GENETICAL AND PHYSIOLOGICAL STUDIES INTO THE BASIS OF COLICIN E2 SUSCEPTIBILITY IN ESCHERICHIA COLI K12

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INDEX

INTRODUCTION

Cell Surface Structure

The envelope surrounding the bacterial cell may be mechanically separated into an outer wall and an inner cytoplasmic membrane. This artificial division of the cell envelope is further emphasised by differences in the structure and function of the two entities. Thus the outer wall is composed of a firm rigid layer underlying two relatively soft, flexible layers (Weidel, Frank and Martin, 1960). The outermost layer of this is a protein-lipid complex, the middle entirely polysaccharide, whilst the innermost layer, the R-layer, obtains rigidity from a composition of glycosaminopeptide and a proteinaceous particulate substance (Martin and Frank, 1962). The R-layer provides the cell with mechanical rigidity, whereas the metabolically inactive outer layers give resistance to the high internal osmotic pressure of the cell contents. The antigenic and endotoxic properties of the cell have been shown to be derived from the outer wall (Weidel and Primosigh, 1958).

The inner cytoplasmic membrane is a protein-lipid complex, and is in intimate contact with the cell cytoplasm (Kellenberger and Ryter, 1958). In earlier studies, the cytoplasmic membrane was thought to be principally involved in osmoregulation in the cell, but evidence accumulated in recent years indicates several additional

essential involvements in cellular metabolism.

Cell Membrane Function

(a) DNA replication, DNA synthesis and cell division

It has been proposed (Jacob, Brenner and Cuzin, 1963; Ryter, Hirota and Jacob, 1968) that the regulation of DNA synthesis in the bacterial cell, and the separation of daughter chromosomes at cell division is dependent on the association of the bacterial chromosome to a specific fraction of the cell membrane. If the membrane does have a role in cell division and chromosome replication in Escherichia coli attachment of the DNA to the membrane may therefore be anticipated. Yet until recently, cytological preparations showing contiguity between membranous infoldings comprising the mesosomes had only been obtained with the gram positive bacterium, Bacillus subtilis (Ryter and Jacob, 1964; Ryter and Landman, 1964). However direct cytological connection between the nucleus and mesosome of E.coli has since been demonstrated (Ryter and Jacob, 1966; Ryter and Jacob, 1967; Ryter, 1968).

Additional evidence in support of these observations has come from Ganesan and Lederberg (1965) with the isolation of a cell membrane bound fraction of bacterial DNA from B.subtilis by gentle breakage of the cell followed by sedimentation of the DNA-membrane complex by sucrose gradient centrifugation. Since the DNA-membrane fraction isolated had DNA polymerase activity, they proposed that the

cell membrane also had an active role in the synthesis of DNA. However Rosenberg and Cavalieri (1968) have indicated that from the unusual shear sensitivity of DNA of the E.coli genome and the location of phospholipid binding, DNA has not one but several attachment points to the cell membrane. They conclude that DNA is not attached to any one specific site on the cell membrane, and no unique cell membrane fraction is implicated in the regulation of DNA replication. In contrast Earhardt, Tremblay, Daniels and Schaechter (1968) have recently isolated a membrane/DNA complex without recourse to the sedimentation properties of the two cell components or the shear sensitivity of the E.coli genome. By their technique, dependent on the ability of the attached membrane fraction to adhere to the hydrophobic surface of detergent crystals, they have biochemically and cytologically demonstrated the attachment of cell DNA to a specific membrane fraction.

A role for the membrane in replication of DNA in B.subtilis has also recently been suggested by Sueoka and Quinn (1968). They demonstrated that the membrane fraction is most enriched after sucrose gradient centrifugation, for DNA transferring ade16, the marker closest to the chromosome origin (Yoshikawa and Sueoka, 1967). This suggests that the origin is, in fact, membrane-attached. Furthermore, they found that the membrane fraction is greatly enriched after

lysis and centrifugation, for tritium used to label the origin, relative to ^{14}C used to uniformly label the DNA of the cell. This supported their genetical results and they concluded that the origin of replication of the chromosome is attached to the membrane.

The cell membrane has also been implicated in the replication of phage DNA. Thus it was found by Knippers and Sinsheimer (1968) that the only molecules of bacteriophage ØX174 which replicated in the cell were attached to a rapidly sedimenting component, presumably membrane. Furthermore Denhardt and Burgess (1968) have suggested that fixation of the DNA of phage ØX174 to the membrane may actually mean that the membrane actively participates in the replication of phage DNA. Similar membrane attachment sites have also been reported for λ (Salivar and Sinsheimer, 1969) and for the DNA of phage T4 in infected cells (Earhardt, Tremblay, Daniels and Schaechter, 1968). In the latter case, electron micrographs of cytological preparations displaying the attachment of phage T4 DNA to a membrane fraction have been successfully prepared (Daniels, personal communication).

(b) Protein synthesis

Evidence now indicates that the cell membrane also has a role in macromolecular synthesis in that the sites of protein synthesis in the cell, the polyribosomes and ribosomes, appear to be bound to the membrane. For example, Schlessinger (1963) has isolated poly-

ribosome/membrane complexes of Bacillus megaterium, and in E.coli, electron micrographs of membrane ghosts (Abrams, Nielson and Thaement, 1964) have indicated the presence of ribosomes in the cell membrane. Furthermore, the membrane fraction isolated by Tremblay, Daniels and Schaechter (1969) has been found to contain polysomes in addition to a DNA/RNA complex. However it is not yet known whether the membrane has a role in protein synthesis other than presumably co-ordinating the activity of the ribosomes.

(c) Oxidative phosphorylation

The isolation of a membrane fraction with associated ATPase activity from Micrococcus lysodeikticus (Munoz, Freer, Ellar and Salton, 1968) suggested an involvement of the membrane in energy metabolism in the cell. Previously, cytochemical studies (Iterson and Leene, 1964; Kawata and Inove, 1965; Steed and Murray, 1966) have demonstrated that various oxidation-reduction reactions in the bacterial cell take place either in the membrane or in membranous infoldings which comprise the mesome of the bacterial cell. Futhermore, Marr (1960) has described the location of cytochromes comprising part of the electron transport system, and respiratory enzymes such as myokinase, ATPase and polynucleotide phosphorylase in the cell membrane. Thus a structural organisation may well be common to both mitochondria and the bacterial membrane, with the membrane possibly co-ordinating redox actions within the cell.

(d) Sugar transport and metabolism

Certain enzymes involved in the phosphorylation of sugars have been found to be associated with the cell membrane. An example is alkaline phosphatase, released by the lysis of bacterial cells after sucrose and EDTA treatment (Nossal and Heppel, 1966), which has been shown to participate in the transport of sugars by isolated membrane preparations of E.coli (Kaback, 1968).

(e) Site of enzyme attachment

Enzymes with a variety of metabolic functions have been found to be associated with, or bound to the cytoplasmic membrane of the cell. These include the degradative enzymes alkaline phosphatase, cyclic phosphodiesterase and 5-nucleotidase. These can be released by sucrose and EDTA treatment of bacterial cells which releases cell surface layers without affecting the integrity of the cell membrane, thereby not promoting the release of cell contents. In addition, alkaline phosphatase (Malamy and Honecker, 1964), the deoxyribonuclease endonuclease I and a yet unidentified exonuclease (Obinata and Mizuno, 1968) have been found to be membrane attached, located in the periplasmic space between the cell wall and the membrane. The surface location of these enzymes contrasts to, for example, exonuclease I (Obinata and Mizuno, 1968), and endonuclease II (Friedberg and Goldthwaite, 1968) which are found within the cytoplasm and are

not released by light osmotic shock of the cell.

(f) Permease synthesis and function

The cell membrane is thought to be the active site of a variety of permeases in E.coli. Thus the membrane has been suggested to be the seat of bacterial permeases (Cohen and Monod, 1967), to participate in the synthesis of, for example, the β-galactosidase permease (Tarlov and Kennedy, 1965) and to contain amongst others the hexose phosphate transport system (Winkler, 1966) and the phosphotransferase system (Kundig, Ghosh and Roseman, 1964).

(g) Lipid synthesis

The cell membrane is also thought to control the synthesis of lipids in the cell. Thus lipid synthetase enzymes are membrane bound (Hildebrand and Law, 1967; Kanfer and Kennedy, 1963) and transmethylation in the biosynthesis of fatty acids is thought to take place in the cell membrane (Law, Zalkin and Kaveshino, 1963; O'Leary, 1965).

Implicit in all these reports is a picture of the cytoplasmic membrane not as a homogenous structure of identical repeating lipid-protein subunits, but as a matrix of repeating subunits containing a mosaic of many different functional sites involving the regulation and activity of major synthetic processes. An analytical study of the organisation of the membrane is therefore critical to an understanding of cell metabolism and the co-ordination of cellular functions.

One approach to the study of membrane organisation may be through the isolation of membrane fractions with associated structures, as developed by Nagata, Shibuya and Marus (1967). A second approach may be through the use of drugs specifically causing damage to the cell membrane. For example, Silver and Levine (1968) have shown that the steroidal diamine IDA acts directly on the cell membrane. Thus alteration of the permeability of the cell to K^+ ions can be attributed directly to effects of IDA on the transport system located in the cell membrane (Silver and Levine, 1968)

A third approach may involve the use of certain basic dyes, for example, acriflavine. The seat of acriflavine resistance in E.coli is thought to be the cytoplasmic membrane, and resistance has been demonstrated to be determined by a chromosomal gene (Nakamura, 1968). Thus mutants of E.coli, sensitive to acriflavine, may be examined morphologically, cytologically and biochemically for cytoplasmic membrane damage.

Yet a further method may be through the medium of agents which specifically interfere with macromolecular processes within the cell, and which, as indicated above, appear to be located in the cytoplasmic membrane. One such class of compound is the group of naturally occurring protein antibiotics, namely colicins.

Colicins

Definition of Colicins

Colicins and other bacteriocins were defined by Jacob, Lwoff, Siminovitch and Wollman (1951) as highly specific antibacterial proteins elaborated by certain strains of bacteria and active against other strains of the same species. They generally consist of lipo-polysaccharide/protein complexes, with the protein moiety as the active agent, and differ from conventional antibiotics by a unique action with sensitive cells and a very limited spectrum of activity. Strains capable of elaborating colicins (Col^+) are said to be colicinogenic.

Historical Background

The first real impetus to a study of bacteriocins came from Gratia in 1925, when he reported the inhibition of growth of Bact. coli phi by a substance in the growth medium of Bact.coli V. The substance, "Principle V", was non-antigenic, inhibitory at a one hundred-fold dilution, and the absence of plaques excluded phage action. In further experiments Gratia (1932) demonstrated that the higher the growth rate of E.coli V, the greater was the production of "Principle V". These pioneer studies encouraged search for other strains capable of the production of similar antibacterial agents. Large numbers of such compounds were found, and the variety of principles isolated were characterised. It was then found that

these principles, namely colicins, were elaborated only from strains carrying an extrachromosomal determinant of colicin production, the col factor.

Colicinogeny and Colicin Induction

These genetic determinants, the col factors, were found to exist as stable plasmids not integrated into the chromosome (Nagel de Zwaig, Anton and Puig, 1962; Clowes, 1963; Nagel de Zwaig and Puig, 1964). The colE factors from respective strains of E.coli have recently been characterised and shown to exist in supercoiled DNA forms, approximately 1/100 the size of the E.coli chromosome (Bazaral and Helinski, 1968). The molecular weights of the E2 and E3 colicinogenic factors were found to be essentially identical, but differed slightly from that of the E1 colicinogenic factor (Bazaral and Helinski, 1968). In non-induced cells only one col factor appears to be present per chromosome (Jacob, Brenner and Cuzin, 1963). However after UV induction, several copies of such plasmids are found to be present per chromosome (Bazaral and Helinski, 1968).

The production of colicin from col^+ cells was shown by Ozeki, Stoker and De Margerie (1959) to be a lethal biosynthesis carried out by only a fraction of the bacterial population. They observed that single cells which released colicin were then incapable of further multiplication, although they did not lyse, and suggested that the actual production of colicin was responsible for cell death. However

Herschman and Helinski,(1967) although confirming that colicin production was a lethal synthesis, found that colE factors actually replicate after colicin induction. They inferred from their experiments that the release of colicin from the cell was a secondary effect and that death resulted from unbalanced replication of col factors.

Ozeki et al.,(1959) demonstrated that only a small fraction of a population of colicinogenic cells undergo spontaneous induction of colicin production. In contrast Jacob, Siminovitch and Wollman (1952; 1953) found a 50-fold increase in the production of colicin E1-ML after UV irradiation; and so drew an analogy between the induction of colicin and temperate phage. Techniques originally used for the induction of temperate phage have now been successfully adapted to increase colicin output from col^+ strains. These include thymine deprivation (Sicard and Devoret, 1962; Luzzati and Chevallier, 1964); treatment with acridine orange (Smarda, Koudelka and Kleinwachter, 1964); with azaserine (Sandoval, Reilly and Tandler, 1965); UV irradiation (Fredericq, 1946; Reeves, 1963). Finally Iijima (1962) observed greatly increased rates of colicin production after incubation of a col^+ strain with minute amounts of mitomycin C. This has since proved to be most effective in the induction of colicins of the E group (Nomura, 1964; Helinski and Herschman, 1967; Hill and Holland, 1967).

Classification of Colicins

After the initialwork of Gratia, a variety of substances with differing degrees of antibacterial activity were isolated. It therefore became of paramount importance to classify these substances before any significant advances could be made in studies into their mode of action. A systematic classification of colicins was originally proposed by Fredericq (1948a), and this has since provided a model for the classification of all bacteriocins. Fredericq tested "antibiotics" produced by 250 strains capable of the inhibition of growth of a suitable indicator strain on the basis of the specificity of resistant mutants of the indicator strain and various morphological criteria. Seventeen different groups of colicins were initially characterised.

This system was later simplified (Fredericq, 1950a) by a further grouping of colicins according to their receptor specificity. Thereby, the colicin types E, F, J, S2, S3 and S5 were condensed into the common group E. However this scheme was not entirely satisfactory, as certain colicins, for example, those of the E group, known to adsorb to a common cell surface receptor, have very specific and different modes of action. Since it was known that colicinogenic cells are immune to biochemical changes induced by the colicin they produce (Fredericq, 1948b), a further sub-grouping was therefore applied, based upon the immunity of Col^+ strains to the colicin they are

capable of producing. The col factors required for the production of colicins of the E and I group were then transferred to sensitive strains, and on the specificity of immunity so conferred, the E group was sub-divided into E1, E2 and E3 (Fredericq, 1956) and the I group into Ia and Ib (Stocker, 1965; 1966).

Chemical criteria involving the antigenic properties of colicin antisera (Bordet, 1948; Goebel, Barry and Shedlovsky,1956), and the electrophoretic mobility of colicins (Ludford and Ledever, 1953), have been utilised in the characterisation of further colicins, and the original seventeen groups proposed by Fredericq (1950) have now been extended to include twenty eight colicins.

It must be emphasised that the system of classification primarily revolves around the specificity of mutational alterations in the cell surface adsorption sites of sensitive strains, so preventing their adsorption. This classification scheme is inadequate for various reasons. As has been pointed out by several workers, resistance may by no means be absolute. For example, physiological conditions may profoundly affect the resistance of mutants to certain colicins, and "resistant" mutants can appear sensitive under extreme conditions (Ben-Gurion, 1963; Holland, personal communication). In addition Fredericq (1965) has described unstable mutants resistant to certain colicins which were difficult to isolate, and Holland (1962) has

indicated a similar problem with mutants resistant to Megacin 216. Secondly mutants have been found to exist which are capable of colicin adsorption, yet which are resistant to the effects of the adsorbed colicin (Nomura, 1964; Clowes, 1965; Hill and Holland, 1967). Finally, the classification scheme does not take into account the different lethal effects of colicins in a common group, as for example is seen with colicins of the E group (Nomura, 1967).

It may subsequently prove possible to classify colicins and the bacteriocins according to their chemical composition and their mode of action, although at the present time the number of colicins and related bacteriocins which have been purified to a sufficient degree of homogeneity for comparisons to be made is limited. However three classes do emerge (Herschman and Helinski, 1967a).

One group includes bacteriocins with a significant proportion of lipid and carbohydrate in association with the protein fraction. For example, the first colicin isolated in the pure state, colicin K-K235 (Goebel, Barry, Jesaitis and Miller, 1955; Goebel, Barry and Amano, 1957) was a lipopolysaccharide-protein complex, consisting of 70% protein and 30% of the associated 'O' somatic antigen of the producing strain E.coli K235 (Goebel and Barry, 1958; Goebel, 1962). Colicin V(Hutton and Goebel, 1961; 1962), colicin A (Barry, Everhart and Graham, 1963; Barry Everhart, Abbot and Graham, 1965), colicin I (Keene, 1966) and colicin E2-317 (now colicin F) (Reeves, 1961)

also fall into this same group.

A second group is comprised of bacteriocins composed almost entirely of amino acids. For example Herschman and Helinski (1967b) have purified the colicins E2-P9 (W3110) and E3-W3110, which were found to be composed entirely of amino acids forming simple proteins with molecular weights of approximately 60,000. In addition, they demonstrated that colicin E2 could exist under certain conditions in two interconvertible, electrophoretically distinguishable forms, and that, although the proteins of colicins E2 and E3 contained regions of similar structure, structural elements were unique to each. It is probable that this difference is reflected more by the different modes of action of these colicins after adsorption (discussed later). Other members of this group include a related E-group colicin, E2-CA42 (Reeves, 1963), and possibly Megacin A216 elaborated by Bacillus megaterium 216 (Holland, 1961). This bacteriocin is also a simple protein with a molecular weight of 51,000. However recently Ozaki, Higashi, Satio, An and Amano (1966) have demonstrated that Megacin A-216 has phospholipase activity and thus may be a simple hydrolytic enzyme, and is therefore unlike, for example, colicins of the E group.

A third, contrasting group consists of a chemically heterogenous collection of bacteriocins produced by Pseudomonas aeruginosa (Ishii, Nishi and Egarii 1965; Kageyama, 1964), Listeria monocytogenes

(Bradley and Dewar, 1966), Bacillus subtilis (Bradley, 1967) and various strains of Escherichia coli (Bradley and Dewar, 1966). Under the electron microscope, these "colicins" resemble components of bacteriophages, often with small heads and contractile tails, and consequently may not in fact qualify as colicins.

When all limitations are considered, it becomes apparent that the present colicin classification system is rather inadequate. Indeed Hamon and Peron (1966) have proposed a complete reclassification of the E group, and Lewis and Stocker (1965) have reclassified this group on a basis of the transmissibility of the col factor of colicin E1, and serological properties of E2 and E3. However Nomura (1967) has advocated that no reclassification should be attempted until further knowledge of both the chemical nature and mode of action of colicins has been accumulated.

In an effort to simplify the classification system, Reeves (1965) and Lewis and Stocker (1965) have proposed a system of nomenclature whereby a colicin is referred to by the name of the producer strain and preceded by the original name of the colicin, since it was pointed out by Fredericq (1965) that colicins produced by different strains may never be quite identical. This convention has become increasingly adopted and is now accepted and employed by the majority of colicin workers. Thus E2-P9 refers to colicin E2 produced from Shigella sonnei P9. When the col factor from Sh.sonnei

P9 has been transferred to another strain by cell contact, this strain name then precedes the colicin. For example, W3110 (E2-P9) refers to colicin E2 produced by a derivative of W3110 made colcinogenic by the transfer of the col E2 factor from Sh.sonnei P9. The col factor for E2-P9 is referred to as colE2-P9 by this system.

Mode of Action of Colicins

1. Adsorption to Sensitive Cells

Following the isolation of mutants resistant to the effects of certain colicins from a sensitive strain of E.coli K12, Fredericq (1946b) postulated that colicin molecules were adsorbed to receptors in the bacterial cell surface. In this case colicins, unlike conventional antibiotics, would not be expected to penetrate the surface layers of sensitive cells. Much evidence in support of this hypothesis has since accumulated and will now be presented.

(a) Indirect evidence that colicins were adsorbed by sensitive cells but not by resistant mutants was first provided by Fredericq (1952a and b) and Jacob et al.,(1951). After the addition of colicin K to sensitive and resistant cells, the supernatant obtained from a culture of sensitive cells was found by these workers to have a lower colicin titre than that from the resistant cells. This was ascribed to probable adsorption of the colicin by the sensitive cells. Hamon and Peron (1960) have since similarly demonstrated the adsorption

of a solution of six colicins and five pyocins by sensitive, but not resistant, strains.

(b) It has been reported by several workers that mutation to resistance to certain bacteriophages simultaneously provides resistance to certain colicins. For example, mutation to resistance to the phage BF23 also provides resistance to colicins of the E-group (Fredericq, 1950b), resistance to phage T6 excludes colicin K (Fredericq, 1950b), and similarly resistance to colicin M excludes phage T1 and T5 (Fredericq, 1951a). As phages are known to adsorb to specific receptors in the bacterial cell surface, and in these cases both colicins and phages were found not to be adsorbed to the bacterial cells, it was thought that colicins, like phages, may be adsorbed to specific phage-like receptors in the bacterial cell surface.

(c) Further indirect evidence was provided by the observation that sensitive cells were protected against colicin action by antibacterial antisera in the culture medium (Bordet, 1948). These results indicated that serum acted by blocking the appropriate receptors, and furthermore extracts of sensitive cells have been shown to specifically neutralise colicins, presumably by fixing them (Bordet and Beumer, 1948).

(d) The first evidence of direct adsorption of colicin to cell surface receptors was provided by Maeda and Nomura (1966). They demonstrated that radioactive colicin E2-P9 was adsorbed specifically

to the surface layer of sensitive, but not resistant cells. However the precision of the method employed by them is questionable, since although 95% of accounted radioactive colicin was adsorbed to the cell surface layer, the remaining, unaccounted for 5% would still be quite sufficient to promote lethality via penetration and direct action.

(e) In experiments to investigate the kinetics of colicin action on sensitive cells, Jacob, Siminovitch and Wollman (1951) discovered that the initial rate of kill and final surviving fraction of bacteria treated with colicin E1-ML was proportional to the concentration of the colicin. It was concluded, therefore, that colicin action, like phage action, is a "single-hit" process, with a single colicin particle being capable of killing a single bacterium with a certain probability, and this has since been confirmed by Nomura (1963) and Reeves (1965). Colicins may then be thought to have a particulate action, with one single adsorbed particle potentially lethal to the sensitive cell. However the probability of a single particle promoting lethality may well be low and for example, Holland (1962) found that the probability of successful action of an adsorbed molecule of Megacin A-216 was as low as 0.01. It is not clear whether this figure arises from a low proportion of active bacteriocin molecules in the preparation or from only a low proportion of active receptors amongst many potentially capable of a lethal interaction with the colicin molecule.

(f) It has been shown that the colE1$^+$, colE2$^+$ and colE3$^+$ producing strains can adsorb their homologous colicins to an extent equivalent to that achieved by corresponding sensitive strains (Cavard and Barbu, 1966). As colicinogenic strains are resistant to all but extremely high concentrations of their homologous colicins (Levisohn, Koninsky and Nomura, 1967) it is unlikely that cell penetration has taken place, and the colicin presumably then remains at an extracellular position on the cell surface. Furthermore, the isolation and purification of many colicins, for example colicin K-K235 (Goebel and Barry, 1958), and colicin I (Keene, 1966) has shown that they are intimately associated with the 'O' somatic antigen of the producing strain. Therefore colicin molecules are not only probably adsorbed to the cell surfaces of sensitive strains, but are often found in association with specific surface components in producing strains.

(g) More indirect evidence that colicins are adsorbed to extracellular attachment sites comes from enzymatic digestion experiments on colicin-treated, sensitive cells. Thus Nomura and Nakamura (1962) have found the complete inhibition of macromolecular synthesis of colicin E1 and K can be reversed even after several hours, by treatment of the cells with the proteolytic enzyme, trypsin. This reversal strongly suggests that the colicin molecule itself competes for sites in the electron transport chain of the sensitive cell, and so inhibits macromolecular synthesis. However with colicin E2, trypsin digestion

increases the number of colony formers for only a maximum of 30 minutes following addition of colicin (Nomura, 1963; Reynolds and Reeves, 1963), after which the lethal action is irreversible. Similar results have also been reported with colicin E3 (Nomura, 1963; 1964). These latter results do not necessarily show that the inhibitory effects of colicin E2 and E3 are reversible, but merely indicate a reversible stage after colicin adsorption. On the contrary, it seems likely that the physical presence of these colicins eventually triggers an irreversible phase which may be the production or activation of an intracellular enzyme, after which trypsin digestion has no effect. However the ability of trypsin to remove radioactive colicin E2 from the receptor after adsorption (Maeda and Nomura, 1966) does very strongly imply that colicin molecules remain at, and act from an extracellular receptor.

When taken together, the above experimental evidence does strongly suggest that colicins are adsorbed to cell surfaces, and act from an extracellular position. Several workers have calculated the amount of colicin which adsorbs to sites on a sensitive cell, and results vary from 11 (Mayr-Harting, 1964) to up to 30 (Maeda and Nomura, 1966) killing units of E2-P9 or E2-CA42 at saturating levels. Maeda and Nomura (1966) have taken the molecular weight of colicin E2 as 60,000 (Helinski and Herschman, 1966) and have calculated the actual amount of colicin protein which corresponds to a single killing

unit. From these calculations, 1 killing unit appears to correspond to about 100 colicin molecules, and from this figure, the number of receptor sites was then estimated to be between two and three thousand. This figure differs from that of 30-90 quoted by Reeves (1963), based upon one killing unit corresponding to one molecule of colicin E2, and this discrepancy can only partly be accounted for by variation in experimental conditions. However it is clear that if colicin molecules do indeed adsorb to specific receptors, there are many such receptors present on the cell surface, all perhaps capable of mediating the characteristic intracellular changes following the adsorption of a colicin molecule.

Adsorption studies have also indicated that the number of receptor sites for E3-CA38 is similar to that for E2-P9 (Maeda and Nomura, 1966), as may be expected from their receptor specificity. Furthermore colicins E2 and E3 have been shown by Maeda and Nomura (1966) and by Holland (personal communication) to compete with each other in adsorption experiments, conclusive evidence that these colicins do in fact share a common receptor.

Despite the above evidence, the existence of specific extracellular receptors for the adsorption of colicin molecules has been disputed by various workers, (Reeves, 1965; Smarda and Taubeneck, 1968). Thus it was shown by Smarda and Taubeneck (1968) that L-forms, but not normal Proteus mirabilis cells, were susceptible to colicin E2.

L-forms of this species lack cell walls, and are consequently resistant to phage attack as the necessary phage receptors are not present. Smarda has implied from this result that colicin molecules adsorb directly to the cell membrane and not to the cell wall, and act from this position (Smarda, 1967).

Indeed, certain colicin molecules may well adsorb directly to the cell membrane of certain susceptible bacterial strains, and act directly with the membrane from this position. However, according to the Smarda model, receptor adsorption is unnecessary for all colicin action. In this event, one may anticipate that the sensitivity of spheroplasts prepared from cells sensitive to, for example, colicin E2, would not be reduced. However it was found by Nomura and Maeda (1965) that spheroplasts so prepared were resistant to the effects of this colicin and, moreover, that these spheroplasts still adsorbed the colicin. It was concluded, therefore, that maintenance of the cell surface layers is essential for the successful action of at least colicin E2.

2. Biochemical Effects of Different Colicins

(a) Colicins E1 and K

In colicin E1-treated sensitive cells a rapid inhibiton of all macromolecular biosynthesis in the cell was found by Jacob, Siminovitch and Wollman (1951 and 1952). Thus, these workers demonstrated that a rapid cessation of growth, and inhibition of protein, DNA and RNA

synthesis was a feature of El-treated cells. However the rate of respiration in these cells did not decline for at least 30 minutes after the addition of colicin El, and it was therefore concluded that colicin El was functioning as an energy uncoupling agent in the cell.

Similar lethal effects were observed with colicin K. Thus Fredericq (1953a) demonstrated that the multiplication of intracellular phage ceased in sensitive cells treated with both colicins El and K. In addition, experiments of Levinthal and Levinthal (cited by Luria, 1964) have indicated that both colicins interfere with the oxidative phosphorylation systems in sensitive cells. Now, as the oxidative phosphorylation system lies in the cytoplasmic membrane of the cell (Marr, 1960), colicin El, interacting with the membrane, may act directly as an uncoupling agent in this system. In this event, one may anticipate derangement in the energy levels of certain enzymes concerned with specific aspects of membrane metabolism. Indeed, Luria (1964) has found that permeases specific for β-D-galactosidase, isoleucine and the uptake of potassium ions cease to function "in vivo" in the presence of colicin El, suggestive of some direct colicin /membrane interaction. These biochemical changes in colicin treated El and K-sensitive cells have been further investigated by Fields and Luria (1969), who have found that these colicins produce a reduction in ATP levels in the cell. However it is important to note that subtle changes in the cell membrane would be sufficient

to produce these biochemical changes. For example, a molecular rearrangement in the cell membrane could cause blockage in the electron transport chain without the activation of an intracellular compound or the penetration of the membrane by the colicin molecule.

