, N.Y.* **146**, 533–5.

TEMIN, H. M. (1971). The protovirus hypothesis: speculations on the significance of RNA-directed DNA synthesis for normal development and for carcinogenesis. *J. natn. Cancer Inst.* **46**, iii–vii.

THOMAS, C. A., HAMKALO, B. A., MISRA, D. N. & LEE, C. S. (1970). Cyclization of eucaryotic deoxyribonucleic acid fragments. *J. molec. Biol.* **51**, 621–32.

WALKER, P. M. B. (1971). Origin of satellite DNA. *Nature, Lond.* **229**, 306–8.

WANG, J. C. (1971). Interaction between DNA and an *Escherichia coli* protein *ω*. *J. molec. Biol.* **55**, 523–33.

WARING, M. (1970). Variation of the supercoils in closed circular DNA by binding of antibiotics and drugs: evidence for molecular models involving intercalation. *J. molec. Biol.* **54**, 247–79.

WATKINS, J. F. (1970). The effects of some metabolic inhibitors on the ability of SV_{40} virus in transformed cells to be detected by cell fusion. *J. Cell Sci.* **6**, 721–37.

WATSON, J. D. (1970). The Molecular Biology of the Gene, 2nd ed. New York: W. A. Benjamin.

WEINTRAUB, H., CAMPBELL, G. LE M. & HOLTZER, H. (1972). Identification of a developmental program using bromodeoxyuridine. *J. molec. Biol.* **70**, 337–50.

WESSELLS, N. K. (1964). DNA synthesis, mitosis and differentiation in pancreatic acinar cells *in vitro*. *J. Cell Biol.* **20**, 415–33.

WESTPHAL, H. (1970). SV40 DNA strand selection by *Escherichia coli* RNA polymerase. *J. molec. Biol.* **50**, 407–20.

WHITTAKER, P. A., HAMMOND, R. C. & LUHA, A. A. (1972). Mechanism of mitochondrial mutation in yeast. *Nature, New Biol., Lond.* **238**, 266–8.

WOLPERT, L. (1969). Positional information and the spatial pattern of cellular differentiation. *J. theoret. Biol.* **25**, 1–47.

WORCEL, A. & BURGI, E. (1972). On the structure of the folded chromosome of *Escherichia coli*. *J. molec. Biol.* **71**, 127–47.

YAFFE, D. (1968). Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. natn. Acad. Sci., U.S.A.* **61**, 477–83.

YASUMURA, Y., TASHJIAN, A. H. & SATO, G. H. (1966). Establishment of four functional, clonal strains of animal cells in culture. *Science, N.Y.* **154**, 1186–9.

YUNIS, J. J. & YASMINEH, W. G. (1971). Heterochromatin, satellite DNA and cell function. *Science, N.Y.* **174**, 1200–9.

ZAKHAROV, A. F. & EGOLINA, N. A. (1972). Differential spiralisation along mammalian mitotic chromosomes. I. BUdR-revealed differentiation in Chinese hamster cells. *Chromosoma* **38**, 341–65.

ZIMMERING, S., SANDLER, L. & NICOLETTI, B. (1970). Mechanisms of meiotic drive. *A. Rev. Genetics* **4**, 409–36.

Note added in proof

Linear duplexes of DNA of the bacterial virus λ may be converted *in vitro*, in the presence of intercalating agents, into covalently closed and supercoiled circles. The number of superhelical turns in these circles can be controlled by varying the concentration of intercalating agent in the reaction mixture. In this way, four kinds of circular DNA molecules, possessing different numbers of superhelical turns superimposed upon the same base sequence, have been prepared and used as templates in directing RNA synthesis *in vitro*. The superstructure of the DNA is found to determine both the amount and type of RNA synthesized by RNA polymerase (Botchan, Wang & Echols, 1973).

REFERENCE

Botchan, P. Wang, J. C. & Echols, H. (1973). Effect of circularity and superhelicity on transcription from bacteriophage λ DNA. *Proc. natn. Acad. Sci. U.S.A.* **70**, 3077–81.