Other colicins producing similar biochemical changes in sensitive cells include those of the I group, Ia and Ib. These have been shown to inhibit the multiplication of intracellular phage (Fredericq, 1953a), and to inhibit oxidative phosphorylation in the cell (Levisohn, Koninsky and Nomura, 1967).

(b) Colicin E2

Although high multiplicities of colicin E2 have been reported to inhibit macromolecular synthesis without alteration in the rate of respiration in sensitive cells (Nomura, 1963), low concentrations appear to specifically initiate the degradation, and prevent the synthesis of DNA (Nomura, 1967). The primary action of colicin E2 may well therefore involve some aspect of chromosomal metabolism, especially since phage T^4 and λ multiplication in E2-treated sensitive cells proceeds normally, evidence cited by Nomura and Nakamura (1962) for the integrity of cellular protein synthesising machinery under these conditions.

However the mechanism of DNA degradation remains unclear, although nucleases are presumably involved at some stage, Nomura (1963) has tested the possible formation of a "de novo" DNAase

after E2-addition, and found that pretreatment of sensitive cells with chloramphenicol or streptomycin did not alter the degradation of DNA. Regretably, in his experiments Nomura did not indicate the precise degree of inhibition of protein synthesis achieved by these agents, and Holland (1965) has since suggested, in the case of Megacin B, that small residual levels of protein synthesis may be sufficient for the formation of a bacterial induced DNAase. However Holland (personal communication) has more rigorously prevented protein synthesis in E2-sensitive cells, and found that DNA breakdown still takes place after E2-addition. The formation of a new DNAase is therefore unlikely, and the extracellular presence of colicin E2 then at some stage presumably activates DNAases already present in the sensitive cell.

Despite the lack of involvement of protein synthesis in colicin E2 action, an energy requirement has nevertheless been reported for some stage in the action of colicin E2 on sensitive cells. Thus it was found by Nomura (1963) and Nomura and Maeda (1965) that the respiratory inhibitor 2-4 dinitrophenol could suppress both the induction of E2-induced DNA breakdown and the killing action of the colicin, although it is not known at which stage in the sequence of events energy is actually required. This could be visualised to participate in the adsorption and orientation of the colicin molecule at the receptor site, or in a molecular rearrangement of

some cell components integral in a later stage of colicin action.

A second requirement for the successful action of colicin E2 on sensitive strains of E.coli appears to be an intact cell membrane. Thus colicin E2 had no effect on an in vitro preparation of DNA (Nomura, 1964), and DNA degradation in E2-treated spheroplasts was shown to be greatly reduced (Nomura and Maeda, 1965; Beppu and Arima, 1967). The membrane may well be involved in the transmission of the E2-induced stimulus to the biochemical target, and as will be later discussed, has to be maintained for successful colicin action to take place. There have been no reports that colicin E2 had any effect on cell permeability, or that the colicin directly damaged the cell membrane.

Since Holland (1967) has reported the occurrence of long non-septate filaments of sensitive cells soon after the addition of low multiplicities of colicin E2, it is possible that a further primary manifestation of E2 action is the inhibition of the cell division machinery. Indeed, as discussed previously, if DNA metabolism and cell division are coupled by the participation of some cell membrane component, a possible molecular rearrangement of the membrane induced by colicin E2 may well produce cell division abnormalities as well as destruction of the chromosome.

In more recent studies on the primary action of colicin E2, Holland (1968) has reported a perceptible time lag of up to 20 minutes between the onset of DNA degradation and the cessation of DNA synthesis

in sensitive cells after the addition of colicin. It can be implied from this result that DNA degradation is a primary effect, and the inhibition of DNA synthesis is a secondary effect of colicin E2, and furthermore Holland has suggested that DNA synthesis inhibition may well be a direct consequence of the destruction of the template for DNA polymerase, the DNA. In this respect, the colicin E2 degradation of DNA differs from DNA degradation induced by a variety of other agents which cause direct damage to DNA, for example, Mitomycin C which appears to induce DNA degradation only after the cessation of DNA synthesis (Boyce and Howard-Flanders, 1964).

Several non-specific effects have been attributed to high concentrations of colicin E2, or to a prolonged exposure to the colicin. For example, Persiel (1965) has reported an inhibition of respiration in sensitive cells after exposure to high colicin E2 multiplicities. This is unlikely to be a primary event as both DNA degradation and the inhibition of macromolecular synthesis have been reported when the rate of respiration in E2-treated sensitive cells was unaltered. Similarly, the degradation of ribosomal RNA after exposure to colicin E2 for a prolonged time (Nose, Mizuno and Ozeki, 1966) is unlikely to be a primary effect.

Other colicins with similar modes of action to E2-P9 have also been reported. These include colicin F5 (now called E2-CA42) (Reynolds and Reeves, 1963; Hamon and Peron, 1967), and colicin P (Hamon and

Peron, 1965). These colicins, like E2-P9, induced bacteriophage λ development, and produced both DNA degradation and an inhibition of DNA synthesis in sensitive cells.

(c) Colicin E3

When sensitive cells are treated with a high multiplicity of colicin E3-CA38, both the induction of the enzyme β-galactosidase and the incorporation of $S^{35}O_4$ into total protein was completely inhibited (Nomura, 1963; Nomura and Maeda, 1965). However, as the synthesis of RNA and DNA is not affected it was suggested by Nomura (1963) that this colicin acts specifically by inhibiting protein synthesis.

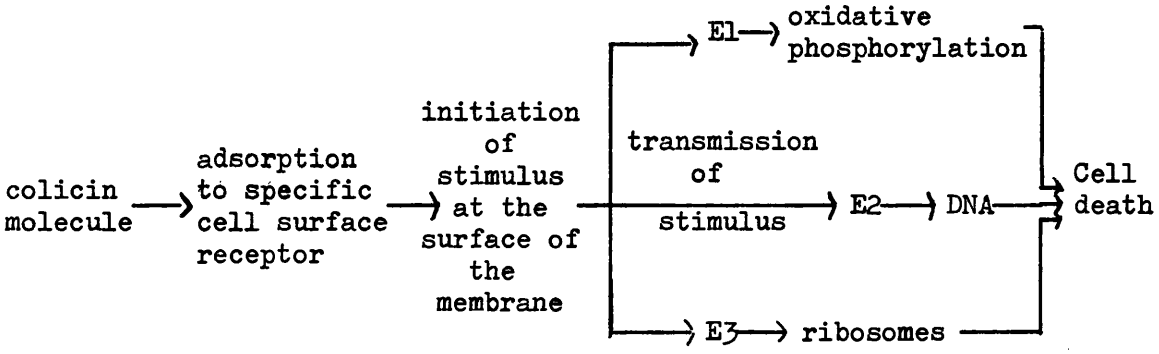
More detailed studies into the primary action of colicin E3 have been carried out by Koninsky and Nomura (1967). They found that ribosomes extracted from both colicin E3 and colicin K treated cells had an altered 23S core of the 30S ribosomal sub-unit, whereas the 50S unit was unaffected. When cells were exposed to high concentrations of E3-CA38 or colicin K for a prolonged time period prior to extraction, the ribosomes were found to have undergone actual physical degradation. Koninsky and Nomura considered it unlikely that ribosomal degradation is a primary effect of colicin E3, and suggested that this is more likely to be a non-specific effect developing after ribosomal inactivation.

Koninsky and Nomura (1967) also investigated the effects of colicin E3-CA38 "in vitro" on ribosomes previously extracted from sensitive cells, but found no inactivation by the colicin. They concluded from this experiment that the general integrity of the cell has to be maintained for the successful action of colicin E3, and in particular that an intact cell membrane at least is essential.

Models of Colicin Action

The biochemical modes of action of different colicins demonstrate that the primary actions of different colicins are entirely specific. Thus there have been no recent reports of non-specific cell damage after exposure to low colicin concentrations and in particular, no membrane disruption has been observed. Gross changes were only apparent either after the addition of high colicin concentrations, or after a prolonged time interval. Secondly, in the cases of colicins E2 and E3 at least, "in vitro" preparations of DNA and ribosomes were not affected by the respective colicins, stressing the importance of the maintenance of the integrity of the cell for the action of colicins. Finally, studies previously discussed have indicated the probability of specific extracellular attachment sites for different colicins. Therefore to correlate these facets of colicin action, it can be imagined that specific intracellular biochemical changes follow the attachment of the colicin molecule to receptors in the cell surface, without the physical penetration of the cell membrane by the colicin.

Various models have been proposed to explain the action of colicins. Thus Nomura (1964) has suggested that a stimulus is initiated by the adsorption of a colicin molecule to a specific cell wall receptor. This transmitted via the cell membrane to a specific biochemical target thereby promoting the observable biochemical change and cell death (see diagram below):



This model has been extended by Hill and Holland (1967) who have suggested that the receptor may have an active role in the initiation of the colicin stimulus in addition to participating simply in the adsorption of the colicin. Since the structure of the bacterial cell surface layers are highly complex, they have suggested that a correct orientation of the colicin molecule at the receptor site may also be an essential prerequisite before stimulus initiation.

An essential aspect of the model (see diagram) is the location and nature of the transmission system, responsible for the "connection" between the extracellular colicin and the intracellular biochemical "target". In addition, the "single-hit" killing kinetics, and the

inactivation of several thousand ribosomes by but a few molecules of colicin E3 (Koninsky and Nomura, 1967) suggest that the transmission system must also be capable of amplifying the initial stimulus many times. When considering the mode of action of colicins of the E group, the colicins E2 and E3, as will be later presented, share a common receptor. However these colicins inactivate unrelated biochemical targets in the cell, and therefore the transmission system for these colicins must diverge at some post-adsorption stage.

The most suitable candidate for the seat of the transmission system in the bacterial cell is the cytoplasmic membrane. Not only is the repeating sub-unit structure of the membrane compatible with the transmission and amplification of an initial change produced by the colicin molecule throughout the cell, but evidence also indicates that cell structures inactivated by particular colicins are physically related to the membrane. Indeed, Changeux and Thiery (1966) have proposed that the transmission of a colicin-invoked stimulus may involve a conformational transition of repeating units of the cell membrane. This may then produce an allosteric alteration in intracellular proteins bdund to the internal surface of the cell membrane. Mutual cooperation between repeating units could be such that small numbers of colicin molecules could provoke alteration sufficient to produce a complete cell membrane transition.

If such a transmission system or multistep pathway of colicin action does exist, and which resides at least partially in the cytoplasmic membrane, then alteration of the membrane of sensitive cells in various ways should interfere with colicin action. In fact protection to the adsorbed colicins E2 and K was found by Beppu and Arima (1964) to be afforded to sensitive cells after their suspension in a medium of high Osmotic Pressure. These workers ascribed this protection to osmotically induced structural changes in the cytoplasmic membrane, which interfered with the transmission of the colicin stimuli. Similarly, after the disruption of the cell wall and cytoplasmic membrane in the formation of spheroplasts, colicin E2 adsorption was found to be unaltered, but the E2-induced degradation of cellular DNA was greatly reduced (Nomura and Maeda, 1965; Beppu and Arima, 1967). In contrast, E1-treated spheroplasts were found to be more sensitive to colicin E1 (Obdrzalek, Smarda, Cech and Adler, 1969). This result should in fact be treated with reservations, since no controls, or times for which the spheroplasts were exposed to colicin E1 were given. Nevertheless, with the possible exception of the latter example, physiological alteration of the cytoplasmic membrane <u>does</u> reduce the sensitivity of the cells to the colicin, presumably by disruption of the transmission system.

In further support of a multistep pathway, many different classes of mutants resistant to colicin have been isolated. This is

indicative of a multistep pathway, and in some cases, as will be discussed later, the mutants have additional properties compatible with altered membranes.

Resistance to Colicins

At least two classes of genetic mutants resistant to the action of colicins can be predicted from the model previously illustrated and discussed. Thus one group could be resistant because of failure to adsorb a specific colicin. In contrast, cell death in a second group could be prevented by mutation in some post-adsorption stage, for example, by mutations preventing (1) the initiation of the "stimulus"; (2) the "transmission" of the stimulus to the "target"; (3) the reception of the stimulus by the "target".

(a) Genetics of resistance

The occurrence of mutants resistant to colicin through the loss or inactivation of a receptor structure was first postulated by Fredericq (1948c) and several examples have since been reported (Fredericq, 1956; 1958). Receptor formation has been shown to be under the control of several independent genes. These have been mapped by Fredericq and Betz-Bareau (1951; 1952), Jenkin and Rowley (1955) and Gratia (1966). For example, the chromosomal locus of a receptor mutation, producing resistance to the colicin E group and to phage BF23 was mapped between thi and met (Fredericq and Betz-Bareau, 1952; Jenkin and Rowley, 1955). However recent studies by

Hill and Holland (1967) have demonstrated that the colicin E group receptor is more complex than was first imagined. Thus although the majority of mutants unable to adsorb to colicins E1, E2 and E3, designated R^- through loss or inactivation of the receptor, map at the chromosomal locus between thi and met (Gratia, 1966), which also excludes the bacteriophage BF23, at least four other R^- phenotypes have been found which have lost the capacity to adsorb colicins E2 and E3, but not E1 (Hill and Holland, 1967). These R^- phenotypes, which differ in their response to temperature and also in their resistance to phage BF23, have not yet however been mapped. In support of this apparent separation of the receptor structure for colicin E1 from that of colicins E2 and E3, it has been shown that prior adsorption of colicin E2 to bacterial cells subsequently inhibits the adsorption of E3, but not of E1 (Maeda and Nomura, 1965; Holland, personal communication). These results therefore indicate the presence of physically distinct fixation sites for colicin E1, and for colicins E2 and E3. Nevertheless, the isolation of single-step mutants which no longer adsorb colicins E1, E2 and E3, demonstrates that although the reception site for colicin E1 may be distinct from that for colicins E2 and E3, certain constituents of the receptor must be common to all.

Examples of a second class of resistant mutants, which still retain the ability to adsorb colicin , have been isolated by several

workers, and given various designations, i.e. "colicin-tolerant" (Nomura and Maeda, 1965; Nagel de Zwaig and Luria, 1967); "mutationally immune" (Clowes, 1965; Reeves, 1966; Stouthamer and Tieze, 1966); "t" (Hamon and Peron, 1967); "Refractory" (Hill and Holland, 1967). As indicated previously, such mutants are to be expected if colicin action involves several "steps" following adsorption of the colicin but prior to the completion of the effect.

Studies into this group of receptor-retaining (R^+) mutants have been carried out almost exclusively with the E group of colicins. Several distinct mutants of this refractory class have been isolated showing refractivity to one or more of the E group colicins and other unrelated colicins, and where possible, the position of the mutation has been mapped. The phenotypes of these mutants are summarised in Table a, and the map location and methods of analyses indicated (see page 37). Several distinct multiply-resistant phenotypes map adjacent to the gal region, although the number of cistrons and their functional relationship has not been precisely determined. However by complementation analysis Nagel de Zwaig and Luria (1967) have demonstrated that the tolII and tolIII mutations are contained in at least two different cistrons. In all probability, the tolII mutants of Nagel de Zwaig and Luria (1967) and Nomura and Witten (1967) are the same. Similarly the tolIII mutants of these workers may well correspond to the refV mutants of Hill and Holland (1967), and tolIV

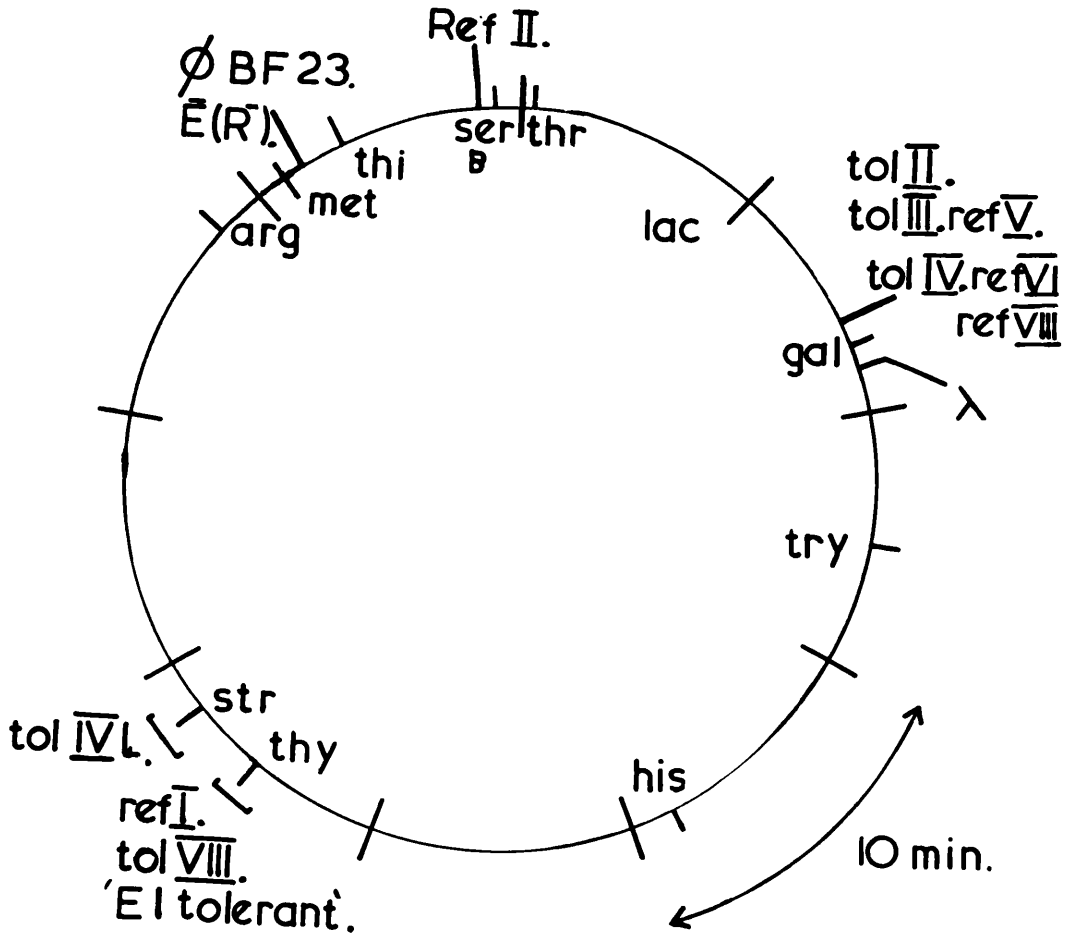
Table a Phenotypes, nomenclature and gene order of R^+, colicin E resistant mutants

	Resistance pattern A	E1	E2	E3	K	Nomenclature	Gene order	Method of analysis	Reference
Multiply resistant R^+ phenotypes	r	r	r	r	r	tolII	tol-gal-bio	cotransduction of gal with P1	de Zwaig & Luria, 1967
	r	s	r	r	r	tolIII	tol-gal-bio	cotransduction of gal with P1	de Zwaig & Luria, 1967
	-	s	r	r	s	RefIV	unknown		Hill & Holland, 1967
	-	s	r	r	r	RefV	100% linkage to gal	Linkage analysis	Hill & Holland, 1967
	-	s^{30^o} r^{40^o}	r	r	r	RefVI	100% linkage to gal	Linkage analysis	Hill & Holland, 1967
	-	s	r	s^{30^o} r^{40^o}	r	RefVII	between arg and his	Linkage analysis	Hill & Holland, 1967
	-	s^{30^o} r^{40^o}	r	r	s^{30^o} r^{40^o}	RefVIII	100% linkage to gal	Linkage analysis	Hill & Holland, 1967
	-	r	r	r	r	tolII	tol-gal-λ	cotransduction of gal with P1	Nomura & Witten, 1967

	-	s	r	r	r	tolIII	tol-gal-λ	cotransduction of gal with P1	Nomura & Witten, 1967
	-	s	r	r	s	tolIV	tol-gal-λ	cotransduction of gal with P1	Nomura & Witten, 1967
	-	s	$s^{30°}$ $r^{40°}$	$s^{30°}$ $r^{40°}$	s	tolIV1	xyl-tol-his	Linkage analysis	Nomura & Witten, 1967
Singly resistant R^+ phenotypes	-	r	s	s	s	RefI	near thy	Interrupted mating	Hill & Holland, 1967
	s	r	s	s	s	tolVIII	near thy	Linkage analysis	de Zwaig & Luria, 1967
	s	r	s	s	s	El tolerant	near thy	Linkage analysis	Clowes, 1965
	-	s	$r^{30°}$ $s^{40°}$	s	s	tolVII	unknown		Nomura & Witten, 1967
	-	s	$r^{25°}$ $s^{40°}$	s	-	RefII	refII-thr	Interrupted mating	Holland & Threlfall, 1969

The positions of the various R^+ mutations on the bacterial chromosome are shown on Figure 1. in relation to the revised linkage map of the E.coli K12 chromosome (Taylor and Trotter, 1967).

Fig. 1 Chromosome Map of E.coli K12 showing in particular colicin E resistant loci.


Ref II.
Ø BF23.
Ē(R⁻).
ser
B
thr
thi
met
arg
lac
tol II.
tol III. ref V.
tol IV. ref VI
ref VIII
gal
λ
try
his
str
thy
tol IV.
ref I.
tol VIII.
'E l tolerant'.
10 min.

mutants to RefIV mutants. Amongst the mutants with refractivity to a single colicin, the RefI, tolVIII and El-tolerant mutants (Clowes, 1965) no doubt correspond, as may the tolVII (Nomura and Witten, 1967) and the RefII mutants (Holland and Threlfall, 1967), although the position of the tolVII locus has not yet been mapped.

It is important to note that no mutants have been isolated simultaneously refractory to both the colicins El and E2 or to El and E3, whereas many mutants simultaneously refractory to the colicins E2 and E3 have been reported. The early separation of the transmission system for colicin El from that of colicins E2 and E3 is therefore suggested. However, although the El receptor, and hence the transmission system, may be quite distinct from that of colicins E2 and E3, the isolation of R^+ mutants simultaneously refractory to all three colicins does suggest that common components in the reception system at a postfixation level also exist.

The colicins E2 and E3 share a common receptor, but their transmission systems must diverge at some stage, in the case of E2 to the chromosome and/or the cell division machinery, in the case of E3 to the ribosomes. It should therefore be theoretically possible to isolate mutants refractory only to colicin E2, and only to colicin E3. In fact, the former class has been isolated, and designated tolVII (Nomura and Witten, 1967), RefII (Hill and Holland, 1967). The isolation of multirefractory mutants refractory to both the

colicins E2 and E3, or the colicins E1, E2 and E3 and the unrelated colicins A and K (de Zwaig and Luria, 1967; Hill and Holland, 1967; Nomura and Witten, 1967) may indicate specific common components in the transmission pathways of these colicins, or non-specific alteration of membranes affecting all the transmission systems. In the former event, the altered component in multirefractory mutants is more likely to participate in an early step in the transmission system, than the component altered in the mutants refractory to a single colicin, for example E2.

Physiology of refractory mutants

Several mutants with multirefractory phenotypes, mapping in the gal region were found by de Zwaig and Luria (1967) to be hypersensitive to deoxycholate (DOC) and EDTA and alteration of some membrane or other surface component was implied. Nevertheless, there were no observable differences between the major phospholipid and lipopolysaccharide components of parental and mutant strains (de Zwaig and Luria, 1967). This indicates that no gross wall or membrane alteration had taken place in these mutants, but does not eliminate a discrete membrane alteration, for example in the conformation of a protein molecule, which would not have been observed.

The permeability of mutants refractory to colicin E1 alone was also shown to be increased. In consequence, this class of mutants is hypersensitive to methylene blue and to acridines (de Zwaig and

Luria, 1967; Hill and Holland, 1967; Luria, 1969). In addition these mutants may be distinguished from multirefractory classes by their map position near thy which is quite distinct from other refractory loci. Again, a change in some cell membrane component can be imagined to have taken place in these mutants which does not lead to gross cell damage.

A second class of mutant, refractory to a single colicin, colicin E2, has also been isolated. These mutants, designated RefII by Hill and Holland (1967) are characterised by all being refractory at 30°C yet sensitive at 40°C. As mentioned above, it is more probable that the altered component in this class participates in some later stage of the transmission system for colicin E2 than does the altered component in the multirefractory mutants. Moreover, Holland (1967) has indicated that certain RefII mutants were deficient in the repair of UV damaged DNA. These results indicate that ~~the activity of~~ the DNAase which is activated by the presence of colicin E2 also participates in the repair of UV damaged DNA (see Howard-Flanders and Boyce, 1966) and this is altered in RefII mutants.

An important step in the elucidation of the action of colicins is the identification of the altered components in refractory mutants. Indirect evidence does indicate the alteration of some membrane proteins in both single- and multirefractory classes of mutants. For example, in several classes of multirefractory mutants, colicin refractivity was

temperature dependent (Hill and Holland, 1967; Nomura and Witten, 1967; de Zwaig and Luria, 1967), and in some cases the mutants showed a rapid change in colicin sensitivity with change in temperature (Nomura and Witten, 1967). This latter property is characteristic of many mutant proteins. Further, more direct evidence that the primary products of at least two "multirefractory genes" are protein comes from the isolation of many amber mutants of the tolII and tolIII types (see Table a) (Tereck Schwarz, personal communication cited by Luria, 1969).

It is interesting to note that adjacent mutations to the multirefractory loci near the gal locus are known to involve the function of enzymes of anaerobic catabolism (Puig, Azoulay and Pichinoty, 1967). These loci control the assembly of several enzymes into a particulate structural element in the cell membrane, and their close linkage to the colicin multirefractory loci may not be coincidental.

Since the RefII mutants were all characterised by rapid acquirement of sensitivity to colicin E2 with change from low to high temperature, it was suggested by Holland (1968) that colicin E2 refractivity involved a conformational alteration in a membrane protein, and it seems likely that in the RefII mutants such a protein is more or less normal at 40°C, but takes on the "mutant" configuration at 25°C.

Immunity

Finally, before concluding this introduction to the genetics of colicin resistance, it must be emphasised that resistance through

genetic mutation differs from the immunity of colicinogenic cells to their homologous colicin (Fredericq, 1948b). Immunity is only conferred by the transfer of the col factor (Maeda and Nomura, 1966) and upon loss of this episome the cells regain their colicin sensitivity. The mechanism of immunity is not understood although Nomura (1964) has postulated the formation of a specific "immunity substance" affecting some post-adsorption stage. "Resistance" is clearly different from immunity, since at high concentrations the homologous colicin has been shown to produce changes in col^+ cells, similar to those obtained with low concentrations on sensitive cells, and killing takes place (Levisohn, Koninsky and Nomura, 1967). In contrast, resistance through receptor inactivation is absolute (Siccardi, 1966).

In summarising the evidence to date for the mode of action of colicins, one may say that:

(a) Colicins attach to specific cell surface receptors, and in certain as yet undetermined circumstances, a stimulus is transmitted from these extracellular attachment sites.

(b) Colicin action, like phage action, is a "single hit" process, and a few colicin particles are capable of inactivating a bacterial cell. However, as a few particles of colicin E3 can inactivate several thousand ribosomes, the colicin stimulus must be amplified within the cell.

(c) The maintenance of the integrity of the cell membrane is necessary for the successful transmission of the colicin effect.

(d) The final lethal and biochemical effects invoked by the colicin molecule from the surface site are quite specific for different colicins.

(e) There is no gross cellular damage produced by the action of colicins. In particular, there is no detectable change in the osmotic properties of the membrane.

To account for the extracellular mode of action of colicins, it is necessary to postulate transmission and amplification of an external stimulus to specific intracellular components, which are thereby inactivated. The postulated repeating sub-unit structure of the cytoplasmic membrane provides a possible means for the transmission and amplification of the stimulus.

The studies into the susceptibility of bacteria to colicin E2 which will now be presented and discussed were therefore initiated to (1) qualify the role of the membrane in colicin E2 action; (2) determine the organisation of the membrane in the transmission and amplification of the colicin E2 stimulus.

It was hoped that these aims would be achieved by the study of various mutants blocked at some step in colicin action, in the hope that different steps and their relationship to the organisation of the cell membrane could thereby be identified.

MATERIALS AND GENERAL METHODS

Bacterial Strains

Colicinogenic and indicator strains employed throughout this study were as follows: the colicinogenic E.coli strains ML (colE1$^+$) (Jacob, Siminovitch and Wollman, 1952), CA38 (colE3$^+$) (Fredericq and Delcour, 1953), the colicin sensitive strain K12-ROW and its colicin E resistant derivative ROW-E were obtained from P.Fredericq. The source of colicin E2 was Salmonella typhimurium LT2, 906 (colE2$^+$) carrying the E2 factor originally derived from Shigella sonnei P9 (Nomura, 1964).

Non-refractory strains used, their origin, colicin E2 sensitivity, response to ultraviolet irradiation (UV) and recombination properties are shown in Table 1. The origin of the Hfr and F-prime strains employed are shown in Figure 2.

Maintenance of Bacterial Strains

Permanent stocks of all strains used were kept in the form of stabs in wax-sealed tubes containing 5 ml NB top agar, at approximately 20°C. Strains in common use were maintained either on slopes of NB agar in 25 ml Universal bottles, or on NB agar plates, and were stored at 4°C. These were renewed at weekly intervals by selection of single colonies following streaking of the strains on NB agar plates.

TABLE

Characteristics of ref$^+$ E.coli

Strain	Mating type	Genotype												
		serB	thr	leu	pro	lac	try	his	arg	thy	met	ileu	λ	str
C600	F	+	-	-	+	+	+	+	+	+	+	+	-	R
ASH10	F$^-$	+	+	-	+	-	+	+	+	-	-	+	+	R
E94	F$^-$	+	-	-	-	-	+	+	+	-	-	+	+	R
ROW	F$^-$	+	+	+	+	+	+	+	+	+	-	+	+	R
ASH5	F$^-$	+	-	-	+	-	-	-	-	+	+	+	+	R
E32	F$^-$	+	+	-	+	+	+	+	+	-	-	+	+	R
158	F$^-$	+	-	-	+	+	+	+	+	+	+	+		S
Rec34	F$^-$	+	-	-	+	+	+	-	-	+	+	-	+	S
KMBL240	F$^-$	+	-	-	+	+	+	-	-	+	+	-	+	S
AB1157	F$^-$	+	-	-	-	-	+	-	-	+	+	+	+	R
JC4457	F$^-$	+	-	-	-	-	+	-	-	+	+	-	+	R
4K	F$^-$	-	-	-	+	+	+	+	+	+	+	+	+	R
4K-T$^+$	F$^-$	-	+	-	+	+	+	+	+	+	+	+	+	R

1

Strains Used in This Study

Hsp	Response to colicin E2	Response to UV	Recombination	Origin	Source
+ +	S	R	rec^+		R.W.Hedges
+ +	S	R	+	W1 x HfrC	R.H.Pritchard
+ +	S	R	+		R.H.Pritchard
+ +	S	R	+		R.H.Pritchard
+ +	S	R	+		R.H.Pritchard
+ +	S	R	+		R.H.Pritchard
+ +	S	R	+	C600	R.H.Pritchard
+ +	S	S	$recA^-$	CR34	A.Rörsch
+ +	S	S	$recA^-$	CR34	A.Rörsch
+ +	S	R	rec^+		A.J.Clark
+ +	S	S	$recB^-$	N.T.G.deriv.of AB1157	A.J.Clark
- -	S	R	rec^+	C600	S.Glover
- -	S	R	+	thr transductant of 4K	

Contd..

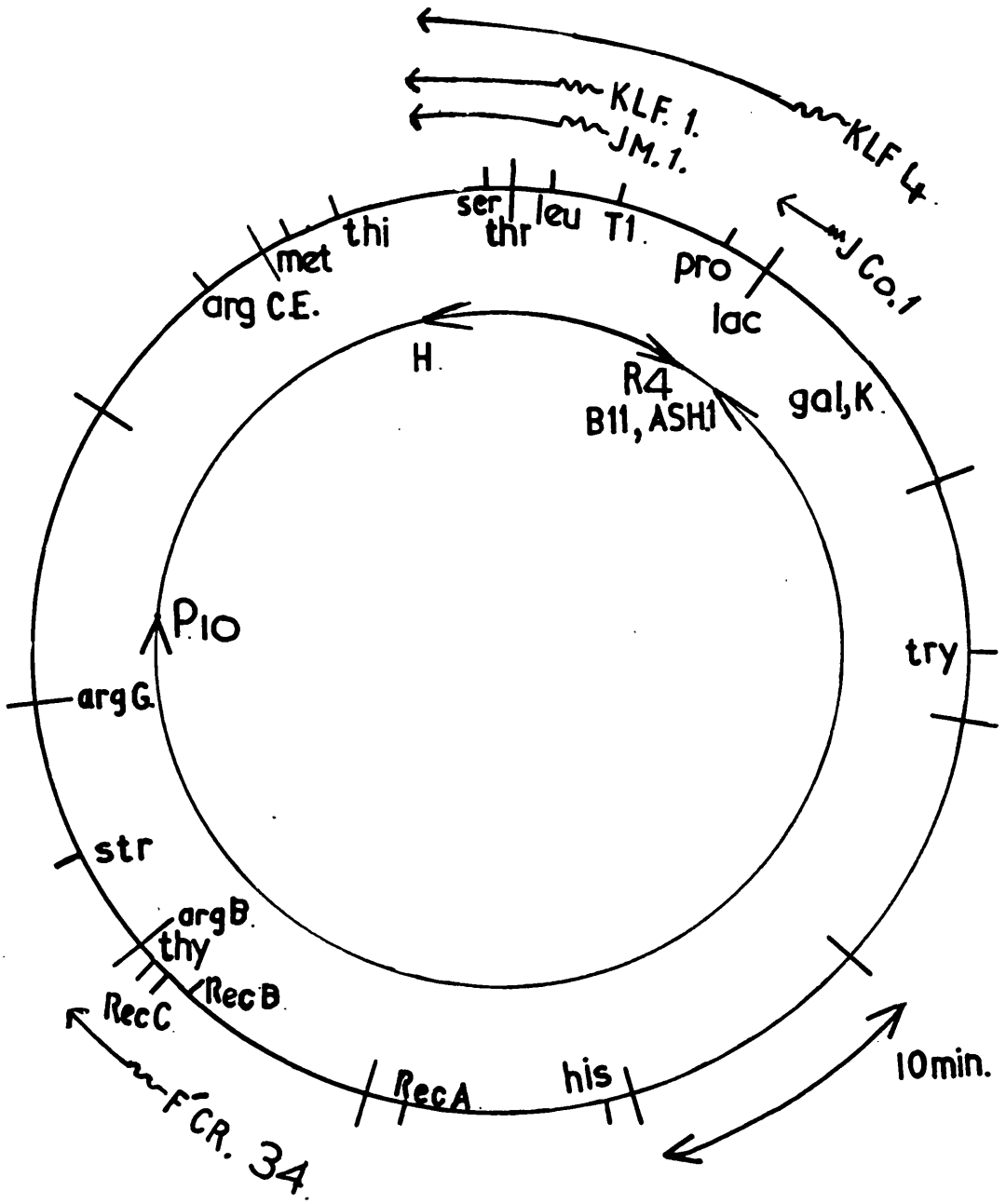
TABLE

Strain	Mating type	Genotype												
		serB	T	L	pro	lac	try	his	arg	thy	met	ileu	λ	str
HfrH202	HfrH	+	+	+	+	+	+	+	+	+	+	+	-	S
ASH3	HfrH	+	+	+	+	+	+	+	+	+	+	+	+	S
ASH4	HfrR4	+	+	+	+	+	+	+	+	+	-	+	+	S
IB11	HfrB11	+	+	+	+	+	+	+	+	+	-	+	-	S
ASH1	HfrB11	+	+	+	+	+	+	+	+	-	-	+	-	R
E6	HfrC	+	+	+	+	+	+	+	+	+	-	+	+	S
P10	HfrP10	+	+	+	+	+	+	+	+	+	+	+		S
KLF	F'	+	TL^+/TL^-		-	-	+	-	-	+	+	+		R
KLF4	F'	+	$\frac{(F'T\ L\ P^+)}{T\ L\ P^-}$		-	+	-	-	+	+	+			R
JM1	F'	+	$\frac{(F^-T\ L^+)}{T\ L^-}$		+	+	+	+	+	+	+			S
CR34(a)	F'	+	-	-	+	+	+	+	+	$\frac{(F'thy^+)}{+}$		+	+	S
J.Co.-1	F'	+	-	-	+	$\frac{F'lac^+}{lac^-}$		+	+	-	+	+	+	

1 (Contd.)

Restrict^{n} and modificat^{n} markers	Response to colicin E2	Response to UV	Recomb-ination	Origin	Source
r^+m^+	S	R	rec^+		R.Walmsley
r^+m^+	S	R	rec^+	λ^+ deriv.of HfrH202	M.Monk
r^+m^+	S	R	rec^+		R.H.Pritchard
r^+m^+	S	R	rec^+		R.H.Pritchard
r^+m^+	S	R	rec^+	IB11 x ASH10	I.B.Holland
r^+m^+	S	R	rec^+		R.H.Pritchard
r^+m^+	S	R	rec^+	HfrP10	H.L.Kornberg
r^+m^+			$recA^-$	AB2463	B.Low
r^+m^+	S		$recA^-$	AB2463	B.Low
r^+m^+	S		rec^+	KLF1 x 158	
r^+m^+	S	R	rec^+	CR34	John Collins
r^+m^+	S	R	rec^+	C600	John Collins

Fig.2 Chromosome Map of E.coli K12, showing in particular the origins of Hfr and F' strains employed.


KLF. 1.
JM. 1.
KLF 4.
JCo.1
ser
thr
leu
T1.
thi
met
arg C.E.
pro
lac
H.
R4
B11, ASH1
gal, K.
P10
try
arg G.
str
argB.
thy
RecB
RecC
F'CR. 34
RecA
his
10min.

Culture Conditions

Unless otherwise indicated, bacterial cultures were grown with aeration at 37°C in a Gyrotory Shaker (New Brunswick Scientific Co., New Jersey, U.S.A.). For bacterial cell counts, 0.05 ml samples of bacterial cultures were diluted into N-saline (Polyfusor, Boots Pure Drug Co. Ltd., Nottingham) and counted in duplicate in a model A Coulter Counter (Coulter Electronics Ltd., England) at cell densities of 2×10^4 to 2×10^6 cells/ml.

Plating Technique for Bacterial Viable Counts

Unless otherwise indicated, 0.1 ml aliquots of bacterial cultures after appropriate dilution were spread onto NB or minimal agar plates.

Colicin Preparation, Assay and Tests

(a) Production of Colicin

Exponential broth-grown cultures (ca 2×10^8 cells/ml) of colicinogenic strains were treated with mitomycin C (0.4 ug/ml), and incubation with aeration was continued for 4 to 6 hours to complete the induction and release of colicin. Following centrifugation, the supernatant was shaken with a few drops of chloroform and assayed as shown below.

(b) Assay of Colicin Preparation

Serial dilutions of the colicin preparations were spotted onto a previously sown lawn of the indicator strain ROW (10^9 cells/ml

mixed with 4 ml of soft agar) and incubated for 14 hours at 37°C. The reciprocal of the highest dilution producing an inhibition of growth was taken as the colicin titre, and was expressed in arbitary units/ml of colicin. This value varied between 10^4 and 5×10^5 units/ml for different preparations, and did not appreciably change after storage for up to four months at 4°C.

(c) Test for colicin E Resistance

To test for colicin E resistance, preparations of colicins E1, E2 and E3 were streaked on NB agar plates, allowed to dry, over-layered with 4 ml top agar, and cross-streaked with test strains and corresponding indicator strains grown to the late exponential phase. Plates were then incubated at the necessary temperatures, and examined after 14 hours.

Media

Oxoid No.2 Nutrient Broth (NB), supplemented with 80 µg/ml thymine when necessary, was used throughout. Nutrient broth agar consisted of NB + 2% Davis agar for plate medium, NB + 1% Davis agar for top layers, and NB + 0.7% agar for NB soft agar. Tryptone Broth (TB) consisted of 1.0% Oxoid tryptone and 0.5% sodium chloride, again supplemented with 80 µg/ml thymine. Magnesium sulphate was added to a final concentration of 0.2% when necessary for phage lambda studies. TB agar contained concentrations of Davis agar as described above.

For the selection of prototrophic recombinants, M9 (Anderson, 1946) + 2% Davis Japanese agar was used, supplemented with appropriate amino acids (20 µg/ml) on lactose (0.4%) and containing 100 µg/ml streptomycin. For the preparation of phage lysates, M9 + 0.7% agar (soft agar) was used.

Unless otherwise indicated, all dilutions were made in 0.07 M phosphate buffer (pH 7.0) (Buffer).

Bacteriophage P1 Preparation, Assay and Transduction Procedure

(a) Preparation of phage P1 lysates

The preparation of phage and the transduction procedure were based on the technique described by Glover (1962). Donor bacteria grown to stationary phase in TB and resuspended in phosphate buffer supplemented with a final concentration of 10^{-3} M $CaCl_2$, were shaken for a further two hours at 37°C; 0.1 ml P1 and 0.2 ml donor bacteria (m.o.i 5-10) were then mixed and allowed to stand for 5 minutes at 37°C; 4 ml of soft agar was added, the suspension poured onto TB agar plates, and incubated for 14 hours at 37°C. Confluent lysis of the donor bacteria was then observed and the top agar was removed, shaken with 2 ml of phage buffer for 15 minutes before centrifugation at 5,000 r.p.m. The supernatant was again shaken with a few drops of chloroform and centrifuged at low speed; the supernatant was finally titred on a stationary phase TB culture of the wild type, strain C600, and retained for transductional studies. Lysates obtained in this

way usually contained ca 10^{10}-10^{11} p.f.u/ml.

(b) Phage Buffer.

The constituents of phage buffer were as follows:

Na_2HPO_4	10.5 gm	0.01 M $CaCl_2$	15.0 ml
KH_2PO_4	4.5 gm	1% gelatin	1.5 ml
NaCl	7.5 gm	1.47 L distilled water	
0.01 M $MgSO_4$	15 ml		

(c) Assay of Phage P1 Lysates

Aliquots of 0.1 ml of phosphate buffer dilutions of the phage P1 lysate prepared as above were mixed with 0.2 ml of a TB culture of a P1 sensitive wild type strain, C600, previously grown to stationary phase in TB, centrifuged and resuspended in an equal volume of phosphate buffer supplemented with a final concentration of 10^{-3} M $CaCl_2$. The phage-bacterial mixture was mixed with 4 ml soft agar after standing at 37°C for 5 minutes, and the suspension was poured onto TB agar plates and incubated for 14 hours at 37°C. From the number of plaques arising, the titre of the phage preparation was calculated.

(d) P1 Transduction

Cultures of the recipient bacteria were grown in TB to ca 2×10^8 cells/ml, and resuspended in 1/5 volume of phage buffer supplemented with 10^{-3} $CaCl_2$. An equal volume of phage lysate was added (m.o.i 10-20) and the mixture incubated at 25°C for 90 minutes.

The adsorption mixture was centrifuged and the pellet resuspended in phosphate buffer supplemented with 0.5% sodium citrate to prevent reinfection on the plates. Aliquots of 0.1 ml of serial dilutions made in buffer again supplemented with 0.5 ml sodium citrate were then plated on the appropriate selective medium for incubation.

Definitions

1. Colicin E2-refractory mutants (Ref-II) were defined by Hill and Holland (1967) as those mutants which, although adsorbing colicin E2 as efficiently as did the wild type, are refractory to colicin E2 at 30°C yet sensitive at 40°C. Ref-II mutants are sensitive to the colicins E1 and E3 at both 30 and 40°C.

2. refA and refB loci. The refA locus is the locus of a gene, refA which in some way participates in the expression of colicin E2-refractivity. This gene has been entirely arbitrarily designated to be "wild-type" ($refA^+$), when refractivity cannot be expressed, and mutant, refIIA, when potentiating the expression of E2-refractivity.

The refB locus is the locus of a second gene, refB, which when mutated (refIIB) directly gives rise to Ref-II phenotypes, i.e. to E2 refractive phenotypes. It is to be noted that, despite mutation at the refB locus to refIIB, E2-refractivity cannot be expressed without the participation of the refIIA allele.

SECTION 1

ISOLATION OF COLICIN E2 REFRACTORY MUTANTS AND GENETIC CHARACTERISATION OF A SECOND LOCUS NECESSARY FOR THE EXPRESSION OF E2-REFRACTIVITY

Introduction

If a multistep pathway is involved in the transmission of the colicin effect to the intracellular target, then mutants refractory to a single colicin are more likely to be altered in a component participating in a later step in the pathway, than are multirefractory mutants. Therefore a study of mutants refractory to a single colicin may assist in the characterisation of later steps in the transmission pathway. For this purpose, mutants refractory to colicin E2 (Ref-II) were isolated.

This class of refractory mutant had previously been isolated from one strain of E.coli K12 (Hill and Holland, 1967). These mutants are characterised by refractivity to colicin E2 at 30°C, whilst remaining sensitive to this colicin at 40°C and to the colicins E1 and E3 at both 30 and 40°C. In this study, selection of Ref-II mutants from a variety of strains of E.coli K12 was attempted.

Materials and Methods

Bacterial Strains: Characteristics and origin of bacterial strains employed in this section are summarised in Table 1 (pp.46-49).

Phage: Transducing phage P1 was obtained from a stock of R.W. Hedges'.

1. Production of colicin E2-refractory mutants (Ref-II mutants)

All the strains shown in Table 3, with the exception of ASH1 were each inoculated into 5 ml of NB containing 500 µg/ml of 2-amino purine (Sigma Chemical Co., London) and incubated statically for 24 hours at 37°C to a final cell concentration of ca 8 x 10^8 cells/ml. Dilutions of the cultures were subsequently plated on NB agar plates containing 10^3-10^4 units/ml of colicin E2, and incubated at 25°C for 24 hours. Duplicate replicas of resistant colonies were then made to NB agar plates overlayered with top agar containing 5 x 10^3 units/ml of either colicin E2 or colicin E3 and incubated at 25°C or 40°C for 24 and 14 hours respectively.

The master and replica plates were then examined and compared. Those colonies present on the colicin E2 plates which did not appear on the colicin E3 plates were isolated, purified and subjected to further examination. Static NB cultures of these strains in late log phase were then prepared and a loopful of each culture streaked over colicin E2 and E3 streaks, previously prepared on NB agar plates, and incubated at 25°C and 40°C. Those mutants resistant to colicin E2, but still sensitive to colicin E3 in such tests were regarded as Ref-II mutants and were retained.

It is important to note that the adsorption of colicin E2 to the Ref-II mutants isolated in this study was not measured. In this

respect, the definition of Ref-II mutants has been relaxed from the criteria originally applied by Hill and Holland (1967), who defined Ref-II mutants as those mutants which actually adsorb colicin E2 yet which are refractive to its effects. However it was considered that since the methods of isolation were the same, it was reasonable to assume that all E2-resistant mutants isolated in this way were refractory. This was apparently confirmed in later genetical studies (Section III), when it was found that all mutants, including some initially isolated by Hill and Holland (1967), were genetically homogenous. The possibility of the existence of both refractory and non-refractory phenotypes amongst the Ref-II mutants isolated in this study can therefore be discarded.

2. P1 transduction (described in General Methods)

(a) Screening of colicin E2-refractive transductants: For the selection of Ref-II transductants, different dilutions of the re-suspended phage-bacterial adsorption mixtures were incubated without shaking in NB for 90 minutes at 37°C to allow the expression of E2-refractivity (see Section IV). The cultures were then plated on NB agar plates supplemented with 5×10^3 units of colicin E2. Plates were incubated for 24 hours at 25°C and the E2-resistant colonies were then again replica-plated to plates containing 5×10^3 units/ml of colicin E2 and to plates containing 5×10^3 units/ml of colicin E3 to confirm the Ref-II phenotype.

Results

A. Isolation of Mutants Refractory to Colicin E2

Hill and Holland (1967) had previously found that the most common type of colicin E resistant mutant isolated by selection against colicin E2 displayed resistance to all E group colicins. Furthermore, adsoprtion tests demonstrated that this class of mutants failed to adsorb E group colicins, and it was presumed that resistance had arisen through loss or inactivation of the specific E group cell surface receptor. This non-refractory, receptor-minus group was designated $\bar{E}$. The second most predominant class of mutants was resistant to colicins E2 and E3 and sensitive to colicin E1 in a variety of phenotypes. These mutants were designated E32. No mutants were found with a combined resistance to the colicins E1 and E2, or to E1 and E3, a point taken as an indication of the early separation of the receptor structure and transmission pathway of colicin E1 from that of colicins E2 and E3. As Ref-II mutants alone were required in this study, screening of E2-resistant mutants by replication to lawns of colicin E3 was considered sufficient to distinguish those mutants refractive to colicin E2 alone from those resistant to at least the two E group colicins, E2 and E3. E2-refractive mutants isolated thus were then screened against colicin E2 at 30°C and 40°C, as a further characteristic feature of Ref-II mutants isolated by Hill and Holland (1967) was refractivity to

colicin E2 at 30°C and sensitivity at 40°C.

In the initial mutant isolation from strain ASH10, complete screening of the mutants was performed by replication of resistant colonies to plates supplemented with the respective colicins E1, E2 and E3, at 30°C and 40°C, to determine the relative proportions of $\bar{E}$, E32 and E2-refractive phenotypes. As can be seen from Table 2, mutants of the $\bar{E}$ and E32 groups remained the predominant classes isolated from ASH10, although their relative distribution was somewhat different to that obtained from ASH1 by Hill and Holland (1967). Ref-II mutants were still the minority class.

Ref-II mutants, as shown in Table 2, are refractory to colicin E2 at 30°C but remain sensitive at 40°C. Such mutants had previously been readily isolated from only one strain, ASH1, out of 4 strains tested (Hill and Holland, 1967). In this study attempts were made to isolate Ref-II mutants from a variety of additional strains, and during the course of these experiments it was observed that by selecting and testing suspect E2-refractory mutants at 25°C rather than 30°C, mutants could be obtained in a limited number of other strains, i.e. 7 out of 15 tested (Table 3)

It should be noted, however, that the comparative frequency of Ref-II mutants in these other strains is lower than that seen in ASH1 (10%) (Hill and Holland, 1967) and in ASH10 (78%). Furthermore, although the numbers of mutants screened derived from 4 strains

TABLE 2

Phenotypes of Colicin E Resistant Mutants Isolated from ASH10

Strain	Group type	Selection incidence of total resistant mutants (%)	Response to colicin			
			E1.37°	E2.25°	40°	E3.37°
ASH10 (total of 449 resistant mutants tested)	- E	42	R	R	R	R
	E32	50	S	R	R	R
	$E2_{25}°$	8	S	R	S	S

Cultures of ASH10 treated with 2-amino purine were plated on NB plates supplemented with 5×10^3 units of colicin E2 and screened for different colicin resistant and refractory mutants as described in Methods (Section I).

Frequency of mutation to colicin E resistance:-

without 2-amino purine 10^{-7}

with 2-amino purine 10^{-4}

TABLE 3

The Incidence of Colicin E2 Refractory (Ref-II) Mutants Isolated from a Variety of Strains of E.coli by 2-Amino Purine Treatment

Strain	No. of colicin resistant mutants isolated	Response to colicin (No. tested)			∴ No. of Ref-II mutants	% incidence of Ref-II mutants
		E2		E3		
		25°C	40°C	37°C		
ASH10	448	345	310	310	35	7.8
C600	145	135	135	135	0	< 0.8
ASH3	220	199	197	197	2	1.0
ASH4	671	606	601	601	5	0.6
E94	198	122	119	119	3	2.5
IB11	270	251	251	251	0	< 0.4
ROW	150	78	78	78	0	< 1.2
ASH5	90	87	84	84	3	3.9
E6	89(a)	47	47	47	0	< 2.0
E32	123(a)	85	85	85	0	< 1.1
rec34	94(a)	63	63	63	0	< 1.3
KMBL240	112(a)	71	71	71	0	< 1.2
4K	281	262	262	262	0	< 0.4
4K-T$^+$	221	198	194	194	4	1.8

(a) The numbers of E resistant mutants screened was insufficient to ensure non-occurrence of Ref-II mutants in these strains.

was rather too low to draw any definite conclusion, 8 strains still failed to produce Ref-II mutants. It was conceivable that the inability to isolate E2 refractive mutants from these strains was the reflection of a further mutation in a second gene, which in some way is essential for the phenotypic expression of E2 refractivity. Further indication of the presence of such a mutation also became manifest in transductional studies (described in Section III) where considerable difficulties were encountered in transducing the refII mutation to particular recipient strains from which Ref-II mutants could not be derived. These difficulties were eventually resolved by the transduction of a second gene into the recipient strain, after which the E2-refractive phenotype was expressed normally (see below).

Investigation into the history of the E.coli strains used revealed that the majority of the strains from which E2-refractive mutants were isolated had previously been exposed to heavy mutagenesis by such severe agents as NMG, and so the random occurrence of such a second mutation is not unlikely. This second gene has tentatively been designated to be in the non-mutated state when present in a strain from which Ref-II mutants cannot be derived, and is referred to as the "refA" gene. On the other hand the gene refB is that which, when mutated by 2-amino purine treatment, produces colicin E2

refractivity. The ref status of various strains is therefore as follows:

Strain 4K - refA$^+$ refB$^+$ - incapable of expressing E2-refractivity

Strain ASH10 - refIIA refB$^+$ - E2-refractivity can be expressed after receipt of the refIIB allele, or mutation of the refIIB gene.

Strain ASH102 - refIIA refIIB - Ref-II mutant of ASH10, i.e. E2 refractivity is expressed.

Strains from which Ref-II mutants could be isolated were presumably therefore initially of the refIIA genotype. It must, however, be emphasised that the method of designation of the second gene, potentiating the expression of E2-refractivity, is entirely arbitrary. For example, it is equally possible that strains such as 4K and IB11, from which Ref-II mutants could not be obtained, carry a mutated refA gene, whereas strains capable of expression E2-refractivity are "wild-type". This point cannot be clarified until the precise function of the refA gene is known, but in this study the above nomenclature will be used.

B. Location of the refA gene on the bacterial chromosome of E.coli

In the course of transduction studies, it was observed that a number of thr$^+$ transductants from a cross between the P1 donor, ASH35 (thr$^+$ leu$^+$ refIIA) and the recipient 4K (thr$^-$ leu$^-$ refA$^+$) were capable

expressing the E2-refractive phenotype after subsequent transduction of the refIIB locus from an E2 refractive mutant. It was apparent, therefore, that the refA locus is linked to thr. To confirm this, and to precisely locate the refA locus, a large number of thr$^+$ transductants from a cross between a refIIA donor and refA recipient were screened for the subsequent ability to receive and express transduced colicin E2 refractivity. For this purpose, strains of known refA and refB status, as shown in Table 4, were employed. In all, 60 thr$^+$ transductants of the recipient strain 4K were then selected after a cross between the donor, ASH3, and the recipient 4K. These transductants were subcultured, and subsequent transduction crosses with these strains as recipients were carried out with P1 grown on the Ref-II mutant, ASH5. Selection for E2-refractivity was made, and the number of the thr$^+$ transductants of 4K with the ability to receive and express E2-refractivity was so determined. This was then taken to indicate the chromosomal position of the refIIA locus.

Following the transduction of thr into 4K from $P1_{(ASH3)}$, 42 out of 60 thr$^+$ transductants were able to receive and express colicin E2-refractivity from the Ref-II mutant, ASH55. Furthermore, this number included 3 thr$^+$ transductants which also had inherited the unselected leu$^+$ marker from ASH3, indicating that the refIIA locus was not only 70% linked to thr, but probably also was positioned to the right of thr on the bacterial chromosome. These results are summarised in Table 5.

TABLE 4

'ref' Status and Genotype of Strains Employed in the Location of the 'refA' Locus

Strain	Response to colicin E2		E2 refractive mutant derivation	∴ ref status and genotype
	25°C	40°C		
4K	S	S	-	refA$^+$ refB$^+$ thr$^-$ leu$^-$
ASH3	S	S	+	refIIA refB$^+$ thr$^+$ leu$^+$
ASH54(a)	R	S	+	refIIA refIIB thr$^+$ leu$^+$

(a) 2-amino purine derivative of ASH3

Preparations of P1 were first made on ASH3 as a donor strain.

"RefIIB" - (E2 refractive) - transductants were scored after plating of transduction lysate on NB plates containing 5×10^3 units of E2/ml, after 90 minutes incubation at 37°C in NB to allow for expression of E2 refractivity (see Methods, Section I). Plates were incubated for 24 hours at 25°C and colonies arising were replicated to lawns of colicins E3 and E2; only those transductants capable of producing colonies refractive to colicin E2 at 25°C, yet sensitive to E2 and E3 at 37°C, were scored as "RefIIA".

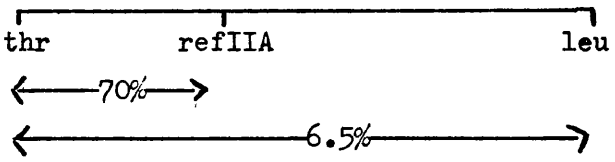
TABLE 5

Linkage between the thr, leu and refA loci

P1 donor	P1 recipient	Selected marker	No. scored	Analysis of transductants (scored as %)		
				thr$^+$	leu$^+$	refIIA
ASH3 (thr$^+$ leu$^+$ refIIA)	4K (thr$^-$ leu$^-$ refA$^+$)	thr$^+$	60	100	6.5	70

Possible classes	No.scored	%
thr$^+$ leu$^+$ refIIA	4	6.7
thr$^+$ leu$^+$ refA$^+$	0	0
thr$^+$ leu$^-$ refA$^+$	18	30
thr$^+$ leu$^-$ refIIA	38	63.3

∴ Probable gene order and linkage



Discussion and Conclusions

Mutants refractive to colicin E2 were isolated from only a limited number of strains of *E.coli*, and likewise the transduction of E2 refractivity to certain recipient strains was found to be impossible. The presence of a second gene potentiating the expression of the Ref-II phenotype was therefore postulated, and mapping studies with the transducing phage P1 have positioned such a gene to the right of, and 70% linked to, the *thr* locus on the *E.coli* chromosome.

The way in which the *refIIA* gene affects the expression of colicin E2 refractivity is not yet known. However this mutation may be ascribed to having one of at least two effects. For example, it may suppress in some way an otherwise lethal consequence of the mutation of the *refB* gene. Alternatively, this gene may control the formation of some further cell constituent which may require alteration before the change wrought by mutation at the *refB* locus can result in refractivity to colicin E2.

SECTION II

PHYSIOLOGICAL PROPERTIES OF COLICIN-E2 REFRACTORY MUTANTS

Introduction

Since a primary observable effect of colicin E2 is the rapid induction of DNA degradation in sensitive bacteria, the activation of one or more deoxyribonucleases is presumably involved. When it is considered that refractory mutants, although still adsorbing the colicin E2 molecule to specific E-group receptors in the cell surface, do not degrade their DNA or show any inhibition of cell division, it is apparent that colicin action is blocked at some stage prior to the initiation of DNA degradation. This failure to transmit the colicin stimulus could be envisaged to occur through changes in components of the cytoplasmic membrane essential to the transmission system, or in the activity of a deoxyribonuclease or deoxyribonucleases normally participating in the enzymic degradation of DNA. In the latter event, the actual loss or defective synthesis of an enzyme, the alteration of the specificity of attachment of the enzyme to the membrane, or subtle alteration of the final substrate, the DNA, could prevent nuclease induced degradation.

Several nucleases are thought to participate in the repair, replication and recombination of DNA, and it is known that a common characteristic of many strains carrying mutations affecting the activity of these enzymes is an increased sensitivity to ultraviolet

irradiation (Howard-Flanders and Boyce, 1966; Clark, Chamberlin, Boyce and Howard-Flanders, 1966). Accordingly, to test the hypothesis that Ref-II mutants have in some way undergone a change in nuclease activity, the response of a number of Ref-II mutants to UV irradiation was determined. Furthermore, in an attempt to determine the nature of the defect in any Ref-II mutants which displayed concomitant UV sensitivity, their response to X-ray irradiation was also determined and compared to that shown by a known Rec$^-$ mutant which is both UV and X-ray sensitive.

The mutation to colicin E2 refractivity may alternatively involve modification of some membrane component or even of the DNA itself which could in turn disturb control of cell growth and cell division (Helmstetter and Pierucci, 1968). In this case, one might anticipate that the mutation might be reflected by abnormalities in the growth patterns of mutant strains, and in extreme cases morphological peculiarities of the cell surface structure may become apparent in particular mutants. The growth characteristics and cellular morphology of selected Ref-II mutants were therefore investigated. De Zwaig and Luria (1967) have in fact previously reported an increased sensitivity of some mutant classes, displaying joint refractivity to colicins E2 and E3, to sodium deoxycholate (DOC) and EDTA, and suggested that this property indicated some alteration in components of the cytoplasmic membrane produced by the mutation. Accordingly, the sensitivity of Ref-II

mutants to sodium deoxycholate was also tested. In addition the possibility that the refA locus, necessary for phenotypic expression of E2 refractivity, also involved alteration in cells surface properties was determined by testing the DOC sensitivity of Ref-IIA strains.

As mentioned in the Introduction, the inhibition of cell division of sensitive cells when exposed to colicin E2 is known to take place almost immediately after induction of DNA breakdown (Holland, 1968). Therefore it is likely that E2-refractivity could also derive from the alteration of some membrane component which is also involved in the normal cell division mechanism. Consequently, any indications of cell division defects in the mutants were particularly looked for.

Materials and Methods

Bacterial strains: The characteristics of relevant ref$^+$ strains referred to are shown in Table 1; the genotypes and derivation of principle Ref-II mutants are shown in Table 6. The origin of other refII mutants will be stated where necessary. Unless specifically mentioned to the contrary, all ref$^+$ strains referred to in this Section are capable of the expression of E2-refractivity and therefore have the genotype refIIA, refB$^+$. These will be simply referred to as ref$^+$.

Phage: The transducing phage P1 was initially obtained from R.W. Hedges. The virulent mutant of phage λ, λgv (Jacob and Wollman, 1953)

TABLE 6

Genotypes of Principal Ref-II Mutants Referred to in Section II

Strain	Origin	Sex	Genotype							
			thr	leu	lac	try	thy	met	str	λ
ASH102	AP[(a)] mutation of ASH10	F^-	+	-	-	+	-	-	R	+
ASH112	AP mutation of ASH10	F^-	+	-	-	-	-	-	R	+
ASH113	AP mutation of ASH10	F^-	+	-	-	+	-	-	R	+
ASH115	AP mutation of ASH10	F^-	+	-	-	+	-	-	R	+
ASH116	AP mutation of ASH10	F^-	+	-	-	+	-	-	R	+
ASH111	AP mutation of ASH1	Hfr	+	+	+	+	-	-	R	-
ASH114	AP mutation of ASH1	Hfr	+	+	+	+	-	-	R	-

(a) 2-amino purine

was obtained from I.B. Holland.

1. UV Sensitivity

(a) Screening of Ref-II mutants for UV sensitivity

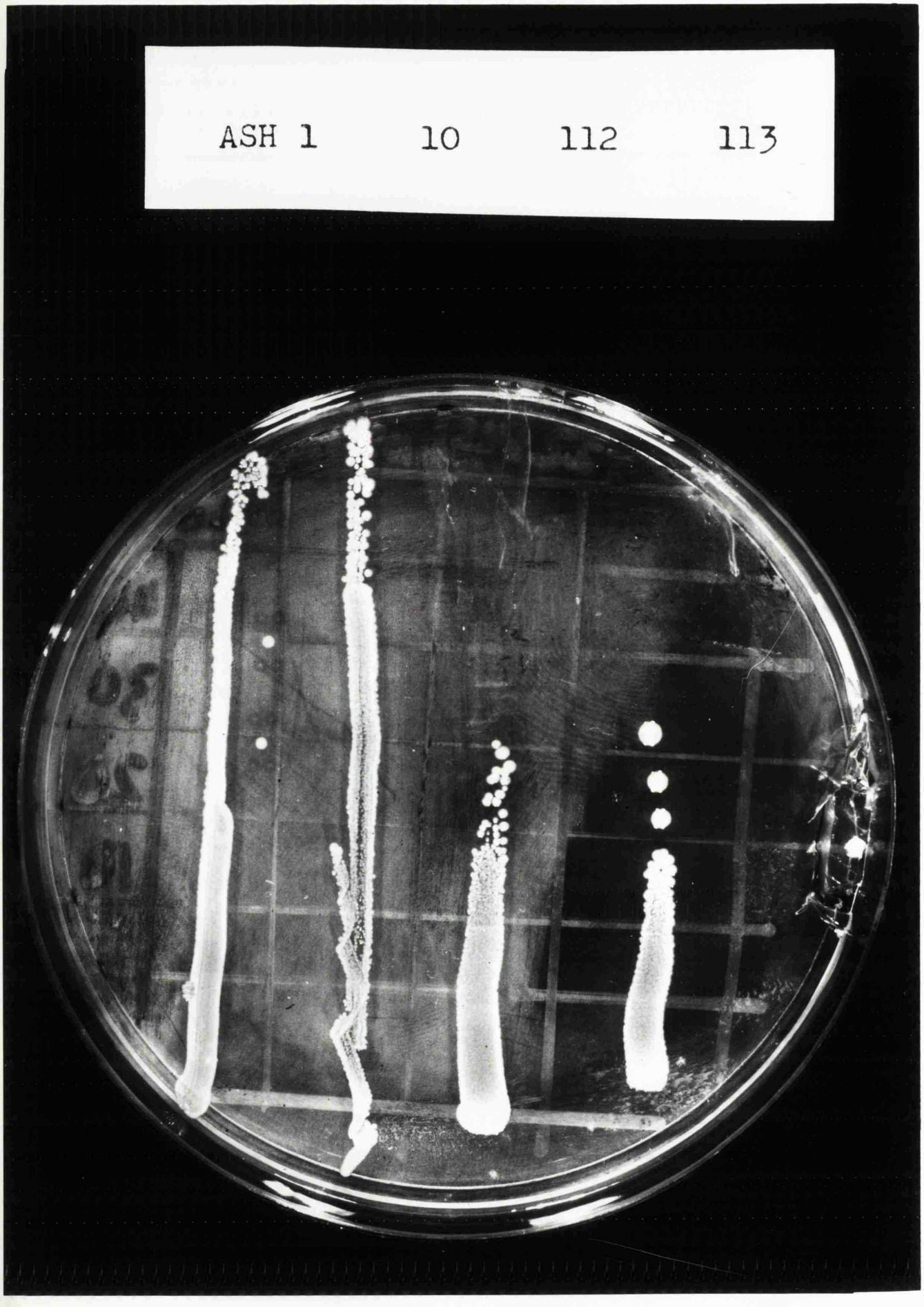
For the detection of UV sensitive strains amongst Ref-II mutants, 10-fold dilutions of late exponential TB cultures of bacterial strains at ca 5 x 10^8 cells/ml were streaked onto duplicate TB agar plates. The source of irradiation was a low-pressure mercury lamp (Hanovia Ltd) with a dose rate at room temperature of approximately 6.5 ergs/ mm^2/sec at 46 cm. Plates were covered with an opaque sheet, which, when placed under the UV source, was progressively withdrawn downwards. In this way, different parts of the streak were exposed to 60, 40, 30, 20, 10 and 5 seconds irradiation, and one portion was not exposed. One plate was then incubated at 25°C and one at 40°C in the dark for 24 hours and 14 hours respectively. The resulting growth of the tested strains was compared to a parental control, resistant to 40 seconds of UV irradiation. All strains gave an identical response at both temperatures, and 6 strains which showed sensitivity to 15 seconds of irradiation or less were selected for further study (Plate 1).

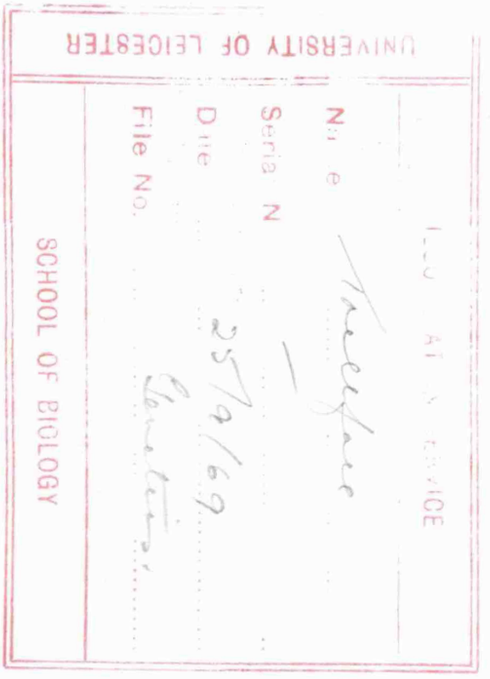
(b) Quantitative determination of survival

For the quantitative determination of the survival of irradiated bacteria, strains were grown in TB to a cell density of ca 5 x 10^8 cells/ml, resuspended in buffer, and plated after appropriate dilution in phosphate buffer onto TB agar plates. The plates were then

Plate 1 Plate Screening Method for UV Sensitivity

Sensitivity of ASH1, ASH10 (UV^R), and the Ref-II, UV^S mutants ASH112 and ASH113, to UV irradiation.


ASH 1 10 112 113


UNIVERSITY OF LEICESTER
SCHOOL OF BIOLOGY
Name
Serial No.
Date
File No.

irradiated over a dose range of 0-400 ergs, incubated in the dark for 14 hours at 37°C, and the surviving fraction counted.

(c) Effect of physiological conditions on the response to UV irradiation

Exponential cultures of bacterial strains in minimal and complex medium at ca 5 x 10^8 cells/ml were adjusted to a final density of 10^8 cells/ml. Aerated growth at 37°C was continued, and duplicate 0.1 ml samples were removed at 30 minute intervals. One such duplicate was diluted through phosphate buffer, plated on TB agar plates and not irradiated; one was similarly diluted and plated and then irradiated for 40 seconds. All plates were incubated for 14 hours at 37°C in the dark, and the resulting colonies counted.

(d) Effect of pantoyl-lactone on UV sensitivity

For the quantitative determination of the effect of pantoyl-lactone on the survival of irradiated bacteria, strains grown in TB to a density of ca 5 x 10^8 cells/ml were resuspended in buffer and plated after appropriate dilutions onto plates of TB agar containing 0.08 M concentrations of DL-pantoyl-lactone (Van de Putte, Westenbroek and Rörsch, 1963) (Calbiochem, Los Angeles, U.S.A.). The plates were irradiated and incubated in the dark for 14 hours at 37°C, and the surviving fraction counted.

2. Measurement of Growth Rate

Measurement of growth rate was examined by three methods.

(a) Turbidity Determination

(i) with an Eel Portable Colorimeter (Eatons Electroselenium Ltd., Halstead, Essex). 0.5 ml of overnight cultures of the various strains examined were re-inoculated into 10 ml of NB. Increase of optical density (OD) of the aerated cultures at 37°C with time was measured directly through a blue filter 621.

(ii) with a Gilford 300 Microsample Spectrophotometer (Wright Scientific Ltd. London). Overnight cultures of the respective strains at ca 10^9 cells/ml were Coulter-counted and 40 ml NB fresh cultures were initiated at 10^7 cells/ml. Aerated growth at 37°C was followed by removal of 0.5 ml duplicate samples at 20 minute intervals; OD was measured at a wavelength of 550 mμ.

(b) Total Bacterial Cell Number was followed by Coulter-counting (see General Methods).

(C) Viable Bacterial Cell Number

0.1 ml samples of bacterial cultures were diluted, plated on NB agar, and incubated at 37°C for 14 hours.

3. Microscopic Characteristics of Ref-II Mutants

NB agar grown colonies and NB cultures of certain Ref-II mutants, after storage for 5 days at 4°C, were examined under a phase contrast microscope (Wild (Heerbruge) Switzerland).

4. Colonial Morphology on Minimal Agar

Late exponential phase cultures of strains to be examined were streaked onto minimal agar plates, incubated for 24 hours at 37°C and

the morphological appearance observed. Capsule formation was tested by staining with a 1 in 20 dilution of Indian Ink and observed under phase.

5. Sodium Deoxycholate (DOC) Sensitivity

(a) Screening for DOC sensitivity

For the detection of increased sensitivity to DOC, late exponential NB cultures of bacterial strains with a cell density of ca 10^9 cells /ml were streaked in duplicate onto 5 duplicate, freshly prepared, NB agar plates containing respectively 2.5%, 1.0%, 0.5%, 0.1% and 0% DOC (Sodium Deoxycholate, BDH Ltd., England). One set of duplicates was incubated at 40°C for 14 hours, and one at 25°C for 24 hours, and subsequent bacterial growth was recorded in terms of growth (R) or non-growth (S) of strains on the various concentrations of DOC. Strains sensitive to less than 1.0% DOC concentrations were selected for further study. It was considered important to read the plates immediately after the set incubation time, particularly with low concentrations of DOC, as background growth in some cases would eventually have been sufficient to mask true resistant growth.

(b) Quantitative determination of survival

For the quantitative determination of the survival of bacterial strains when exposed to concentrations of DOC, exponential NB cultures at ca 5 x 10^8 cells/ml were adjusted to 10^8 cells/ml, and plated after appropriate dilutions onto NB agar plates containing 1%, 0.5%, 0.1%,

0.01% and 0% DOC. Plates were incubated either at 25°C for 24 hours, or 40°C for 14 hours, and the survival fraction counted.

6. X-Ray Irradiation

The source of irradiation was a Gamacell 200 (Atomic Energy of Canada, Ltd.) containing cobalt 60 with an initial content of 1900 curies in March 1962. The current output was 0.13 megarads/hour. Strains to be irradiated were grown to a cell density of ca 5×10^8 cells/ml in TB and adjusted to a cell density of 10^8 cells/ml; 2 ml of culture contained in $1\frac{1}{4} \times \frac{3}{8}$" stoppered tubes were then irradiated, and after subsequent diltuion, 0.1 ml aliquots were plated on TB agar plates. These were incubated for 14 hours at 37°C, and the surviving fraction counted.

7. Preparation and Assay of λ

(a) Preparation

Stocks of λgv were prepared as follows. The contents of a single plaque, suspended in 0.05 ml phosphate buffer, were added to 0.1 ml of stationary phase bacteria grown in TB supplemented with a final concentration of 10^{-3} M $MgSO_4$. After incubation at 37°C for 10 minutes, 4 ml of soft agar was added and the mixture overlayered on TB agar plates containing a final concentration of 10^{-3} M $MgSO_4$. The plates were then incubated at 37°C for 14 hours, after which the top layer was removed, shaken with 2 ml of buffer for 15 minutes, centrifuged, and a few drops of chloroform added to the final supernatant.

(b) Assay

For the assay of λgv prepared as above, 0.1 ml aliquots of various dilutions of the phage in phosphate buffer were mixed with 0.2 ml of indicator bacteria. The latter were previously grown to stationary phase in TB containing a final concentration of 0.25% maltose and 10^{-3} M Mgso4, centrifuged, resuspended in buffer and starved for two hours at 37°C before use. After 10 minutes at 37°C for adsorption, the phage-bacterial mixture was plated with 4 ml of soft agar on TB plates supplemented with a final concentration of 10^{-3} M MgSO4, and incubated for 14 hours at 37°C. The resulting plaques were counted, and the titre calculated.

(c) Release of phage from lysogens

Cultures of bacterial strains were grown to late exponential phase (ca 10^9 cells/ml) and adjusted to a final density of 10^8 cells/ml. The cultures were centrifuged, resuspended in phosphate buffer and irradiated for different time intervals in a thin layer (2mm). Following irradiation, the cultures were again centrifuged, resuspended in NB and incubated at 37°C for 60 minutes for phage release. After subsequent centrifugation, 0.1 ml aliquots of the supernatant were diluted through phosphate buffer and assayed for free phage λ on a λ^-, λ^s bacterial indicator strain, ASH¶.

(d) Adsorption

Bacterial strains, grown to a density of ca 5 x 10^8 cells/ml in

TB supplemented with a final concentration of 10^{-3} M $MgSO_4$ and 2.5% maltose, were adjusted to a density of 2 x 10^8 cells/ml. One ml of bacterial culture was mixed with 1 ml of phage λgv at a multiplicity of 1:1, and the phage-bacterial mixture was allowed to stand at 37°C for 15 minutes before centrifugation and subsequent assay of the supernatant fraction for free phage on the $\lambda^-\lambda^s$ indicator strain, ASH1. As a control, the adsorption of λgv to a lambda receptor-minus Salmonella typhimurium strain, 906, was also determined.

(e) Spot test of λgv resistance

Cultures of the strains to be tested were grown in TB, supplemented with a final concentration of 10^{-3} M $MgSO_4$ and 2.5% maltose, to a density of ca 5 x 10^8 cells/ml; loopfuls of the culture were spotted onto TB agar plates and allowed to dry. These were half overlayed with loopfuls of phosphate buffer dilutions of λgv, and allowed to incubate for 8 hours. Resistant bacterial mutants were then observed to give a full circle of confluent growth at a given dilution of the phage, whereas with sensitive mutants, only a characteristic half-moon of growth had occurred on the area of the culture not overlayered by the phage.

8. Infection and Assay of P1

Stationary phase cultures of bacterial strains grown in TB supplemented with a final concentration of 10^{-3} $CaCl_2$ were infected with dilutions of P1 as described in General Methods, and the plating efficiency calculated after incubation for 14 hours at 37°C.

Results

A. Irradiation Sensitivity of Ref-II Mutants

1. Qualitative response of mutants to UV

To investigate the hypothesis that Ref-II mutants were refractory because of modification in the activity of enzymes concerned with UV repair processes and/or the recombination of DNA, twenty two mutant strains were screened on TB agar plates for increased sensitivity to UV. The results are shown on Table 7.

Of the strains tested, seven did indeed display increased sensitivity to UV. These UV sensitive mutants were characteristically sensitive to fifteen seconds or less of UV irradiation, whereas parental controls and all other Ref-II mutants showed normal growth after 30 seconds of irradiation. It is important to note that the strains were sensitive to UV at both 25° and 37°C, and thus UV-sensitivity, unlike E2-refractivity is not conditional upon temperature.

2. Quantitative Response of Mutants to UV

The UV sensitivity of various Ref-II UV^s mutants was now determined more precisely and compared with that of a known RecA strain, rec34 (Van de Putte, Zwenk, and Rörsch, 1966). Exponential and stationary phase cultures of various strains grown in TB broth were therefore plated and irradiated as described in Methods, Section II and the results obtained are shown in Figures 3 and 4.

(a) = 2-amino-purine

(b) Isolated after AP treatment of ASH100, a $\underline{str}^r$ $\underline{met}^-$ recombinant obtained from an ASH10 x HfrH cross.

Streaks of cultures were irradiated as described in Methods, Section II, on TB agar plates. Those Ref-II mutants sensitive to 15 seconds or less of UV irradiation were considered sensitive, and selected for further study (see Plate 1)

TABLE 7

Response to UV Irradiation of Some E.coli K12 Ref-II Mutants

Strain	Sex	Origin	Response to UV at 25°C and 37°C
ASH50	F^-	$AP^{(a)}$ mutation from ASH10	R
ASH51	F^-	ditto	R
ASH62	F^-	ditto	R
ASH63	F^-	ditto	R
ASH64	F^-	ditto	R
ASH66	F^-	ditto	R
ASH67	F^-	ditto	R
ASH80	F^-	ditto	R
ASH102	F^-	ditto	R
ASH112	F^-	ditto	S
ASH113	F^-	ditto	S
ASH115	F^-	ditto	S
ASH116	F^-	ditto	S
ASH101	Hfr	AP mutation from ASH1	R
ASH111	Hfr	ditto	S
ASH114	Hfr	ditto	S
$ASH110^{(b)}$	Hfr		S
$ASH117^{(b)}$	Hfr		R
$ASH118^{(b)}$	Hfr		R
ASH54	Hfr	AP mutation from ASH3	R
ASH55	Hfr	AP mutation from ASH4	R
ASH57	F^-	AP mutation from ASH5	R

Fig. 3. The response of exponential cultures of certain Ref-II, UV^s mutants and a RecA mutant to UV irradiation

Exponential cultures of various strains in TB at densities of ca 5 x 10^8 cells/ml were adjusted to 10^8 cells/ml, resuspended and diluted in phosphate buffer and plated on TB agar plates. After irradiation, plates were incubated in darkness at 37°C for 14 hours and the surviving fraction determined.

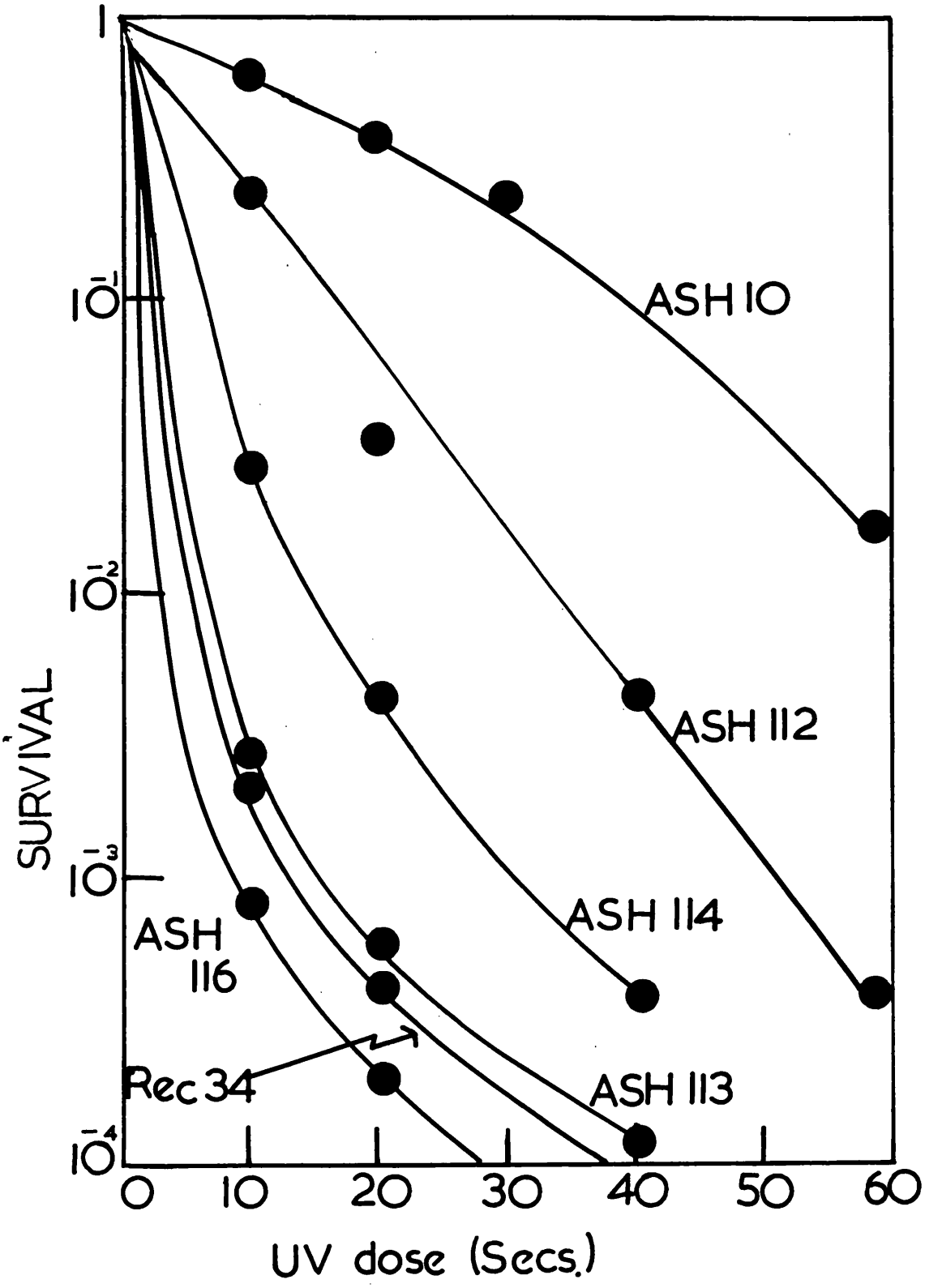
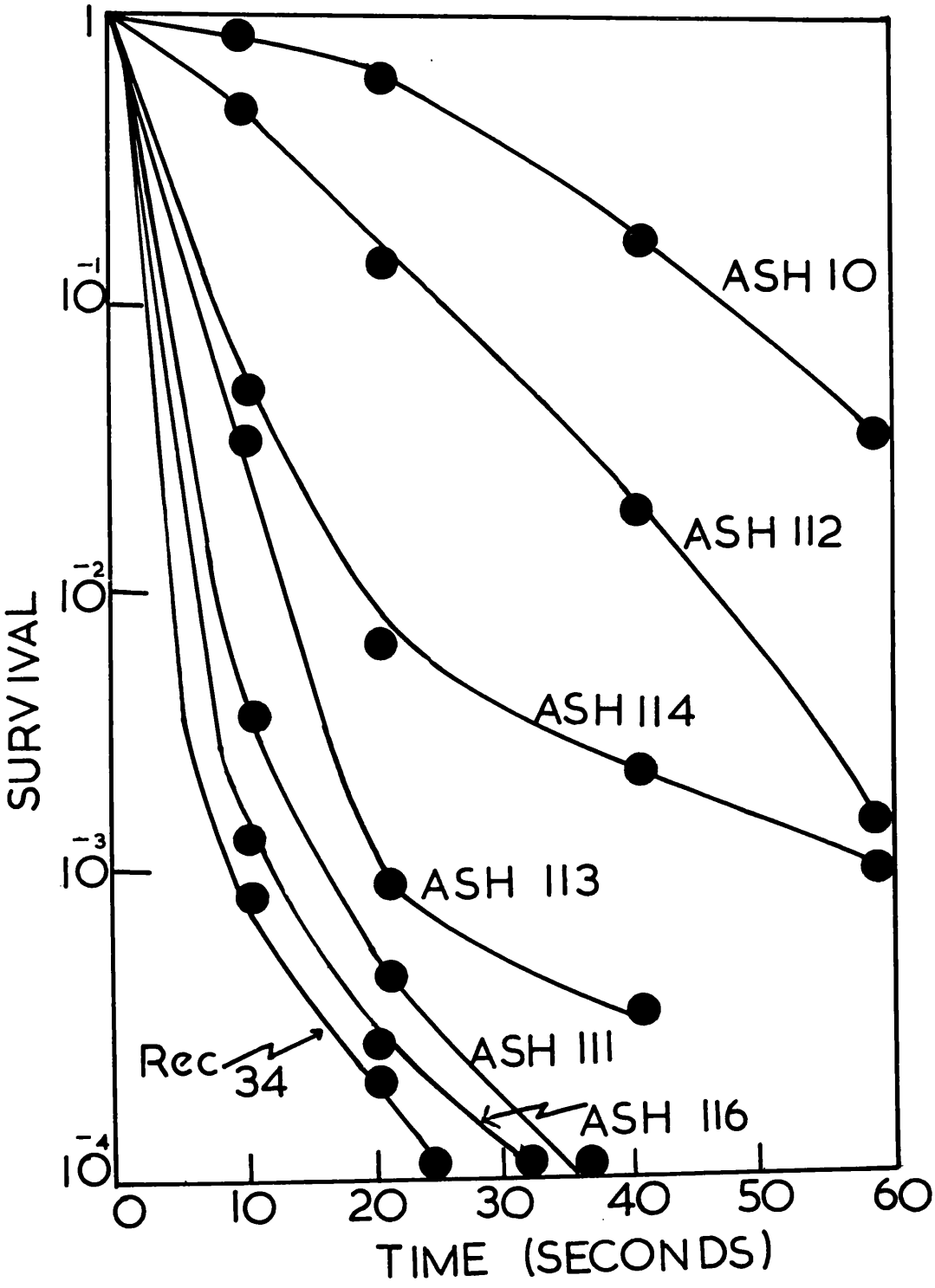

1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
SURVIVAL
ASH 10
ASH 112
ASH 114
ASH 113
ASH 116
Rec 34
0
10
20
30
40
50
60
UV dose (Secs.)

Fig. 4. The response of stationary phase cultures of certain Ref-II, UV^S mutants and a RecA mutant to UV irradiation

Stationary phase cultures of various strains in TB were resuspended and diluted in phosphate buffer, and plated on TB agar plates. After irradiation, plates were incubated in darkness at 37°C for 14 hours and the surviving fraction determined.


1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
SURVIVAL
ASH 10
ASH 112
ASH 114
ASH 113
ASH 111
ASH 116
Rec 34
0
10
20
30
40
50
60
TIME (SECONDS)

As anticipated, all exponentially growing Ref-II UV^S strains showed increased sensitivity over the parental *ref*$^+$ strain, ASH10, yet, with the exception of ASH116, all mutants were more resistant to UV than the RecA strain, *rec*34. The response of the Ref-II UV^S strains to irradiation was by no means identical - ASH116 was the most sensitive and ASH112 the most resistant. Similar results were obtained with both stationary and exponential cultures, with the relative sensitivity of different mutants the same under both conditions. With stationary phase cultures, however, the UV sensitivity of all Ref-II UV^S mutants was seen to decrease in contrast to the RecA strain, *rec*34, which was unaltered. When the quantitative response of stationary phase Ref-II UV^S mutants was repeated at 25°C, results were very similar to those obtained at 37°C. This observation confirms that seen in the initial screening of mutants for UV sensitivity. Thus UV sensitivity of these Ref-II UV^S mutants, unlike E2-refractivity, is *not* a conditional property.

3. Effect of Physiological Conditions on the Response of Ref-II UV^S Mutants to UV Irradiation

(a) Influence of medium and stage of growth

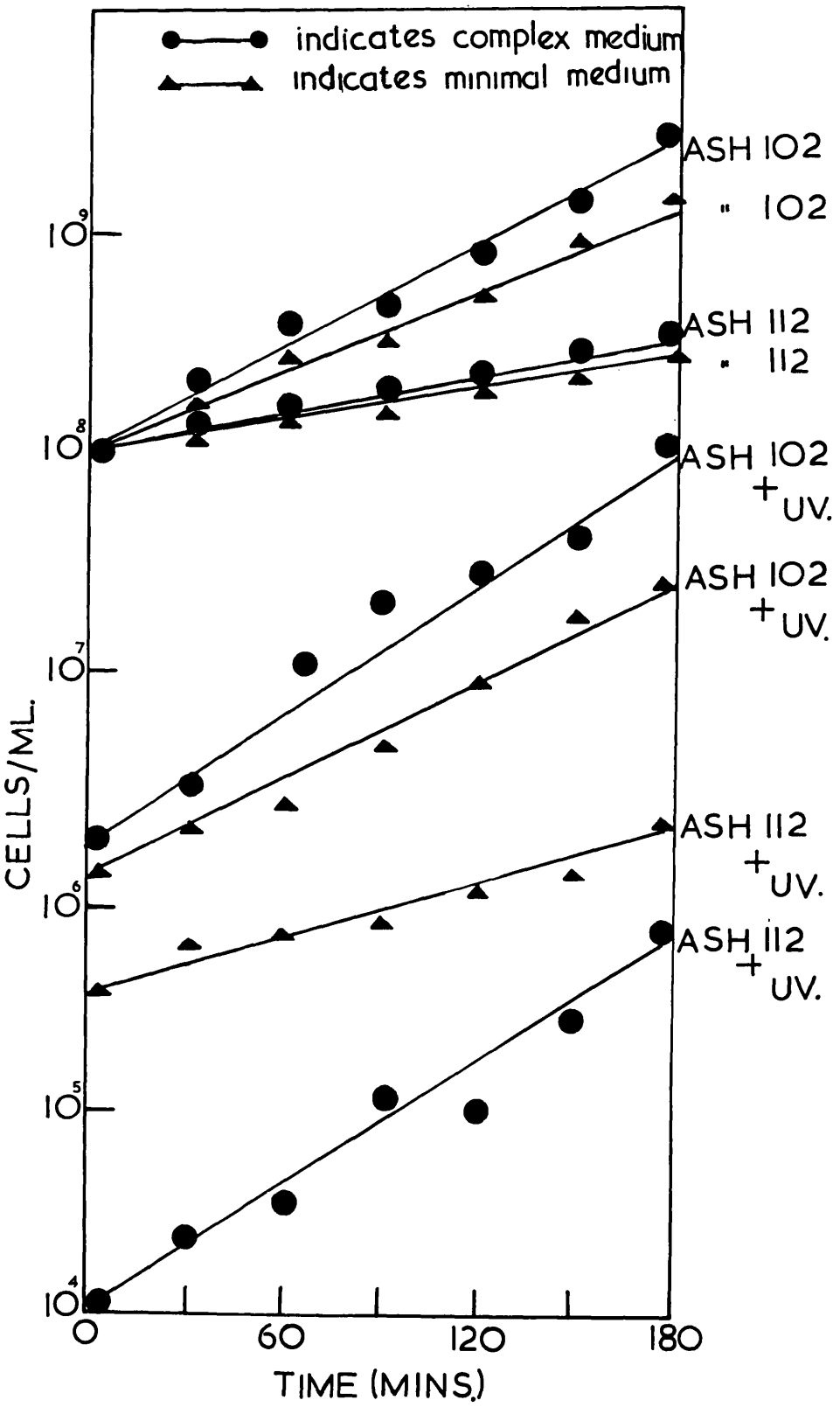
When loopfuls of cultures of Ref-II mutants were cross-streaked over colicins E2 and E3 on NB agar plates and incubated at 25°C and 40°C, only one out of 435 mutants tested (Section I) was refractory to colicin E2 at both temperatures. Additional Ref-II mutants,

however, displayed refractivity at both temperatures when tested in NB liquid culture whilst in contrast many Ref-II mutants largely lose their refractivity if grown in a minimal glucose medium (Holland, personal communication). Similarly, in this study, when cross streak tests were performed on minimal agar containing E2, all Ref-II mutants were found to be completely sensitive at both temperatures. The results indicate that colicin refractivity was dependent upon the physiological condition of the cells in some unknown way. Experiments were therefore initiated to determine the influence of external and internal physiological conditions on the concurrent UV sensitivity of some Ref-II mutants. Figure 5 shows the UV sensitivity of the Ref-II UV^r mutant ASH102 and the Ref-II UV^s mutant ASH112, grown in complex or minimal media. The effect of age of the cultures on UV senstivity was also investigated, and the results are also shown in Figure 5.

The decrease in UV sensitivity previously shown by the Ref-II UV^s mutant in stationary phase (Fig. 4) was confirmed, and was found to occur in minimal media as well as complex broth. However when grown in minimal medium, ASH112 displayed a 50-fold decrease in sensitivity whilst the sensitivity of ASH102 was the same in both media. This change in UV sensitivity with physiological conditions of ASH112 may well correspond to the similar shift in colicin E2 refractivity previously observed. These results do emphasise the importance of the maintenance of exact physiological conditions both in the expression of colicin E2 refractivity and UV sensitivity of certain Ref-II mutants.

Fig. 5. Influence of stage of growth, and growth medium on the UV sensitivity of certain Ref-II mutants

Cultures of the strains ASH102 (Ref-II UV^r) and ASH112 (Ref-II UV^s) at ca 5 x 10^8 cells/ml in minimal and complex medium were re-inoculated at an initial density of 10^8 cells/ml into fresh medium. Samples were removed at 30 minute intervals, plated on TB agar after appropriate dilution, and immediately irradiated for 40 secs; plates were incubated in darkness for 14 hours at 37°C and the survival fraction determined.


indicates complex medium
indicates minimal medium
ASH 102
" 102
ASH 112
" 112
ASH 102 + UV.
ASH 102 + UV.
ASH 112 + UV.
ASH 112 + UV.
CELLS/ML.
10^9
10^8
10^7
10^6
10^5
10^4
0
60
120
180
TIME (MINS.)

4. Influence of DL Pantoyl Lactone

Pantoyl lactone in the growth medium was first reported by Grula and Grula (1962) to reverse the inhibition of division produced by D-amino acids, penicillin or UV in a species of Erwinia. Subsequent observations by Van de Putte, Westenbroek and Rörsch (1963) and Donch, Green and Greenberg (1968) revealed that the addition of appropriate concentrations of pantoyl lactone to the medium after irradiation reduced the formation of filaments in the Fil^+ and Lon^- strains of E.coli, and therefore increased the survival of these strains to a level almost equal to that of the control wild-type strains. Pantoyl lactone is thought to increase the UV resistance of such Fil^+ and Lon^- by overcoming the failure of the cell division mechanism. Pantoyl lactone presumably does not have any effect on repair enzymes and consequently does not influence the sensitivity to UV shown by Uvr^- and Rec^- mutants. (Van de Putte, Westenbroek and Rörsch, 1963)

The effect of pantoyl lactone in the post-irradiation growth medium on the UV sensitivity of certain Ref-II UV^S mutants was therefore determined.

Cultures of various strains, including a UV^S RecB mutant, JC4457, were incubated on TB agar plates with and without pantoyl lactone, and the survival fraction counted after incubation. The results, shown in Figure 6, demonstrate as anticipated that pantoyl lactone does not influence the survival of the recB strain, JC4457. In contrast, two

out of three of the Ref-II UV^S strains tested did show significant recovery on TB plates supplemented with pantoyl lactone after a 40 second dose of UV. The survival of ASH112 was 6-fold enhanced but the effect was ~~not~~ most marked with ASH113, where surivival increased approximately 20-fold. However the filamentous Ref-II UV^S mutant ASH111 showed little or no pantoyl lactone recovery. Possibly the extreme cell division defect evident without irradiation in this strain (see Section II, growth characteristics) could not be retrieved with pantoyl lactone.

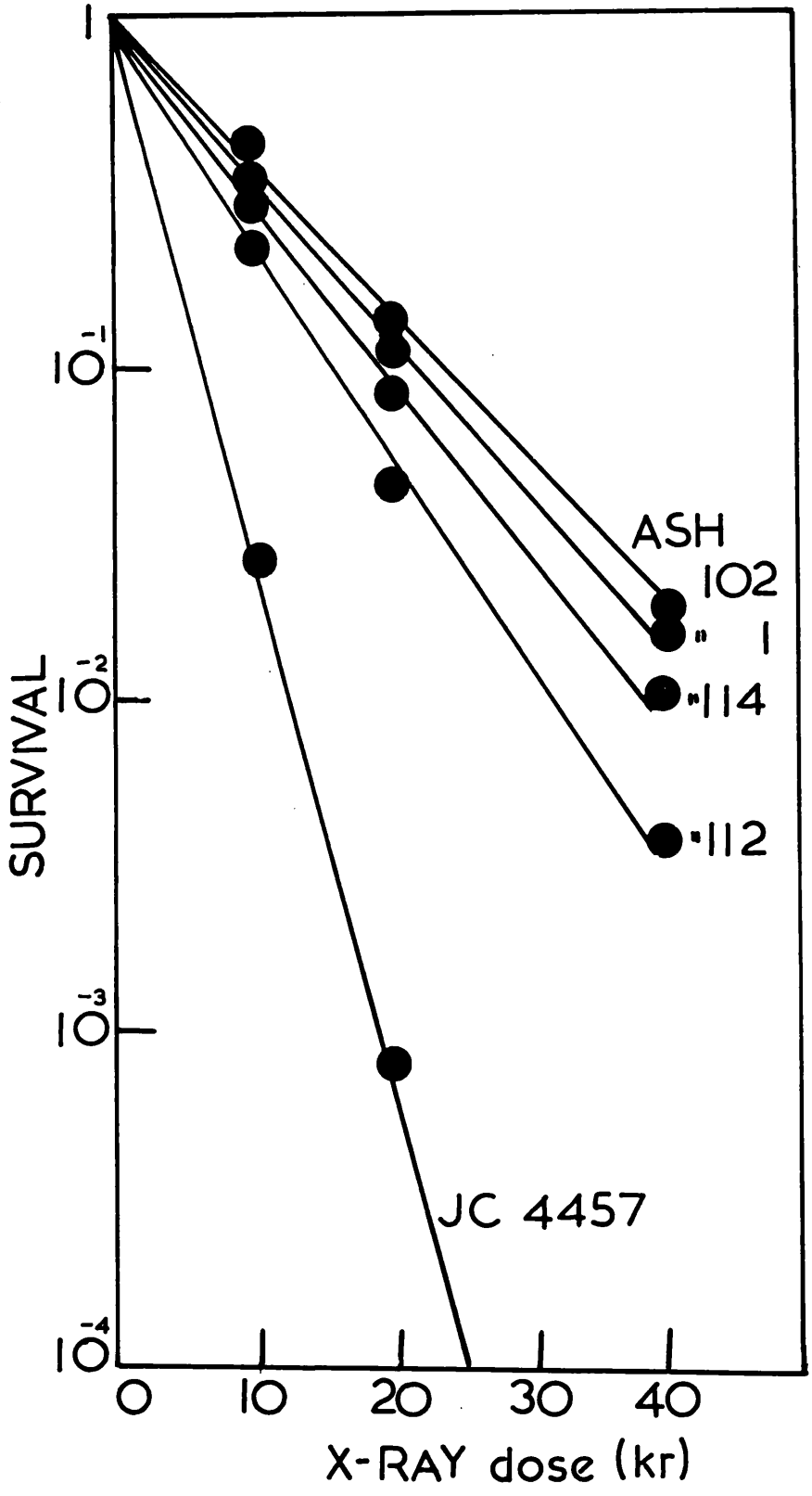
5. Quantitative Response of Ref-II Mutants to X-Irradiation

As will become evident from the genetic analysis of Ref-II mutants described in Section III, the majority of the Ref-II UV^S mutants produced significantly lowered numbers of recombinants after crosses with suitable male strains. Now recombination deficiency is a characteristic of certain previously isolated classes of UV sensitive mutants (Clark and Margulies, 1965), and such Rec^- mutants were found to be also sensitive to X-irradiation (Van de Putte, Zwenk and Rörsch, 1966). To compare the Ref-II UV^S mutants to this class of previously isolated UV^S Rec^- mutants, the X-ray sensitivity of certain Ref-II UV^S mutants and the *recB*$^-$ strain JC4457 was therefore determined and compared, and the results are shown in Figure 7.

It can be seen that in contrast to JC4457, the Ref-II UV^r strain and Ref-II UV^S strains were only slightly sensitive to X-irradiation,

Fig. 7. X-ray survival curves for Ref-II UV^s, Ref-II UV^r and a $recB^-$ UV^s strain

Strains were grown in TB to a density of ca 5 x 10^8 cells/ml, adjusted to a density of 10^8 cells/ml and X-irradiated as described in Methods, Section II. After subsequent dilution, 0.1 ml aliquots were plated on TB agar plates and incubated for 14 hr at 37°C.


1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
SURVIVAL
ASH
102
" 1
"114
"112
JC 4457
0
10
20
30
40
X-RAY dose (kr)

a further difference between Ref-II UV^S and conventional UV sensitive Rec mutants. This suggests that the repair defect in the RecB mutant does not correspond to that in the Ref-II UV^S mutant. This result, together with the pantoyl lactone recovery of UV irradiated Ref-II UV^S mutants, clearly distinguishes these mutants from the classes of UV^S recombination deficient mutants previously isolated.

B. Growth Characteristics of Ref-II Mutants

1. Growth Rates of Ref-II Mutants

During the course of genetical and physiological studies, it was noticed that certain Ref-II mutants had reduced rates of growth. Experiments were therefore initiated to investigate and quantify the growth rates of these mutants.

Initial studies were carried out using the Eel colorimeter to determine the optical density changes during growth. However inaccuracies in this method were such that it was possible only to differentiate the Ref-II mutants into two broad classes - those with a growth rate similar to that of the parental ref^+ strain, ASH10, and a minority with a reduced growth rate. Yet one significant fact did emerge - all the various Ref-II UV^S mutants fell into this latter category.

More accurate measurements of growth rates were obtained using a Gilford 300 microsample spectrophotometer. A typical Ref-II UV^r mutant ASH102, and the various Ref-II UV^S strains were grown in NB at 37°C

and the growth followed by OD changes. The results obtained are shown in Figure 8, and the growth rates calculated from these curves in Table 8.

From the curves obtained, two points are evident; not only did the cell "doubling time" of the Ref-II UV^S mutants show a significant increase over that of $\underline{ref}^+$ and Ref-II UV^r mutants (Table 8), but stationary phase was reached at a substantially lower cell density than with other strains. Thus the maximum cell density in terms of absolute cell number in the culture was 10^{10} cells/ml with ASH10 and ASH102, whereas with ASH112 this was reduced to 10^9 cells/ml. Mutation to colicin E2 refractivity may well then be partially lethal, at least in the case of Ref-I^I UV^S mutants, and as such is reflected by poor growth rates and depleted final cell concentrations of mutant cultures.

The growth rates of two strains, ASH111 and ASH115 were also studied in more detail, and growth followed by the determination of increases in both total and viable cell number and in addition to the mass increase (OD). Results are shown in Figure 9.

In the parental control ASH10 there was little discrepancy between total and viable cell count after 200 minutes (Fig. 9a). Presumably only a small proportion, less than 10% of the cells, were non-viable and the final turbidity of the culture was accurately reflected by the total number of cells present. In contrast, the two Ref-II UV^S mutants ASH111 and ASH115, showed very poor plating efficiencies (Fig. 9b) -

Fig. 8. Rate of growth of certain Ref-II Mutants

Exponential cultures of strains in NB at ca 5 x 10^8 cells/ml were adjusted to a density of 10^7 cells/ml; subsequent increases in turbidity with time were measured with the Gilford microsample spectrophotometer as described in Methods, Section II.

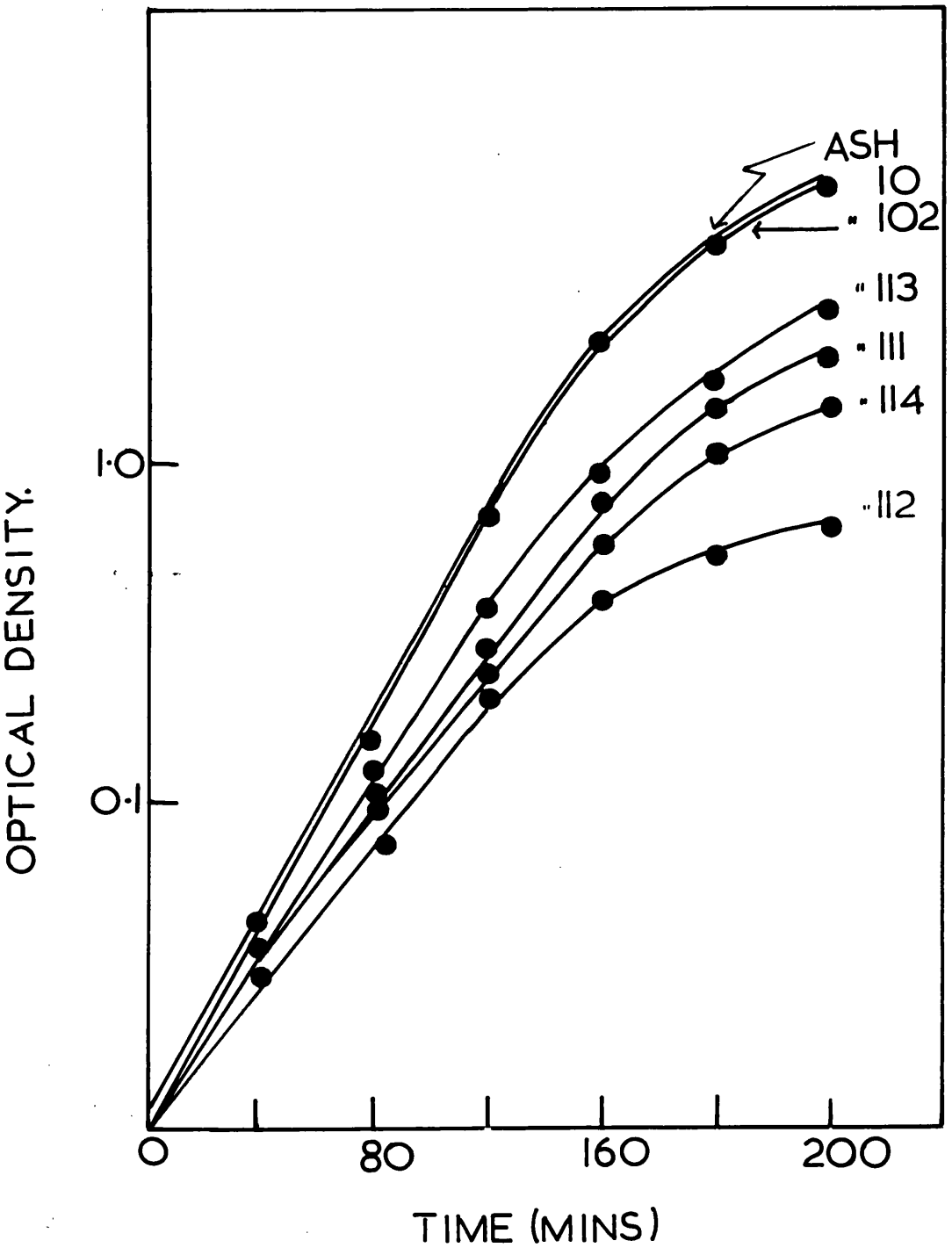

ASH
10
102
113
111
114
112
1·0
0·
OPTICAL DENSITY.
0
80
160
200
TIME (MINS)

TABLE 8

Mean Generation Time of Certain Ref-II Mutants in NB Culture

Strain	ref	Mean generation time[a] (min)
ASH10	ref$^+$	36
ASH102	Ref-II	36
ASH111	Ref-II UV^S	42
ASH112	Ref-II UV^S	60
ASH113	Ref-II UV^S	42
ASH114	Ref-II UV^S	45
ASH115	Ref-II UV^S	46

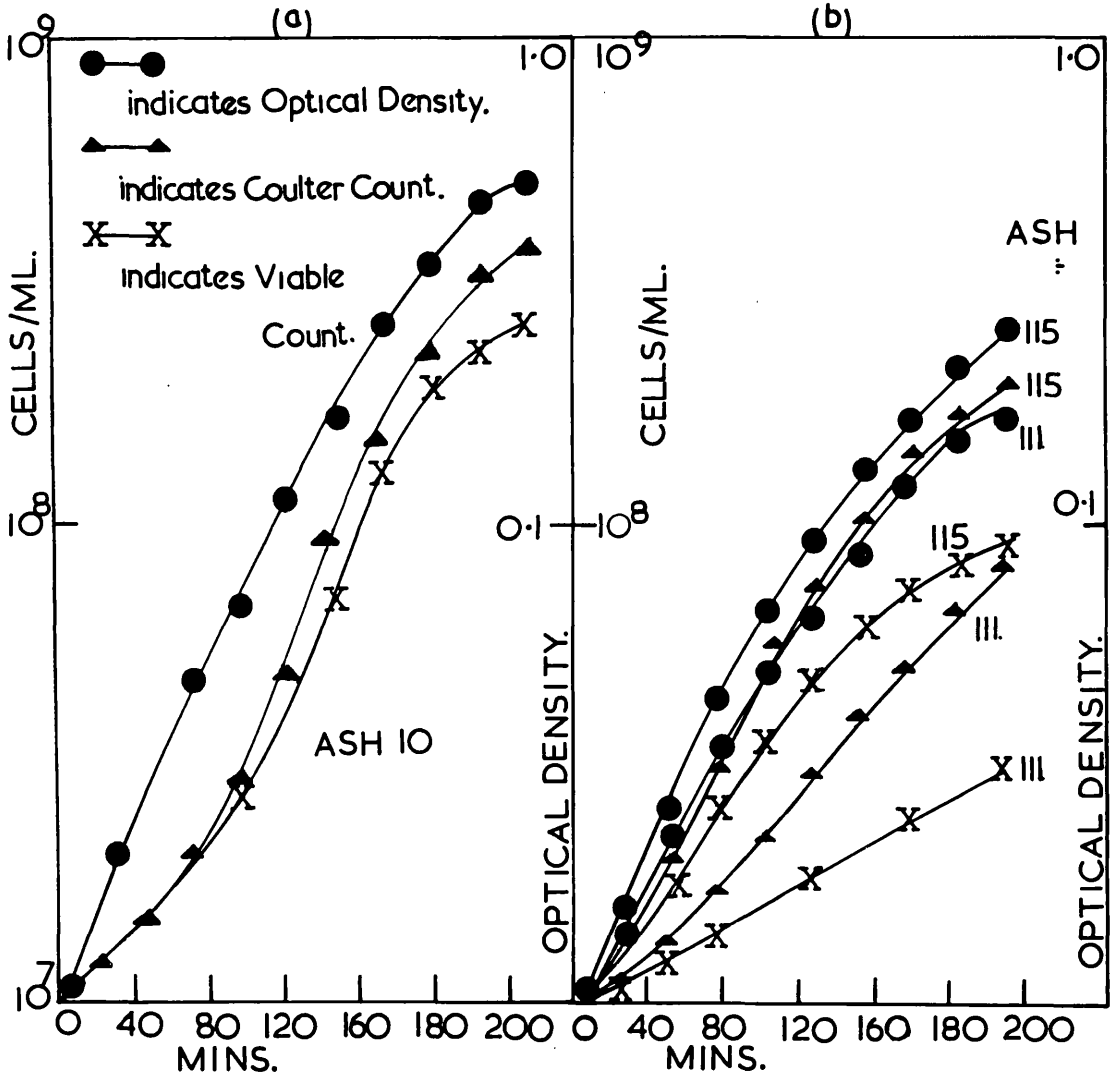
(a) cell generation time was calculated from optical density increase (Fig. 8) over a time interval from 20-80 minutes.

Fig. 9. Comparison of increase of turbidity and total cell count and viable cell count of:

a. ASH10 (ref^+)

b. ASH111 and ASH115 (Ref-II UV^S)

Strains were inoculated into NB at 37°C at an initial density of 10^7 cells/ml and grown with aeration. Turbidity was measured with a Gilford microsample spectrophotometer, total cell count with a Coulter Counter, and viable count by plating on NB agar after appropriate dilutions as described in Methods, Section II.


(a)
(b)
indicates Optical Density.
indicates Coulter Count.
indicates Viable Count.
ASH 10
ASH
115
115
111
115
111.
111
CELLS/ML.
OPTICAL DENSITY.
MINS.
0 40 80 120 160 180 200
1·0
0·1

only 40% and 20% respectively of the total cells present after 200 minutes actually gave rise to viable colonies. Furthermore, the plating efficiency of these mutants varied with the age of the culture, the cell viability apparently decreasing as cells aged. With ASH115, although there was good correlation between turbidity and total cell number, only one out of five stationary phase cells gave rise to viable colonies, indicative of either a high proportion of non-viable cells in the culture, or fragility of cells during dilution through phosphate buffer. A similar low plating efficiency was found with ASH111, with the viable count only 40% of the total count. In addition, in this mutant there was a marked discrepancy between turbidity and total cell count, suggestive of filament formation. The discrepancies between total and viable cell count in these Ref-II UV^S mutants is reminiscent of lethal sectoring displayed by RecA, B and C mutants (Haefner, 1968), and may well correspond to this character.

2. Microscopic Examination of Ref-II UV^S Mutants

From the growth rates of the Ref-II UV^S mutants, it was apparent that a number of mutants were defective in some aspect of growth. Furthermore, as the discrepancy between total and viable cell counts and the cell mass suggested morphological peculiarities in the mutants, phase examination of the mutants was initiated. Results were various; cultures of the Ref-II UV^S strain ASH111 particularly, and to a far lesser extent ASH116 and ASH112, contained numbers of filaments of up

to 30 μ in length (Plate 2), most evident when slides were prepared from colonies taken from solid NB agar (Plate 3). Stationary cultures of ASH111, 113, 114, 115, and to a lesser extent ASH112 also contained many cells showing clear indications of cell surface defects. Many free spheroplasts, cells showing large cytoplasmic protrusions and completely lysed cells were common, and examples are shown on Plate 2. Occasionally even branched cells were observed. Ref-II UV^r strains and RecA, B and C mutants similarly examined by phase contrast microscopy revealed no such defects.

Extensive filaments as found with ASH111 have also been observed in the UV^s Lon^- mutants after UV irradiation (Walker and Pardee, 1967; Donch, Green and Greenberg, 1968), presumably through inhibition of septum formation. An additional characteristic of Lon^- mutants is the formation of excess polysaccharide, particularly when cultures are grown on minimal agar (Donch and Greenberg, 1968). In addition a mutation affecting the cell envolope (envA) has been described by Normark, Boman and Matsson (1969) which also interferes with septum formation, and strains carrying such a mutation typically produce mucoid filamentous colonies on minimal agar. To test the possibility that the Ref-II UV^s strains were also defective in polysaccharide synthesis in this way, NB grown cultures of Ref-II UV^s strains, the Ref-II UV^r strain ASH102 and the parental ref^+ strain ASH10 were streaked onto minimal agar plates, and the morphological appearance of the bacterial

Plate 2 Phase Examination of ASH111

Magnification x 800. NB grown culture. Filament of c. 30 μ in right hand corner. Examples of cytoplasmic protrusions arrowed. Lysed cells and free spheroplasts present.

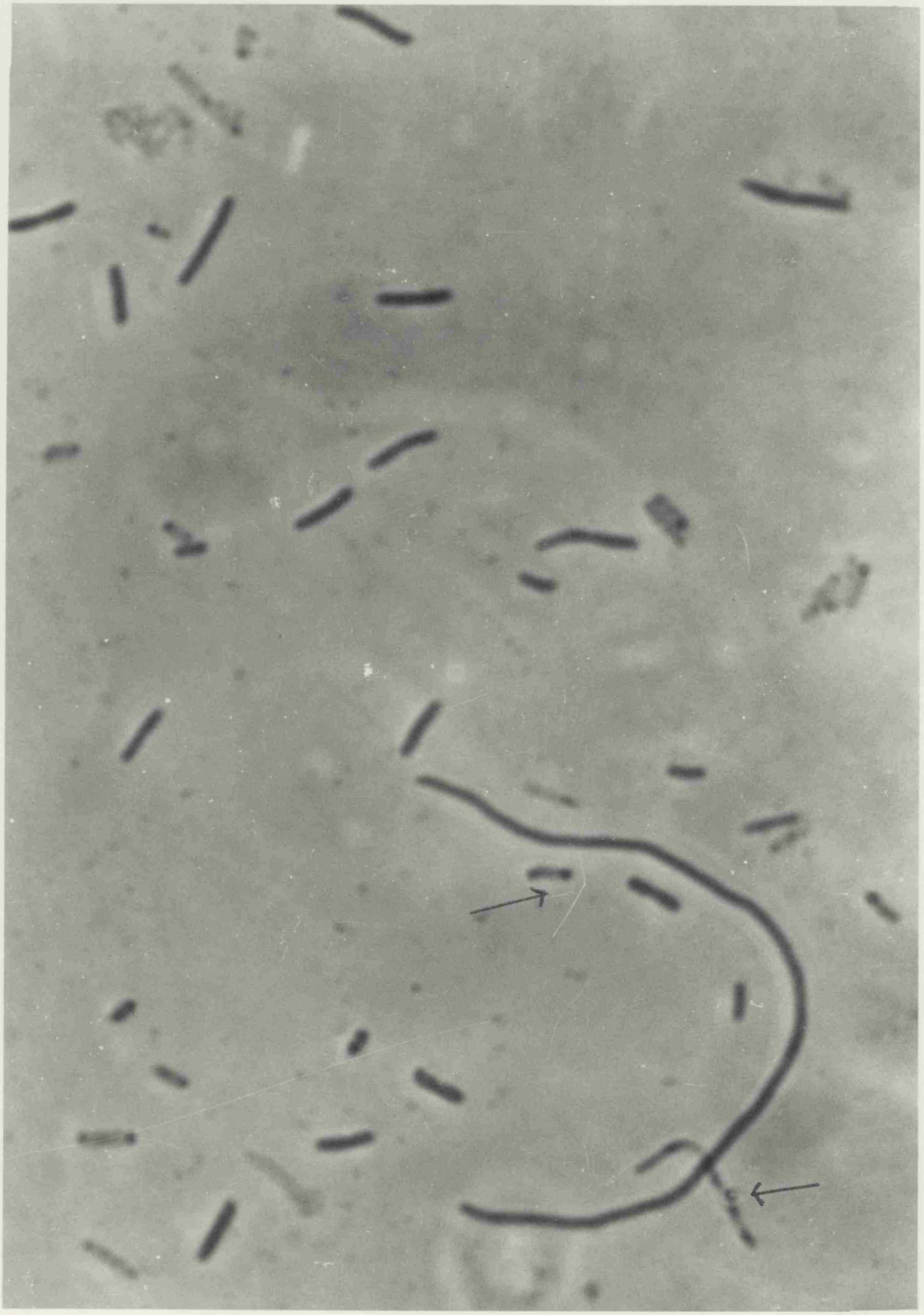
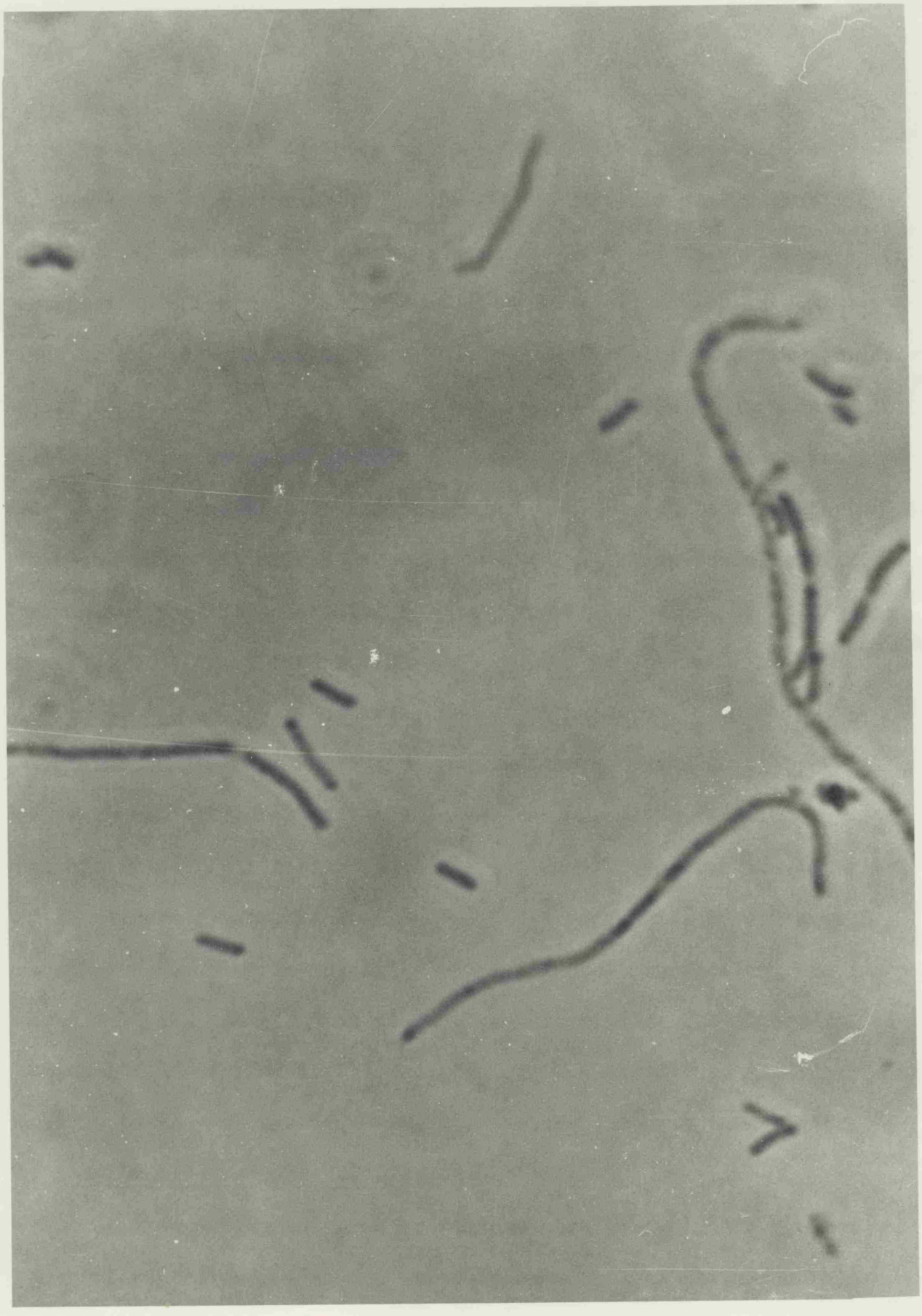


Plate 3 Phase Examination of ASH111

Magnification x 800. NB agar clone. Several filaments are present, all displaying cytoplasmic protrusions.



streaks was observed after 24 hours. The results, shown on Plate 4, demonstrated that most of the Ref-II UV^S mutants grow rather slowly, but abnormal polysaccharide synthesis could only clearly be ascribed to one strain, ASH116. When this strain was stained with Indian Ink and examined by phase contrast microscopy, the ink particles could be seen to define a diffuse but distinct capsule surrounding the cells. The property of capsule formation displayed by this strain, has not been investigated further, and it is not yet known whether this character has arisen directly from the mutation to colicin E2 refractivity or is associated with an independent mutation at, for example, the lon or envA loci.

C. Detergent Sensitivity of Ref-II Mutants

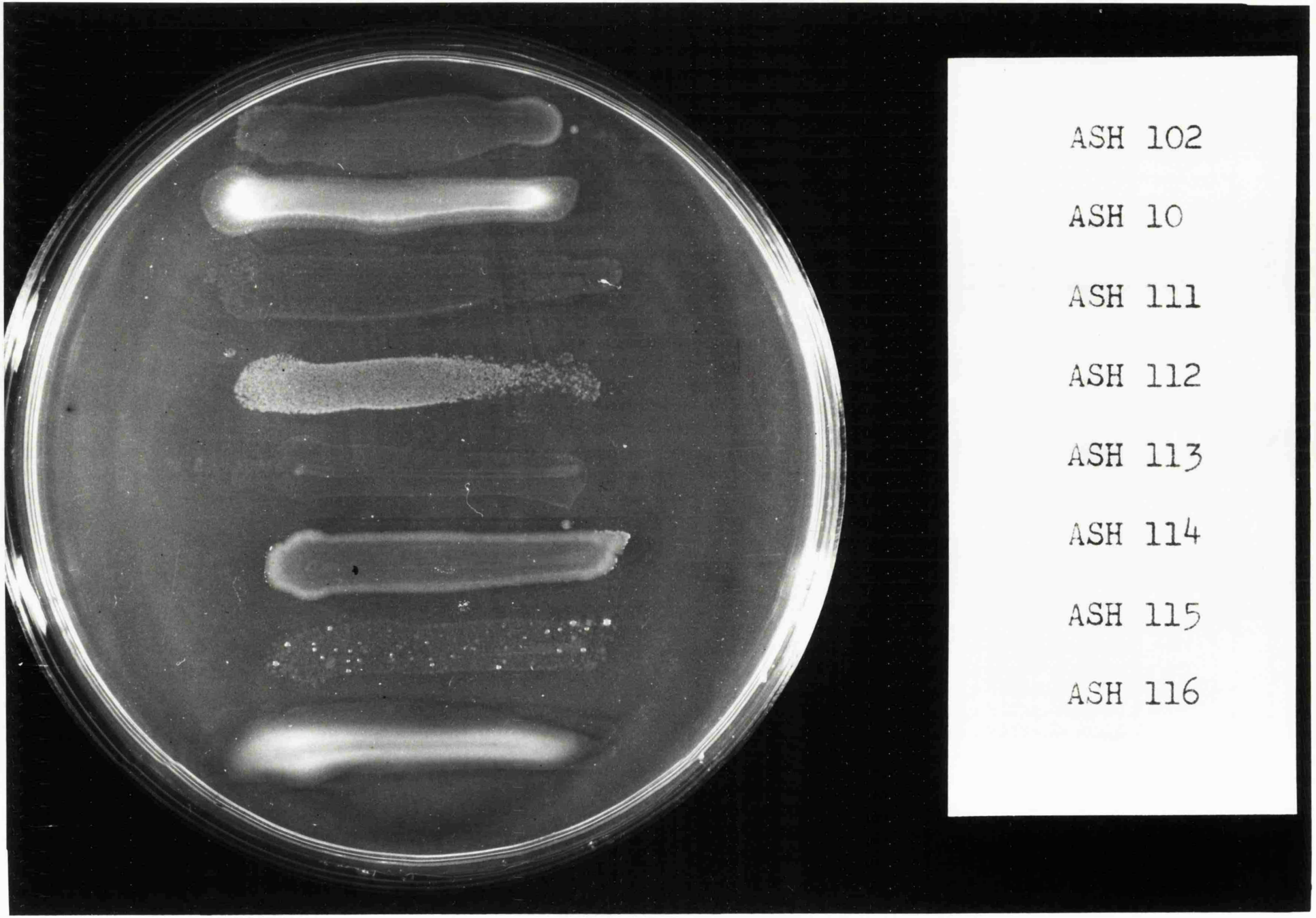
The poor growth rates and cell wall defects displayed by Ref-II UV^S mutants imply that at least in certain cases the mutation to colicin E2 refractivity is particularly lethal. However the majority of E2 refractory mutants isolated were not UV sensitive and growth rates were normal. To investigate possible cell surface changes in the Ref-II UV^S mutants, and to further characterise the altered components in the Ref-II UV^S mutants, the sensitivity of the various strains to the detergent sodium deoxycholate (DOC) was investigated.

1. Qualitative response to DOC

NB exponential cultures of various strains including two ref^+ parental control strains were streaked onto NB agar plates containing

Plate 4 Colonial Morphology of Ref-II UV^S Mutants

Late exponential cultures of ASH10 (*ref*$^+$), ASH102 (Ref-II UV^r) and the Ref-II UV^S mutants ASH111, 112, 113, 114, 115 and ASH116 streaked on minimal agar and incubated for 24 hours at 37°C. Note the weak growth of Ref-II UV^S mutants particularly, and the mucoid appearance of ASH116.


ASH 102
ASH 10
ASH 111
ASH 112
ASH 113
ASH 114
ASH 115
ASH 116

a range of DOC concentrations and plates were incubated at 25°C and 40°C for 24 and 14 hours respectively. The detergent sensitivity of the RecA and RecB strains rec34 and JC4457, the Ref-IIA strain 4K-T$^+$ and the ref$^+$ strains 4K and C600 was similarly tested.

As may be seen from Table 9, all Ref-II UV^s strains displayed a marked sensitivity to DOC in comparison to the Ref-II UV^r and parental ref$^+$ strains. However almost all Ref-II UV^r strains were sensitive to 2.5% DOC, in contrast to the Rec strains, the Ref-IIA refB$^+$ strain 4K-T$^+$, and to the wild-type strains. In no case did the response of any strain differ with temperature. Thus sensitivity to DOC, like sensitivity to UV irradiation but unlike refractivity to colicin E2, was not temperature dependent.

2. Quantitative response to DOC

The response of the Ref-II UV^s strains to DOC was also determined quantitatively at both 25°C and 40°C. To ascertain the influence, if any, of the Ref-IIA mutation on DOC sensitivity, the responses of the Ref-IIA refB$^+$ strain 4K, and the refA$^+$ refB$^+$ strains C600 and 4K were also examined at the two temperatures (Figure 11).

The results shown in Figures 10 and 11 confirmed the results obtained from the initial screening of the mutants (Table 9). The sensitivity of all the Ref-II UV^s mutants was considerably greater than that displayed by the Ref-II UV^r mutant ASH102, and the parental controls, ASH1 and ASH10. Furthermore, the quantitative responses of

Exponential cultures of bacterial strains were streaked on duplicate NB agar plates containing 2.5, 1.0, 0.5, 0.1 and 0% DOC. One set of plates was incubated at 25°C for 24 hours, one at 40°C for 14 hours, and sensitivity recorded in terms of +/- growth.

TABLE 9

The Response of Certain Strains of E.coli K12 to Various Concentrations of Sodium Deoxycholate (DOC) at 25° and 40°C

Strain	ref genotype	Response to UV	Response to DOC (concentration (%))				
			2.5	1.0	0.5	0.1	0
ASH1	refIIA $refB^+$	R	R	R	R	R	R
ASH10	refIIA $refB^+$	R	S	R	R	R	R
ASH102	refIIA refIIB	R	S	R	R	R	R
ASH112	ditto	R	S	S	S	S	R
ASH113	ditto	S	S	S	S	R	R
ASH115	ditto	S	S	S	S	R	R
ASH116	ditto	S	S	S	R	R	R
ASH111	ditto	S	S	S	S	R	R
ASH114	ditto	S	S	S	S	R	R
ASH50	ditto	R	S	R	R	R	R
ASH51	ditto	R	S	R	R	R	R
ASH62	ditto	R	R	R	R	R	R
ASH63	ditto	R	S	R	R	R	R
ASH64	ditto	R	R	R	R	R	R
ASH66	ditto	R	S	R	R	R	R
ASH67	ditto	R	S	R	R	R	R
ASH80	ditto	R	S	R	R	R	R
rec34	$refA^+$ $refB^+$	S	R	R	R	R	R
JC4457	$refA^+$ $refB^+$	S	R	R	R	R	R
C600	$refA^+$ $refB^+$	R	R	R	R	R	R
4K	$refA^+$ $refB^+$	R	R	R	R	R	R
4K-T^+	refIIA $refB^+$	R	R	R	R	R	R

Fig. 10 The DOC sensitivity of certain Ref-II UV^r and Ref-II UV^s strains at 25°C

Exponential cultures of various strains, in NB at 37°C at ca 5 x 10^8 cells/ml were adjusted to 10^8 cells/ ml and plated after appropriate dilutions onto NB agar plates containing various concentrations of DOC. Plates were incubated at 25°C for 24 hours and the survival fraction counted.

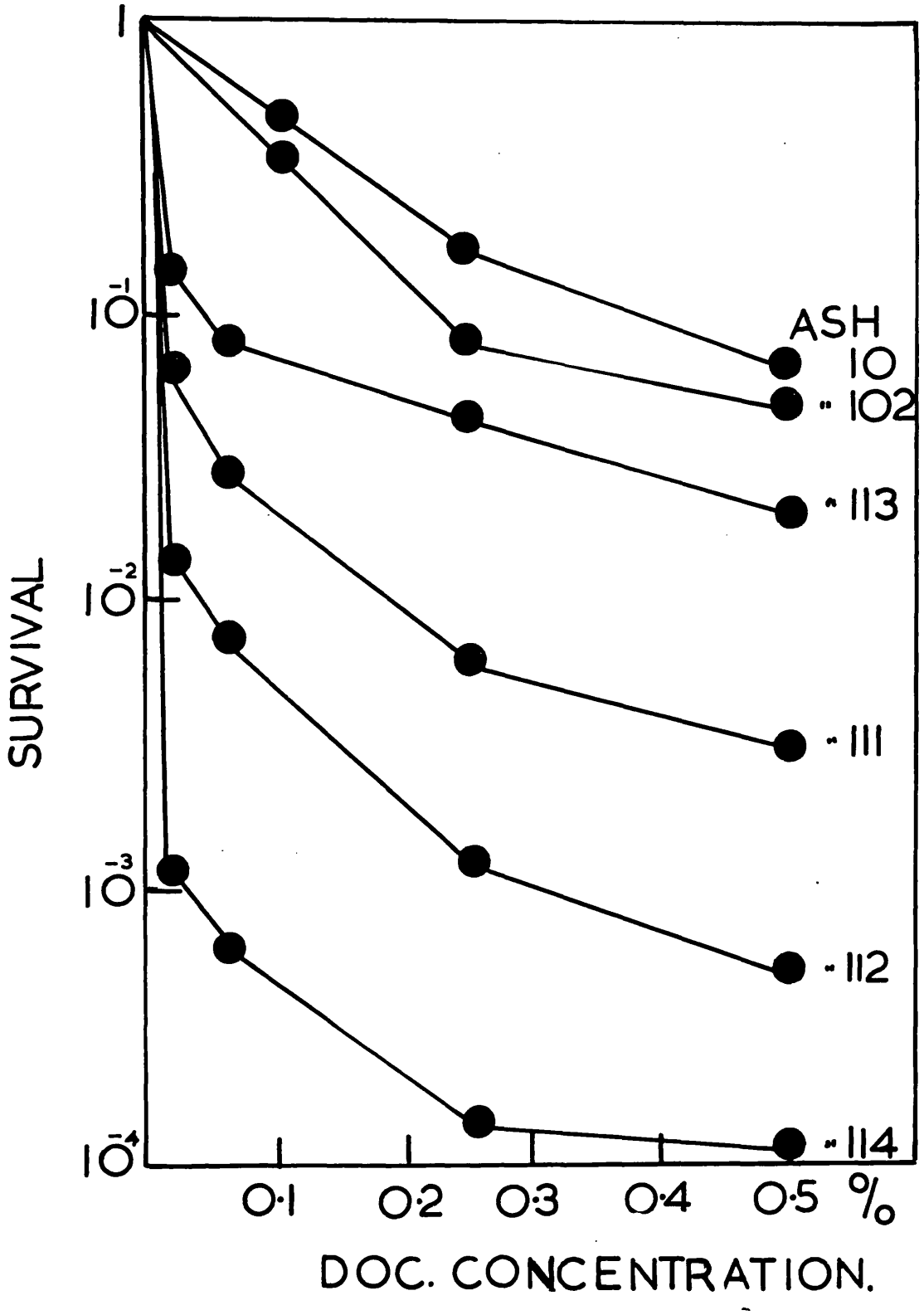
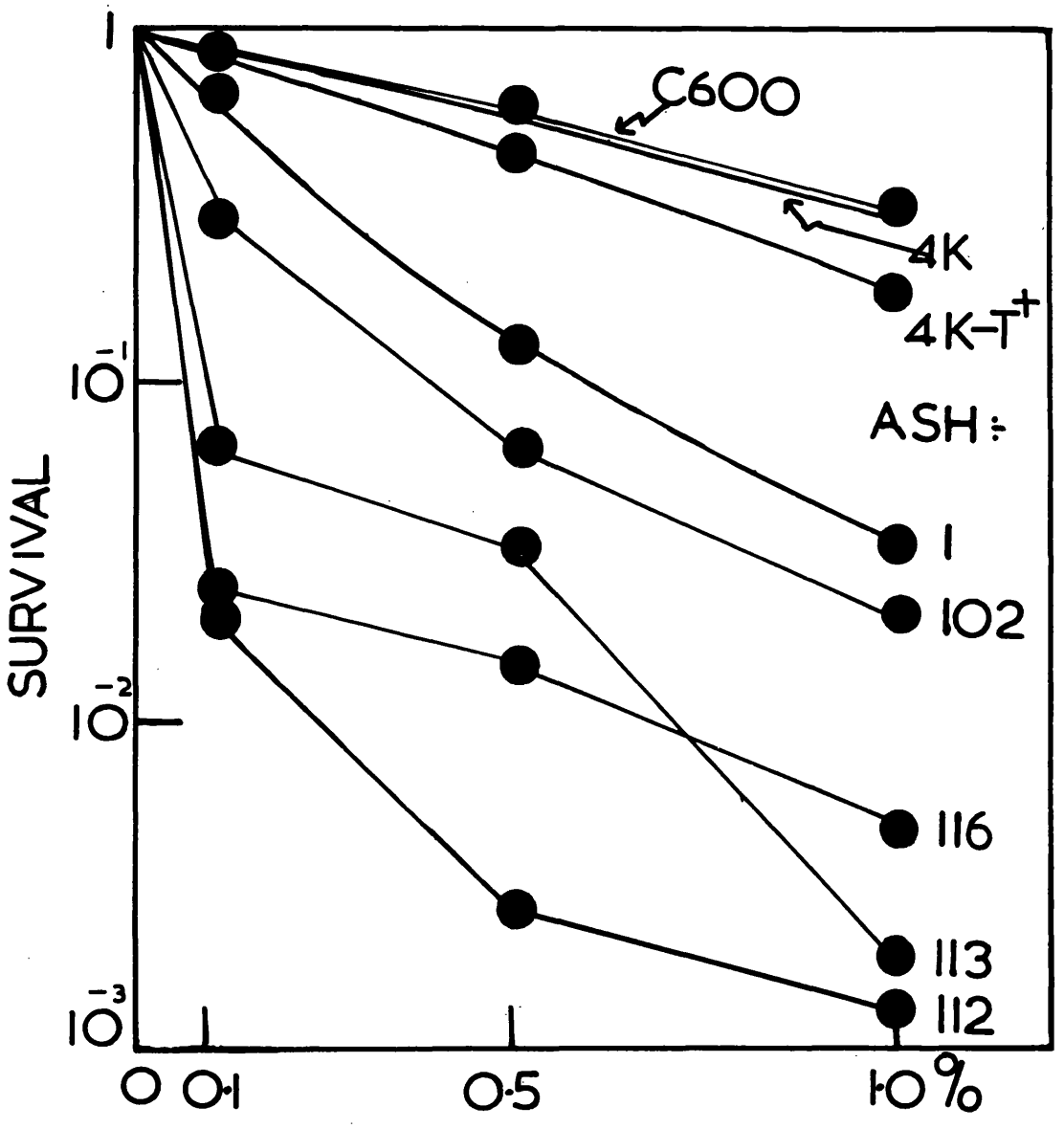

1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
SURVIVAL
ASH
10
102
113
111
112
114
0·1
0·2
0·3
0·4
0·5
%
DOC. CONCENTRATION.

Fig. 11 The DOC sensitivity of certain Ref-II UV^r and Ref-II UV^s strains at 37^oC

Procedure as in Figure 10, except that cells were incubated at 37^o after plating.


1
10^{-1}
10^{-2}
10^{-3}
SURVIVAL
C600
4K
4K-T+
ASH÷
1
102
116
113
112
0
0·1
0·5
1·0%
D.O.C. CONCENTRATION

the Ref-II UV^S mutants to DOC were all dissimilar, but did not correlate in any simple way with any similar diversity shown in their response to UV or in their growth rates in complex media. No differences were evident after incubation at the two temperatures.

The DOC sensitivity of ASH102 was not significantly greater than the refB$^+$ parental strains ASH1 and ASH10. Similarly, the sensitivity of the refIIA refB$^+$ strain, 4K-T$^+$, and the refA$^+$ refB$^+$ strains 4K and C600 were all virtually identical (Fig. 11). However the DOC sensitivity of the refIIA refB$^+$strains ASH1 and ASH10 was significantly greater than that of C600, 4K and 4K-T$^+$. This result may imply that although the refIIB mutation had no demonstrable effect on detergent sensitivity, ASH10 and derivatives of ASH10 may have contained some additional cell surface property which led to an increased sensitivity to DOC.

A point of interest was also provided by the biphasic nature of the survival curves, particularly apparent with the Ref-II UV^S mutants. A high initial rate of kill with low concentrations of DOC of up to 0.1% was not continued with increase of concentration to 1.0%. Indeed, after this initial phase, the rate of kill was approximately equal for Ref-II UV^S, Ref-II UV^r and RefIIA refB$^+$ parental strains.

D. Prophage Induction and Phage Sensitivity in Ref-II Mutants

1. Prophage λ Induction

Despite Ref-II UV^S mutants differing from Rec$^-$ UV^S mutants in

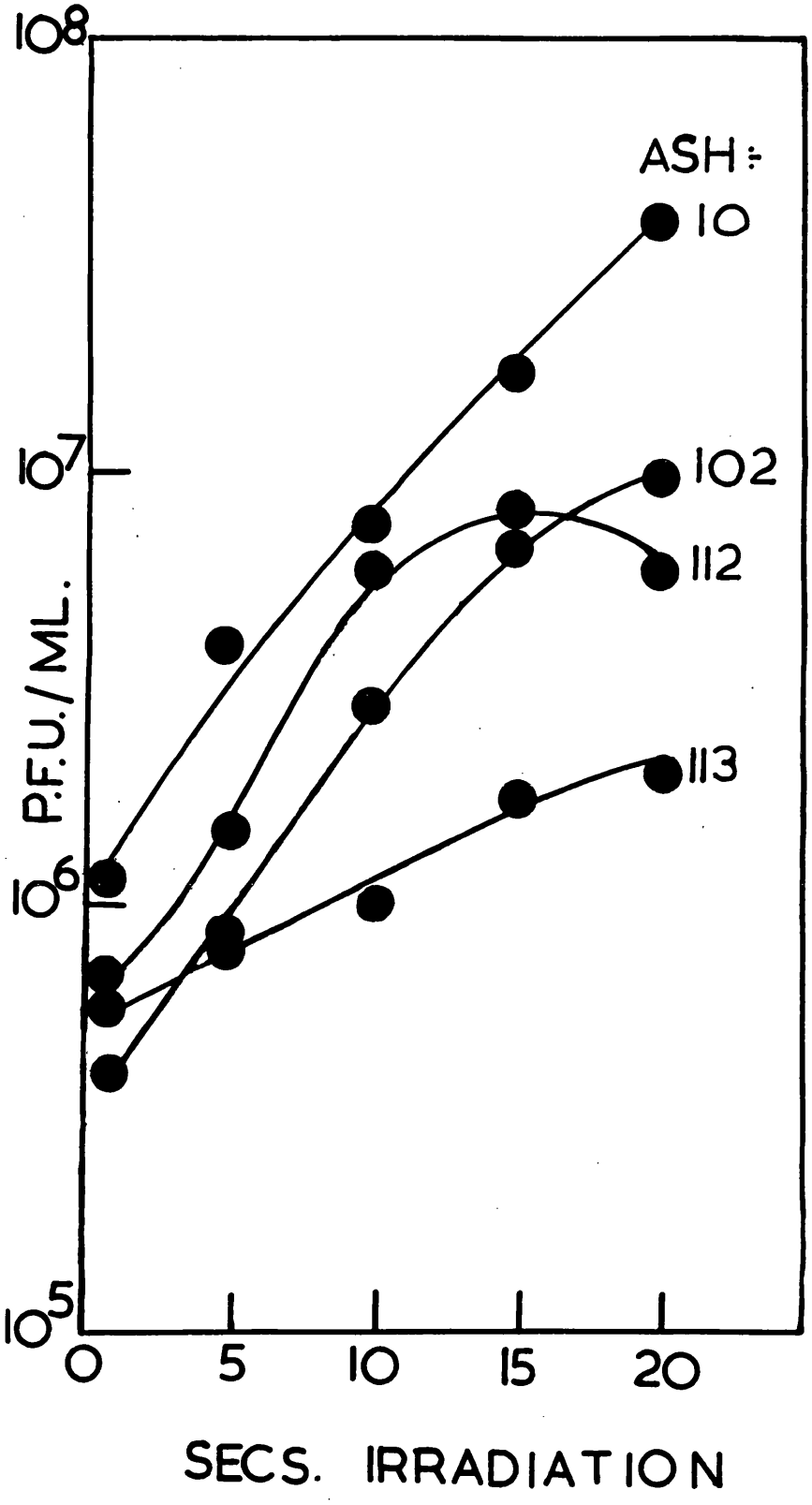
several ways, genetical studies described in Section III do demonstrate however, that Ref-II UV^s mutants may also be recombination deficient.

A characteristic of some RecA mutants, for example the lysogenic rec34 and rec36 strains of Van de Putte, Zwenk and Rörsch (1966), is an inability to induce prophage λ after UV irradiation. These mutants also only had an extremely low spontaneous λ induction frequency, 10^{-4} to 10^{-6} below that of the wild type. A further RecA mutant isolated by this group, rec35 and the RecC mutant, rec38, also had spontaneous levels of λ induction 10 to 100-fold lower than parental strains, although UV induction of λ in these mutants was similar to the control rec^+ strain. To further compare the Ref-II UV^s mutants with these Rec mutants, the prophage λ induction patterns of certain Ref-II UV^r and Ref-II UV^s mutants were therefore determined.

Stationary phase TB cultures of lysogenic Ref-II mutants and the ref^+ parental strain ASH10 were centrifuged, resuspended in buffer and irradiated for varying time intervals as described in Methods, Section II. These were again centrifuged, and resuspended in TB for a further 60 minutes incubation in TB at 37°C to allow liberation of free phage particles into the culture supernatant. These were assayed using the λ^- λ^s strain ASH1 as indicator, and the prophage induction curves obtained, presented in Figure 12, showed little difference either in the spontaneous level of induction, or in the UV induced induction between ASH10, ASH102 and ASH112. Although prophage induction

Fig. 12 The induction of prophage λ in Ref-II UV^s and Ref-II UV^r mutants by UV-irradiation

UV irradiation in buffer was as described in Methods, Section II. Indicator strain ASH1 (λ^- λ^s).


10^8
10^7
10^6
10^5
P.F.U./ML.
ASH:
10
102
112
113
0
5
10
15
20
SECS. IRRADIATION

in ASH113 was slightly reduced, it was not similar to the induction patterns displayed by the RecA mutants, rec34 and rec 36 (Van de Putte, Zwenk and Rörsch, 1966).

Thus although tests carried out on the prophage induction pattern of Ref-II mutants were limited to two Ref-II UV^S and one Ref-II UV^r strain, results indicated no real difference between these strains and the parental control. This property further distinguished these Ref-II UV^S mutants at least from RecA mutants.

2. Phage Sensitivity

(a) Sensitivity to phages λ and P1

The replication of λ and ØX174 DNA has been reported to take place at specific membrane sites (Knippers and Sinsheimer, 1968; Salivar and Sinsheimer, 1969). In addition, Denhardt, Dressler and Hathaway (1967) have suggested that UV^S Rep^- mutants are deficient in the ability to replicate ØX174 DNA due to alteration in some membrane component to which the ØX174 genome is attached in the cell as a pre-requisite to replication. Another notable property of these Rep^- mutants is that they also appear to be Rec^-. Since the mutation to colicin E2 refractivity might involve alteration in some cell membrane component, and that such mutants can also be Rec^- (Section III), it was thought possible that some Ref-II mutants might display a reduced ability to support the growth of, for example, bacteriophage λ. The sensitivity of various Ref-II and Ref-II UV^S mutants to a virulent

mutant of λ, λgv, was therefore determined.

Loopfuls of exponential cultures (ca 5 x 10^8 cells/ml) of 30 Ref-II and Ref-II UV^S mutants were therefore spotted onto NB agar plates and allowed to dry. These were half-overlayered with different dilutions of the phage λgv as described in Methods, Section II, and incubated. Three mutants ASH112, 114 and 116 did in fact display a significant degree of resistance to the phage. These and other Ref-II UV^S mutants were then selected for further quantitative study of their ability to support the growth of λ gv. At the same time, since the efficiency of plating of phage P1 on certain Rec mutants had been reported to be reduced by a factor of about 350 (Hertman and Luria, 1967; Emmerson and Howard-Flanders, 1967), the plating efficiency of this phage on Ref-II UV^S mutants was also investigated. In all, 30 Ref-II mutants were tested (Table 10), and in the Ref-II UV^r mutants the efficiency of plating of λgv was no different than on wild type strains. The "resistance" of the Ref-II UV^S mutants ASH112, 114 and 116 to λgv , found in the initial screening of Ref-II mutants was, however, confirmed. Plating efficiencies in these strains were in fact reduced by factors ranging from 2 x 10^{-3} to 4 x 10^{-3}. In contrast, in the majority of Ref-II UV^S mutants investigated, the plating efficiency of the transducing phage P1 was only reduced 10 to 50-fold.

As many of the Ref-II UV^S mutants had been observed to contain morphological cell surface defects (see Growth Characteristics, Section

TABLE 10

Plating Efficiencies of the Bacteriophages λgv and P1 on Ref-II UV^s Mutants

Bacterial Host	Plating Efficiency	
	λgv	P1
ASH1 (*ref*$^+$ UV^r)	1	1
ASH10 (*ref*$^+$ UV^r)	1	1
ASH102 (Ref-II UV^r)	1	1
ASH111 (Ref-II UV^s)	1	2×10^{-2}
ASH112 (Ref-II UV^s)	4×10^{-3}	1.7×10^{-1}
ASH113 (Ref-II UV^s)	1	1.4×10^{-1}
ASH114 (Ref-II UV^s)	3×10^{-3}	4.0×10^{-2}
ASH115 (Ref-II UV^s)	1	1.2×10^{-1}
ASH116 (Ref-II UV^s)	2×10^{-3}	1.0×10^{-1}

Efficiency of plating of λgv and P1 on *ref*$^+$, Ref-II and Ref-II UV^s mutants. Plating efficiencies shown are relative to those obtained with the parental *ref*$^+$ strains ASH1 and ASH10. Procedure as described in Methods, Section II.

II), and in the case of ASH116 to exhibit a mucoid colonial morphology particularly after growth on minimal agar, it was thought that the adsorption of λgv to cell wall receptors may have been impaired in some way in these mutants. The ability of the various Ref-II UV^S mutants to adsorb λgv was therefore compared with that of a conventional Ref-II mutant, and that of the parental ref^+ strains. Results (Table 10a) showed that with the exception of ASH116, adsorption capacities of the Ref-II UV^S mutants and the control strains were in fact virtually identical.

Although the adsorption of λgv to ASH116 is reduced by approximately 90%, this should still not be sufficient to account for the reduced plating efficiency of λgv on this strain. "Resistance" may well therefore arise from a mechanism identical to that of the two other "resistant" mutants, ASH112 and ASH114, and not be correlated with the formation of a mucoid capsule by this mutant.

It is unlikely that this "resistance" to λgv displayed by ASH112, 114 and 116 is associated with a prophage immunity mechanism, since although ASH112 and ASH116 are lysogenic for λ, ASH114 is non-lsyogenic. However bacteriophage λ "resistance" in E.coli could also arise through an alteration in the restriction-modification system of the host cell. Genes responsible for the control of such host specificity mechanisms are closely linked to the refIIB locus, as will become evident in the genetical analysis described in Section III. It was therefore decided

TABLE 10a

Adsorption of Bacteriophage λgv to Ref-II UV^r and Ref-II UV^s Mutants

Bacterial Strain	Phage Titre after adsorption
ASH1 (*ref*$^+$)	6.0×10^6
ASH102 (Ref-II UV^r)	4.2×10^6
ASH111 (Ref-II UV^s)	7.4×10^6
ASH112 (Ref-II UV^s)	1.8×10^7
ASH113 (Ref-II UV^s)	5.4×10^6
ASH114 (Ref-II UV^s)	6.1×10^6
ASH116 (Ref-II UV^s)	6.1×10^7
S.typhimurium 906	3.0×10^8

Adsorption of phage λ*gv* to Ref-II, Ref-II UV^s and *ref*$^+$ strains, and *Salmonella typhimurium* 906 (λ^r). Equal multiplicites of phage and bacterial cultures mixed, and free phage λ*gv* assayed after adsorption on the λ^- λ^s indicator strain, ASH1, as described in Methods, Section II.

to test the possibility that the bacteriophage λ "resistance" displayed by ASH112, 114 and 116 was due to a mutation affecting the restriction system.

For this purpose, a single plaque of bacteriophage λgv obtained after growth through the λ "resistant" mutant, ASH114, was mixed with a fresh culture of ASH114, and a lysate of λgv.ASH114 so prepared. The efficiency of plating of λgv.ASH114 on wild type and Ref-II mutant strains was then determined, and compared with that of a preparation of λgv grown through the λ sensitive ref$^+$ strain, ASH1. Results, shown in Table 11 indicate that the virus λgv.ASH114, obtained at low frequency after the growth of λgv wild type on ASH114, would, when used to re-infect ASH114 again, now grow normally on this strain. In contrast, a single plaque of λgv grown through the wild type host, ASH1, still exhibited a reduced efficiency of plating on the Ref-II UV^S mutant, ASH114. The new property of λgv.ASH114 was, however, not a transient modification which could be lost on re-cycling phage λgv.ASH114 through the ref$^+$ wild type host, ASH1. Thus λgv.ASH114 appears to have acquired a mutational change which allows it to overcome the "resistance" of the host ASH114 to λ, but which does not affect its ability to grow on wild type, ref$^+$ hosts.

TABLE 11

Plating Efficiencies of Bacteriophage λgv after Growth through ref$^+$ and Ref-II Strains

Phage	Bacterial Host	Plating Efficiency
λgv.ASH1	ASH1 (ref$^+$)	1
	ASH114 (Ref-II UV^S)	3×10^{-3}
λgv.ASH114	ASH1 (ref$^+$)	1
	ASH114 (Ref-II UV^S)	1

Phage λgv added to stationary phase cultures of bacterial strains at a multiplicity of 10. Plating efficiencies relative to that obtained with phage λgv.ASH1 on the host strain, ASH1.

Discussion and Conclusions

Colicin E2 refractory mutants isolated by Hill and Holland (1967) and in this study may be divided into two major phenotypic classes on the basis of their response to UV irradiation. The majority of Ref-II mutants displayed normal sensitivity when exposed to UV, but 7 out of 22 did show significantly increased sensitivity.

Of this latter class, all tested were found to be abnormally sensitive to the detergent sodium deoxycholate, and showed reduced growth rates in complex media. On further investigation a small number proved to have additional pleiotropic characters. Thus out of the 7 Ref-II UV^S mutants tested, 5 were deficient in the formation of prototrophic recombinants after crosses with male strains, as will be shown in Section III. One mutant particularly grew in characteristically long filaments of up to 30 μ in length, and three had greatly reduced capacities to support the growth of the bacteriophage λ. Two out of three of the latter strains were also distinguished by refractivity to colicin E2 at 37°C when grown and tested in NB culture (Holland, personal communication). The third bacteriophage λ "resistant" strain also produced a mucoid capsule when grown on minimal media. Furthermore this mutant, although Rec^+ in crosses with HfrH male strains appeared to have a reduced capacity to either accept or replicate episomal DNA. Finally, most Ref-II UV^S mutants, when subjected to microscopic examination, displayed indications of cell wall defects,

and free spheroplasts, lysed cells, cells with extrusions of spheroplasts, and even branched cells were evident.

The pleiotropic characters displayed by different Ref-II UV^S mutants are summarised in Table 12.

Ref-II UV^S mutants differ from all UV^S mutants previously isolated in several ways. Thus although Holland (1967) has reported that these mutants resembled Rec^- rather than Uvr^- mutants in the reactivation of irradiated phage (Howard-Flanders and Boyce, 1966), in this study Ref-II UV^S mutants were found to be quantitatively less sensitive to UV than Rec^- mutants,(see Howard-Flanders and Theriot, 1966b, Van de Putte, Zwenk and Rörsch, 1966). Nevertheless, the majority of the Ref-II UV^S mutants so far tested did show recombination deficiency, as will become evident in Section III. The UV sensitivity of Ref-II UV^S mutants was in fact similar to that reported by Donch and Greenberg (1968) for Lon^- mutants, and by Denhardt, Dressler and Hathaway (1967) for Rep^- mutants. The Ref-II UV^S mutants further resembled Lon^- mutants, but not Rec^- mutants, by exhibiting only a slight sensitivity to X-irradiation, and one, possibly two Ref-II UV^S mutants out of three tested were also capable of a significant degree of UV irradiation recovery when plated on agar supplemented with pantoyl lactone. In Lon^- strains, pantoyl lactone appears to promote the ability of UV irradiated cells to divide by the induction of septum formation (Donch, Green and Greenberg, 1968) and it appears plausible that the UV sensi-

TABLE

Pleiotropic Characters Associated

	Response to UV		Response to DOC		Response to E2 (plates)		Response to E2 (liquid)(c)	
	25°	37°	25°	37°	25°	37°	25°	37°
ASH1	R	R	R	R	S	S	S	S
ASH10	R	R	R	R	S	S	S	S
ASH102	R	R	R	R	R	S	R	S
ASH111	S	S	S	S	R	S	R	S
ASH112	S	S	S	S	R	S	R	R
ASH113	S	S	S	S	R	S	R	S
ASH114	S	S	S	S	R	R	R	R
ASH115	S	S	S	S	R	S	R	S
ASH116	S	S	S	S	R	S	R	S
rec34	S	S	R	R	S	S	S	S
JC4457	S	S	R	R	S	S	S	S

(c) the response to colicin E2 when in liquid culture at 25° and 37° was provided by I.B. Holland (personal communication)

12

with Colicin E2 Refractivity

Recomb^{n}(a) proficiency	Filament$_{n}$ form (+/-)	Capsule$_{n}$ form	X-ray sensitivity	Pantoyl lactone recovery	Resistance to λgv	Pl e.o.p.	Ability to receive episomes(b) (+/-)
rec^{+}	-	-	R	not tested	S	1	+
rec^{+}	-	-	R	"	S	1	+
rec^{+}	-	-	R	"	S	1	+
rec^{-}	+	-	R	+	S	1	not tested
rec^{-}	-	-	R	+	R	1.7×10^{-1}	+
rec^{-}	-	-	R	+	S	1.4×10^{-1}	+
rec^{-}	-	-	R	not tested	R	4×10^{-2}	not tested
rec^{+}	-	-	not tested	"	S	1.2×10^{-1}	+
rec^{+}	-	+	"	"	S	1.0×10^{-1}	-
rec^{-}	-	-	"	"	S	1.4×10^{-2}	+
rec^{-}	-	-	S	-	S	2.1×10^{-1}	+

(a) and (b) are shown in detail in Sections III and IV respectively.

tivity of at least some of these Ref-II UV^S mutants also results primarily from some defect of the cell division mechanism. However, in this context it is important to note that the Ref-II UV^S strains ASH115 and ASH116 have not yet been tested for the effect of post-irradiation growth with pantoyl lactone. As will be described in Section III, these mutants, unlike the majority of Ref-II UV^S mutants, are not Rec^-, at least in crosses with an HfrH male strain, and a different defect may be responsible for UV sensitivity in these mutants. Further experiments to test the effect of pantoyl lactone on the UV sensitivity of these, and other Ref-II UV^S mutants, are required. It is also important to note that pantoyl lactone had little effect on enhancing the recovery of ASH111, the filament forming Ref-II UV^S mutant. Presumably the extreme cell division defect in this strain cannot be remedied by pantoyl lactone.

Ref-II UV^S mutants, however, may be distinguished from Lon^- mutants by, with one exception, a failure to form mucoid polysaccharide colonies when grown on minimal agar, and to form extensive filaments after UV irradiation, characteristics of Lon^- mutants. They are also genetically distinct, since the lon locus lies to the right of leu (Donch, Green and Greenberg, 1968), whereas the refIIB UV^S locus is co-transducible with serB (Section III). The significance of the single mucoid Ref-II UV^S mutant, ASH116, and the location of the mucoid capsule mutation, have not yet been investigated.

Ref-II UV^S mutants also differ from all classes of UV^S mutants previously studied by their increased sensitivity to the detergent, sodium deoxycholate (DOC), and by various cell surface defects observable upon microscopic examination. A curious feature of the DOC sensitivity of these mutants is the characteristic biphasic survival curves (Fig. 10 and 11). However DOC is initially believed to dissolve the outer lipoprotein and polysaccharide layers of the cell wall (Gilby and Few, 1960) and then possibly to induce separation of lipids from membrane protein (Salton and Schmitt, 1967). The extreme sensitivity at low concentrations shown by Ref-II UV^S mutants may reflect an altered membrane component increasing the rate of the second process. It is interesting to note that the *refB*$^+$ *refIIA* parental strains ASH1 and ASH10 were more sensitive to DOC than the *refB*$^+$ *refA*$^+$ wild type strains C600 and 4K, and, moreover, the *refIIA* transductional derivative of 4K,4K-T$^+$. It may well be that although the *refIIA* gene did not measurably alter detergent sensitivity, ASH10 and derivatives already contain an additional cell surface property, manifest by increased DOC sensitivity.

It is also not yet known whether the expression of the various pleiotropic characters associated with E2-refractivity in the Ref-II UV^S mutants are potentiated by the *refIIA* gene. Unlike E2 refractivity, these pleiotropic characters were evident at both 25° and 40°C, and their expression may not involve the participation of the *refIIA* allele.

Thus it would be interesting to determine whether, in fact, DOC and UV sensitivity are lost along with colicin E2-refractivity after the transduction of the *refA*$^{+}$ allele into a Ref-II UV^{S} mutant (*refIIB* *refIIA*). Such experiments, as will be later discussed, may help clarify the role of the *refIIA* gene in colicin E2 refractivity.

Finally in this Section, the reduced ability of bacteriophage λ*gv* to replicate on certain Ref-II UV^{S} mutants ASH112, 114 and 116 was investigated. It was found that adsorption of λ*gv* to these mutants was not impaired despite the readily demonstrable cell surface peculiarities of these strains. The possibility that bacteriophage λ "resistance" was due to a mutation affecting restriction was also tested and the fact that λ*gv*.ASH114 was capable of normal growth on both ASH114 and the wild type parental strain ASH1 was compatible with the hypothesis that ASH114 does restrict wild type λ*gv* in some way.

SECTION III

GENETICAL STUDIES ON COLICIN E2 REFRACTORY MUTANTS

Introduction

Several classes of multirefractory mutants have previously been mapped at two or more loci all closely linked to the gal operon (Hill and Holland, 1967; Nomura and Witten, 1967; de Zwaig and Luria, 1967). Further classes of mutants refractory to single colicins include a class refractive to E1 alone (Ref-I), mapped at an apparently single locus closely linked to thy (Hill and Holland, 1967; Nomura and Witten, 1967), and the hitherto unmapped class, refractory to colicin E2, initially isolated by Hill and Holland (1967).

Mapping of the refII locus was initiated to determine the realtionship, if any, between this and previously mapped colicin-refractory loci. Secondly, since at least two phenotypic classes of Ref-II mutants have been isolated in this study, Ref-II UV^r and Ref-II UV^s, it was hoped to carry out fine-structure mapping to see if these classes belonged to separate cistrons. Finally, the genetic origin of pleiotropic effects associated with Ref-II UV^s mutants was also investigated.

A. Mapping of the refII locus in K12 Strains

Materials and Methods

Bacterial Strains: ref^+ strains are shown in Table 1. Ref-II mutants were isolated from ASH10 (F^- leu^- thy^- met^- lac^- str^r) by 2-amino purine treatment. The genotype of all ref^+ strains referred to in this Section, unless otherwise stated, was $refB^+$ refIIA - i.e. capable of potentiating the expression of E2 refractivity.

Phage: The transducing phage P1 was prepared from a sample supplied by R.W. Hedges.

1. Mating Conditions

(a) Linkage Analysis Exponential cultures of an Hfr strain (10^8 cells /ml) and F^- Ref-II mutants (2×10^8 cells/ml) were mixed in equal proportions and incubated with gentle shaking; after 90 min at 37°C the mixture was diluted through buffer and plated on appropriate M9 agar selective plates, plus streptomycin. For the selection of $T1^R$ recombinants, samples were plated directly onto NB plates previously spread with 10^7 phage particles. Plates were incubated for 36 hours at 37°C, and the proportion of unselected markers, including E2-sensitivity, amongst the recombinants obtained, was determined by replica plating. In the case of E2-sensitivity as an unselected marker, the procedure was as follows: NB agar plates were overlayered with top agar plus 5×10^3 units/ml of colicin E2 or colicin E3; master plates were then replicated to lawns of both colicin E2 and

E3 which were incubated at 25°C and 40°C. Those colonies sensitive to both colicins after incubation at 25°C and 40°C were scored as non-refractory, E2 sensitive.

(b) Interrupted Mating Hfr and F^- strains were first grown in NB to 5 x 10^7 cells/ml and 5 x 10^8 cells/ ml, respectively; equal volumes were mixed and incubated at 37°C with gentle shaking for 5 min. The culture was then diluted 10-fold in NB to reduce further pairing; 0.5 ml samples were removed at intervals, diluted 10-fold in ice cold phosphate buffer, and blended for 1 minute in a flask shaker (B.T.L. Laboratory Centre, Birmingham, England) before plating for recombinants. For the direct selection of E2-refractive recombinants when E2-refractive males were used, samples from the mating mixture were blended in ice-cold NB plus streptomycin. These samples were then incubated at 37°C for 90 minutes in NB plus 80 µg/ml streptomycin, without shaking, to allow expression of E2 refractivity before plating on NB agar plates plus 5 x 10^3 units of colicin E2. Plates were incubated at 25°C, and the E2-refractive colonies were then replica plated to plates containing colicin E2 and colicin E3 at 25 and 40°C to confirm the Ref-II phenotype.

For the direct selection of UV-resistant recombinants, blended samples were also allowed 90 min of expression time before plating, in this case at 37°C, on TB agar plates plus streptomycin. The plates were then irradiated as described in Methods, Section II. The presence

of streptomycin (80 µg/ml) in samples during incubation for expression of the UV^r and E2 refractivity markers prevented further growth and initiation of transfer by the $\underline{str}^s$ male strain.

2. Transductional Analysis

The preparation of lysates of the transducing phage P1 and the transduction procedure was as described in General Methods. Screening for non-auxotrophic markers was performed as follows:

(a) Colicin E2 refractivity: For the selection of Ref-II transductants the method described in Section I was used. To determine the proportion of unselected markers, E2-refractive colonies were picked off the master plate, and inoculated in a "pattern" formation on a fresh NB agar plate (25 Ref-II colonies per plate). After growth at 37°C for 8 hours "pattern" plates were replicated.

(b) UV sensitivity: For the determination of the proportion of the unselected UV^s marker amongst transductants, the plate screening method as described in Methods, Section II was used.

(c) DOC sensitivity: For the determination of the proportion of the unselected DOC^s marker amongst transductants, the plate screening method as described in Methods, Section II was used. In this case, growth of cultures after incubation on NB agar containing 1% DOC was scored as R, no growth as S.

(d) Classification for "hsp": Suspensions of cultures of strains to be tested were spotted in duplicate on NB agar, allowed to dry, and

then half overlayered with a loopful of a suspension of the virulent bacteriophage λgv containing ca 10^4 pfu/ml, previously grown on an hsp$^-$ host. The dilution of λgv employed was such that after 14 hours incubation at 37°C, hsp$^-$ hosts showed a clear zone of inhibited growth whereas hsp$^+$ strains showed confluent growth. Plates were incubated for 14 hours at 37°C, observed and scored for hsp$^+$ or hsp$^-$.

(e) Bacteriophage λ resistance Suspensions of cultures to be tested were spotted as above, allowed to dry, and half overlayered with a loopful of λgv (10^6 pfu/ml) grown through a wild-type host, and incubated for 14 hours at 37°C. The dilution of λgv employed was such that, after 14 hours incubation at 37°C, λ"s" strains gave a clear zone of inhibited growth whereas known λ"r" strains displayed confluent growth. Plates were observed after 14 hours, and strains scored as λ"r" or λ"s".

(f) Filament formation Suspensions of cultures were observed under phase as described in Section II, at a x400 magnification, and scored for the presence or absence of characteristic filaments of bacterial cells.

3. Isolation of UV^r revertants:

The method employed was based upon that described by Ganesan and Smith (1968). TB cultures of UV^s strains were grown to stationary phase, 0.1 ml aliquots were spread onto plates of TB agar and irradiated with a 20 sec UV dose as described in Section II. After incubation

in the dark for 14 hours at 37°C, each plate, containing approximately 100 colonies, was replica plated to two further plates of TB agar; these were irradiated for 80 seconds and incubated for 24 hours at 37°C. Colonies which appeared UV resistant from their response on the irradiated replicas were picked from the original plate; purified by at least two single colony isolations, and tested for plate UV resistance by the technique described in Methods, Section II. Only revertants obtained from different clones of the original UV^s strains were considered to be of independent origin.

The screening of UV^r revertants isolated for E2 refractivity, DOC sensitivity, growth and recombination proficiency was undertaken by techniques described in Methods, Section II.

A. Genetic Analysis of the ref-II Locus

Results

1. Linkage Analysis of Ref-II Mutants

Twenty one independently isolated Ref-II mutants of strain ASH10 were crossed with the male strain HfrH-202 (Table 1). Selection was made for *leu*$^+$ T1 and *lac*$^+$ recombinants, and, in each case, the recombinants were replica plated to colicin E2 and E3 lawns at 25°C and 40°C to determine the proportion that were still resistant to E2 at 25°C. The distribution of the E2 sensitivity marker among selected markers for the 21 Ref-II mutants tested is shown in Table 13.

(a) An HfrH strain ($E2^s$ Tl^r λ^- $\underline{str}^s$) was crossed with several Ref-II derivatives of the F^- strain ASH10 ($\underline{met}^-$ $\underline{leu}^-$ $\underline{lac}^-$ $\underline{thy}^-$ λ^+ $\underline{str}^r$). Exponential cultures of the male (10^8 cells/ml) and the F^- (2×10^8 cells /ml) were mixed; after 90 min selection was made for $\underline{leu}^+$ $\underline{str}^r$, Tl^r $\underline{str}^r$, and $\underline{lac}^+$ $\underline{str}^r$ recombinant classes; subsequently, the proportion of the unselected E2 sensitivity marker was determined by replica plating to lawns of 5×10^3 units/ml of colicins E2 and E3.

(b) The low proportion of selected recombinants of ASH112 was primarily due to a hitherto unknown tryptophane requirement in this strain. Subsequently crosses (c) were made with this mutant in the presence of tryptophane in the selective medium.

TABLE 13

Linkage Analysis of Ref-II Mutants of ASH10[a]

Ref-II Mutant (Total of 21 tested)	Recombinants			$E2^s$ %
	Selected marker	Input male %	No. tested	
ASH50	*leu*$^+$ *str*^{r}	2.7	199	72
	T1^r *str*^{r}	1.6	96	56
	lac$^+$ *str*^{r}	0.8	228	34
ASH51	*leu*$^+$ *str*^{r}	2.0	200	74
	T1^r *str*^{r}	1.2	124	49
	lac$^+$ *str*^{r}	0.9	98	33
ASH52	*leu*$^+$ *str*^{r}	2.6	128	80
	T1^r *str*^{r}	1.5	17	50
	lac$^+$ *str*^{r}	0.9	61	29
ASH53	*leu*$^+$ *str*^{r}	2.1	76	72
	T1^r *str*^{r}	1.2	21	51
	lac$^+$ *str*^{r}	0.6	48	28
ASH58	*leu*$^+$ *str*^{r}	2.6	211	75
	T1^r *str*^{r}	1.8	82	61
	lac$^+$ *str*^{r}	1.0	194	36

contd.

Ref-II Mutant (Total of 21 tested)	Recombinants			$E2^s$ %
	Selected marker	Input male %	No. tested	
ASH59	*leu*$^+$ *str*^{r}	2.3	84	70
	T1^r *str*^{r}	1.5	79	48
	lac$^+$ *str*^{r}	0.9	134	31
ASH60	*leu*$^+$ *str*^{r}	2.5	260	71
	T1^r *str*^{r}	1.1	229	54
	lac$^+$ *str*^{r}	0.7	132	38
ASH61	*leu*$^+$ *str*^{r}	2.4	130	79
	T1^r *str*^{r}	2.0	90	54
	lac$^+$ *str*^{r}	0.7	121	30
ASH64	*leu*$^+$ *str*^{r}	2.1	152	68
	T1^r *str*^{r}	1.1	16	50
	lac$^+$ *str*^{r}	0.5	55	38
ASH65	*leu*$^+$ *str*^{r}	2.8	170	77
	T1^r *str*^{r}	1.4	31	52
	lac$^+$ *str*^{r}	1.1	49	31
ASH66	*leu*$^+$ *str*^{r}	2.1	90	80
	T1^r *str*^{r}	1.0	94	48
	lac$^+$ *str*^{r}	1.1	31	34

contd.

Ref-II Mutant (Total of 21 tested)	Recombinants			$E2^s$ %
	Selected marker	Input male %	No. tested	
ASH67	$\underline{leu}^+$ $\underline{str}^r$	2.8	148	71
	Tl^r $\underline{str}^r$	0.9	20	60
	$\underline{lac}^+$ $\underline{str}^r$	0.9	114	41
ASH68	$\underline{leu}^+$ $\underline{str}^r$	2.0	218	71
	Tl^r $\underline{str}^r$	1.2	33	49
	$\underline{lac}^+$ $\underline{str}^r$	0.7	19	38
ASH69	$\underline{leu}^+$ $\underline{str}^r$	3.4	53	60
	Tl^r $\underline{str}^r$	0.4	21	42
	$\underline{lac}^+$ $\underline{str}^r$	0.9	44	39
ASH70	$\underline{leu}^+$ $\underline{str}^r$	3.1	234	65
	Tl^r $\underline{str}^r$	-	-	-
	$\underline{lac}^+$ $\underline{str}^r$	1.1	46	34
ASH73	$\underline{leu}^+$ $\underline{str}^r$	2.3	180	76
	Tl^r $\underline{str}^r$	1.5	132	54
	$\underline{lac}^+$ $\underline{str}^r$	0.9	147	25
ASH102	$\underline{leu}^+$ $\underline{str}^r$	2.3	46	77
	Tl^r $\underline{str}^r$	1.7	81	46
	$\underline{lac}^+$ $\underline{str}^r$	0.3	93	39

contd.

Ref-II Mutant (Total of 21 tested)	Recombinants			$E2^s$ %
	Selected marker	Input male %	No. tested	
ASH112[b]	*leu*$^+$ *str*^{r}	0.06	134	74
	T1^r *str*^{r}	0.009	91	56
	lac$^+$ *str*^{r}	0.001	118	28
ASH112[c]	*leu*$^+$ *str*^{r}	0.4	72	73
	T1^r *str*^{r}	0.12	24	59
	lac$^+$ *str*^{r}	0.09	96	32
ASH113	*leu*$^+$ *str*^{r}	0.005	46	68
	T1^r *str*^{r}	-	-	-
	lac$^+$ *str*^{r}	0.002	21	30
ASH115	*leu*$^+$ *str*^{r}	2.7	112	78
	T1^r *str*^{r}	1.6	25	51
	lac$^+$ *str*^{r}	0.8	165	34
ASH116	*leu*$^+$ *str*^{r}	3.0	90	70
	T1^r *str*^{r}	1.3	17	53
	lac$^+$ *str*^{r}	0.8	28	40
MEAN	*leu*$^+$ *str*^{r}		3073	72.5
	T1^r *str*^{r}		1323	52.5
	lac$^+$ *str*^{r}		2020	33.6

The results indicated that the refIIB locus was linked to leu. This was confirmed in crosses where selection was made for recombinants for the distal marker, lac, when the proportion of the unselected markers, leu^+ $T1^r$ and E2 sensitivity among lac recombinants was determined by replica plating. The results (Table 14) clearly indicated the presence of a single refII B locus closely linked with and to the left of the leu locus (Fig. 1).

2. Mapping of the Tryptophane Requirement of ASH112 by Interrupted Mating

Initial crosses between the Ref-II mutant, ASH112 and the male strain HfrH gave rise to only very low number of recombinants (Table 13). On testing the growth requirements of this mutant, it was found to have acquired an additional requirement for tryptophane. Now two try loci have previously been mapped on the bacterial chromosome; one (Fig. 2) is not linked to the refIIB locus; a second, tryP, lies to the left of serB (Taylor and Trotter, 1967) and may conceivably be linked to, and associated with, the refIIB locus. Although it was not anticipated that the mutation producing a tryptophane requirement in ASH112 had arisen other than independently from the Ref-II mutation after mutagen treatment, confirmation was sought by interrupted mating experiments. The male strains HfrH 202 and HfrB11 were therefore both crossed with the F^- Ref-II mutant ASH112, and the time of entry of the tryptophane requirement determined in each case. As can be seen from Figure 13a and b, the try locus entered after approximately 25 minutes

TABLE 14

Linkage Analysis of Ref-II Mutants of ASH10[a]

Ref-II Mutant (Total of 24 tested)	Marker selected, *lac*$^+$ *str*^{r}			
	No. tested	Proportion of unselected markers		
		T1 *str*^{r} %	*leu str*^{r} %	E2^s %
ASH51	161	82	73	57
ASH52	333	86	79	54
ASH53	224	83	72	56
ASH58	202	87	74	43
ASH59	184	89	71	52
ASH60	192	78	67	55
ASH62	164	85	74	49
ASH63	181	87	76	56
ASH65	103	81	79	53
ASH66	127	82	80	58
ASH69	111	83	74	59
ASH71	194	79	74	57
ASH72	262	84	80	53
ASH73	154	84	74	58
ASH74	197	81	72	53
ASH75	102	79	77	58
ASH76	94	76	69	51

contd.

Ref-II Mutant (Total of 24 tested)	Marker selected, *lac*$^+$*str*^{r}			
	No. tested	Proportion of unselected markers		
		T1 *str*^{r} %	*leu str*^{r} %	E2^s %
ASH77	190	87	79	54
ASH78	84	82	76	51
ASH79	113	84	80	55
ASH102	103	85	75	59
ASH112	202	90	81	26
ASH115	83	79	64	53
ASH113	84	-	76	44
MEAN	3847	83	75.3	54

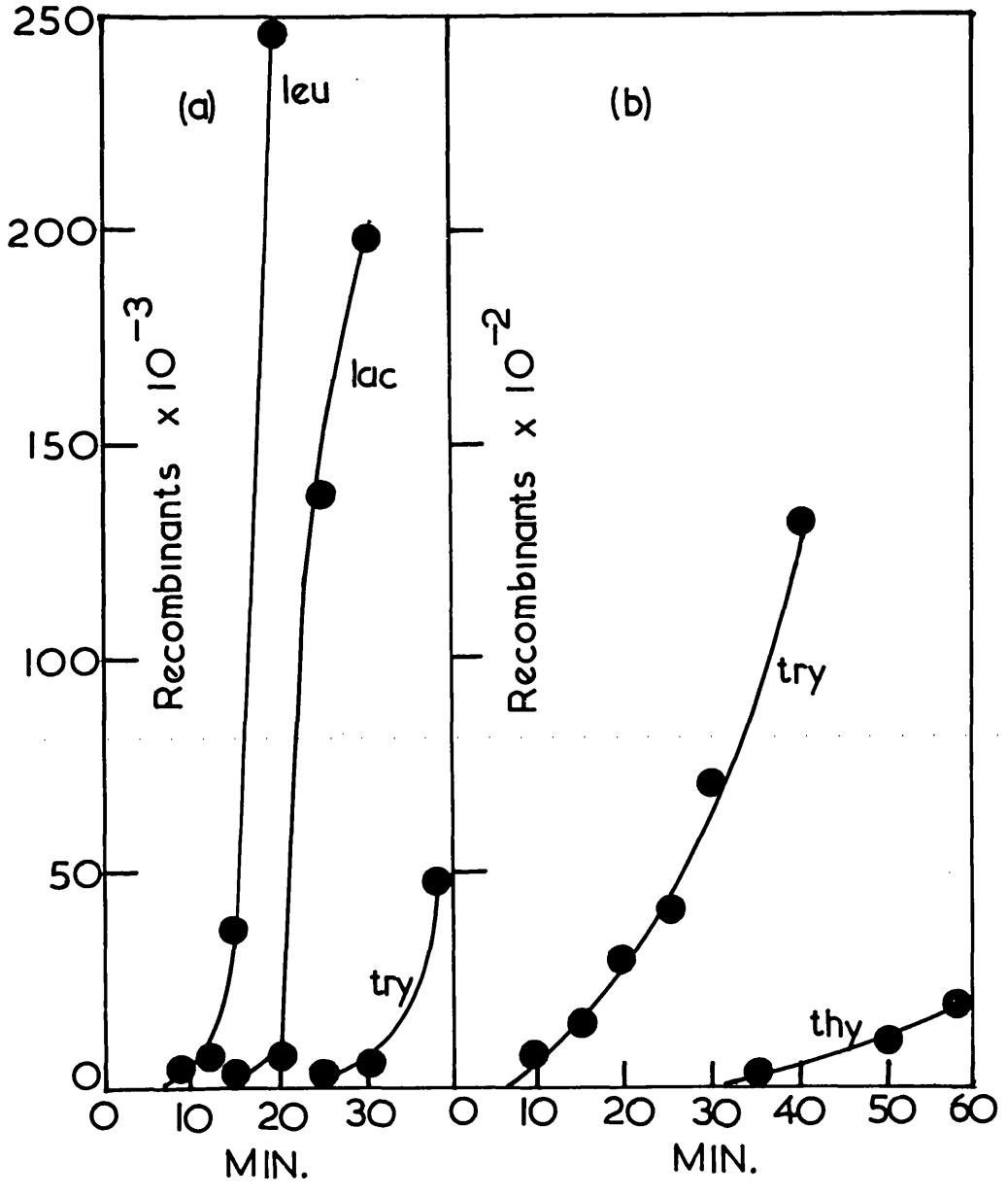
(a) An Hfr H strain (E2^s T1^r λ^- *str*^{s}) was crossed with several Ref-II derivatives of the F$^-$ strain ASH10 (*met*$^-$ *leu*$^-$ *lac*$^-$ *thy*$^-$ λ^+ *str*^{r}). Exponential cultures of the male (10^8 cells/ml) and the F$^-$ (2×10^8 cells/ml) were mixed; after 90 min selection was made for *lac str*^{r} recombinant class; subsequently the proportion of unselected markers, including E2 sensitivity, was determined by replica plating.

Fig. 13 Time of entry of the try locus in the colicin E2-refractory mutant, ASH112

(a) HfrH 202 crossed with ASH112

(b) HfrB11 crossed with ASH112

The mating was carried out as described in Methods, Section III, and samples removed at intervals, blended and plated for leu$^+$ lac$^+$ try$^+$ and thy$^+$ prototrophic recombinants.


250
200
150
100
50
0
(a)
leu
lac
try
Recombinants x 10^-3
0 10 20 30
MIN.
(b)
try
thy
Recombinants x 10^-2
0 10 20 30 40 50 60
MIN.

in the cross with the HfrH male, and after 8 minutes in the cross with the HfrB11 male. These results indicated a map position 15 minutes clockwise from the lac locus, and which corresponds to the position of the try locus originally mapped by Ito and Crawford (1965). This mutation therefore presumably arose from 2-amino purine treatment of ASH10 quite independently of E2-refractivity.

3. Mapping of the Ref-II Locus by Interrupted Mating

Confirmation of the position of the refIIB locus was sought from a series of interrupted mating experiments. Mutants refractory to colicin E2 (Ref-II) were isolated in an HfrH strain, ASH3 and in an HfrR4 strain ASH4. These Ref-II mutants, which were designated ASH54 and ASH55 respectively, were crossed with the F^-, colicin E-sensitive strain E94 (Table 1) and the time of entry of E2-refractivity was determined as described. The results are shown on Figure 14, which, as can be seen, indicated a position of the refIIB locus 3-4 minutes to the left of the leu locus.

To more accurately locate the refIIB locus, the interrupted mating was repeated in crosses with the F^- colicin E-sensitive strain ASH5 (Table 1), which was thought to be more applicable than E94 because of an additional requirement for threonine. The time of entry of the refIIB locus was again determined, and the results indicated a map position 1 to 2 minutes counter-clockwise to the thr locus (see Fig.15). The position of the refII locus on the chromosome is shown

Fig. 14 Time of entry of the colicin E2-refractory locus

(a) ASH54 (HfrH) crossed with the E2-sensitive F^- strain E94($\underline{leu}^-\underline{pro}^-\underline{lac}^-\underline{str}^r E2^s$)

(b) ASH55 (HfrR4) crossed with the E2-sensitive F^- strain E94

The mating was carried out as described in Methods, Section III, and samples removed at intervals, blended, and plated for $\underline{leu}^+$, $\underline{pro}^+$, $\underline{lac}^+$ and E2-resistant recombinants.

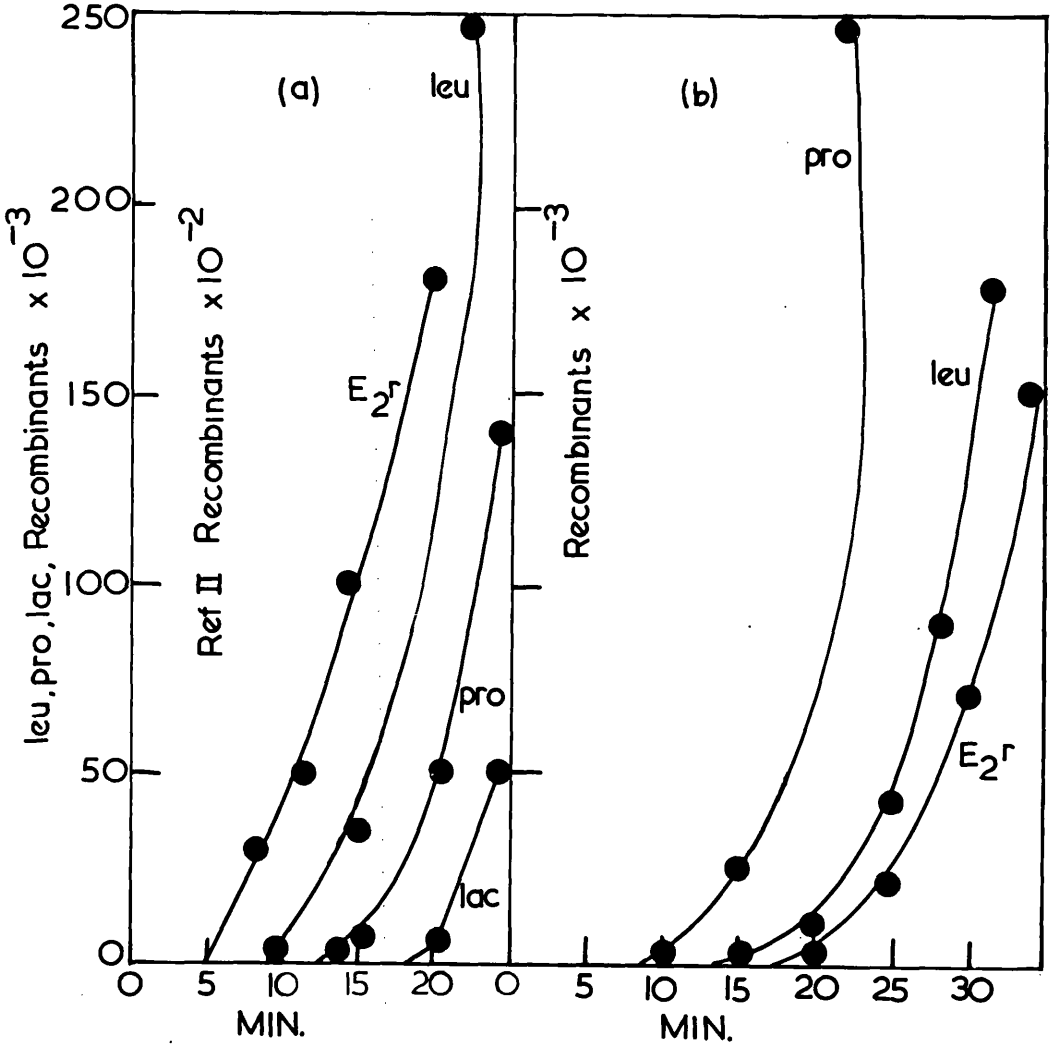
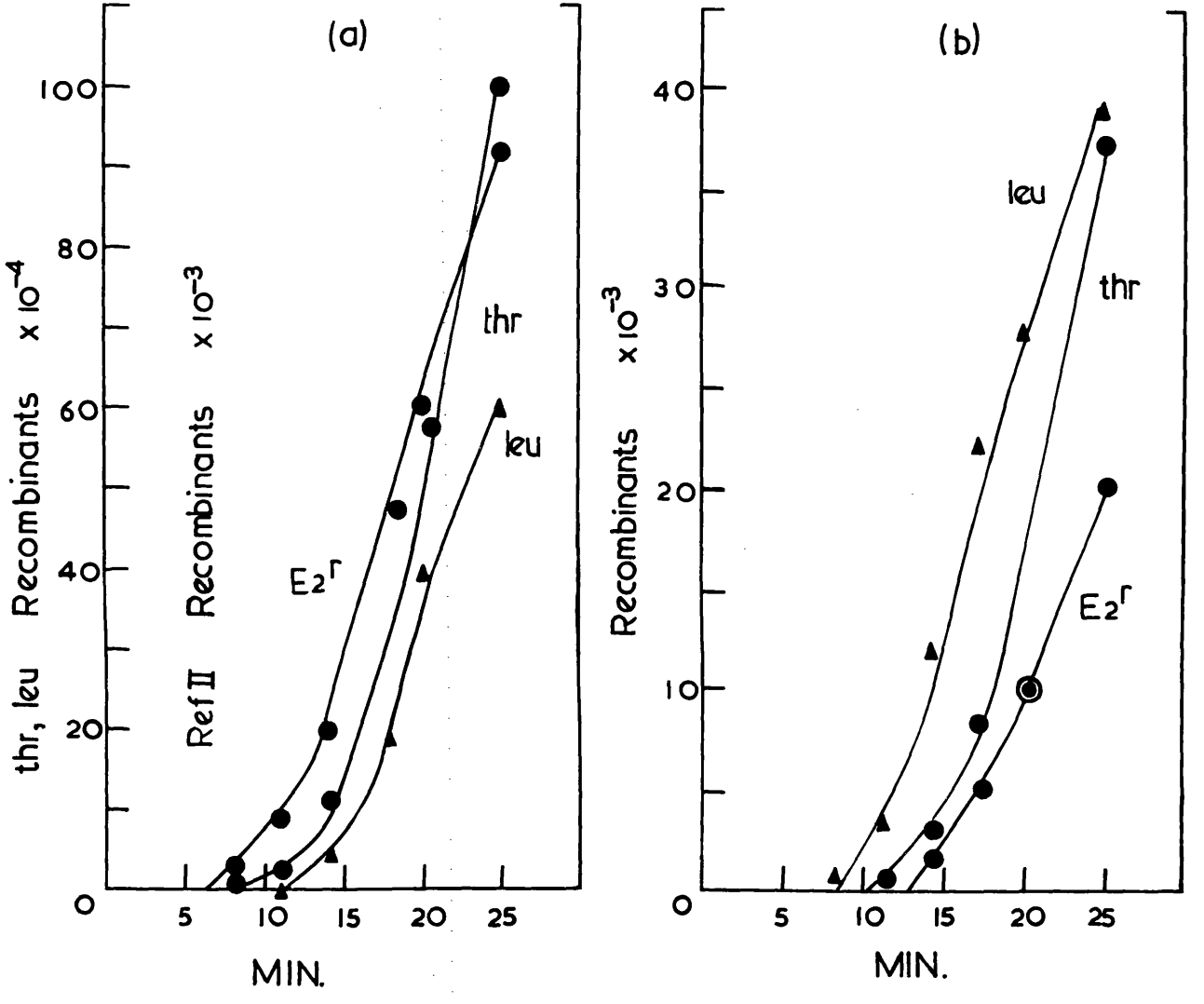

250
200
150
100
50
0
leu, pro, lac, Recombinants x 10^-3
Ref II Recombinants x 10^-2
(a)
leu
E2r
pro
lac
0 5 10 15 20
MIN.
(b)
pro
leu
E2r
Recombinants x 10^-3
0 5 10 15 20 25 30
MIN.

Fig. 15 Time of entry of the colicin E2-refractory locus

(a) ASH54 (HfrH) crossed with E2-sensitive strain ASH5

(b) ASH55 (HfrR4) crossed with E2-sensitive strain ASH5

The mating was carried out as described in Methods, Section III, and samples removed at intervals, blended, and plated for *thr*$^+$, *leu*$^+$, *lac*$^+$ and E2-resistant recombinants.


(a)
thr, leu Recombinants x 10⁻⁴
Ref II Recombinants x 10⁻³
100
80
60
40
20
0
5
10
15
20
25
MIN.
thr
leu
E2r
(b)
Recombinants x 10⁻³
40
30
20
10
0
5
10
15
20
25
MIN.
leu
thr
E2r

in Figure 1. The different scales used in Fig. 14 and 15 should be noted; in several experiments using ASH54 as donor, reduced numbers of Ref-II recombinants relative to other classes were obtained. Similar results have been reported by Low (1965) for markers very near the origin of the transferred chromosome.

4. Transductional Analysis of Ref-II Mutants

Previous genetic studies (Taylor and Trotter, 1967) have described a serB locus to the left of thr in E.coli K12. This is apparently homologus to the structural gene for phosphoserine phosphatase in Salmonella. In a transductional cross between the P1 donor strain ASH3 (refIIA, serB$^+$ thr$^+$ leu$^+$ and the recipient strain 4K (refA$^+$ serB$^-$ thr$^-$ leu$^-$), the linkage between serB and thr was found to be 45.5%, and that between serB and leu, 0.5% (Table 15).

The relationship between the refIIB and serB loci was then determined for both the Ref-II UV^r strain ASH102 and the Ref-II UV^s strain ASH112. Thus the P1 donor strains ASH102 and ASH112, both of which carry the serB$^+$ marker, were crossed with the serB$^-$ recipient strain 4K-T$^+$ (see footnote). Ref-II transductants were analysed, and in each case 21% of the Ref-II transductants inherited the donor serB$^+$

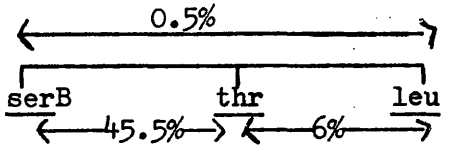
(Footnote. The serB$^-$ recipient strain 4K-T$^+$ was originally derived from a transductional cross between P1 grown on ASH3 (thr$^+$ refA$^+$). A thr$^+$ transductant was selected (4K-T$^+$) which was capable of expressing E2-refractivity on receipt of the refIIB allele.)

TABLE 15

Linkage between the serB, thr and leu Loci

P1 Donor	P1 Recipient	Selected Markers	Transductants per 10^6 P1	No. scored	Analysis of transductants (scored as %)		
					thr$^+$	leu$^+$	serB$^+$
ASH3	4K	serB	9.8	200	44	0.5	100
(refIIA serB$^+$	(refA$^+$ serB$^-$	thr	6.8	200	100	6.5	47
thr$^+$ leu$^+$)	thr$^-$ leu$^-$)	leu	1.8	41	6	100	0

∴ compatible gene order and linkage



Transductional procedure was as described in General Methods; the proportion of unselected markers was determined by replica plating.

marker, and conversely 21-22% of the serB$^+$ transductants inherited the donor Ref-II allele (Table 16).

Since similar results were obtained with either the UV sensitive or resistant Ref-II donor, this indicated that if mutants of the Ref-II UV^r and Ref-II UV^s classes do in fact reflect different cistrons both affecting E2-refractivity, these must be extremely closely linked.

To clarify the order of the serB and refIIB loci, 250 serB$^+$ transductants of strain 4K-T$^+$ were now selected, and the linkage of serB to the refIIB locus and to the outside marker hsp (host-controlled modification) (Taylor and Trotter, 1967) was determined (Table 17). In addition, 100 Ref-II transductants of 4K-T$^+$ were selected in the same cross and the proportion of these now showing host-controlled modification was determined. The results, shown in Table 17, indicated a close linkage between the refIIB and hsp loci, and confirmed the 21% linkage between the serB and refIIB loci originally shown in Table 16. This result also clearly demonstrated that the refIIB locus lies to the left of serB. Furthermore, since 18.4% of serB$^+$ transductants selected exhibited host-controlled modification, whereas 20.8% were refractive to colicin E2, this suggested that the hsp locus was distal to the refIIB locus.

In an attempt to determine relative order of the closely linked refIIB, hsp and serB loci a three factor transductional cross was carried out. SerB$^+$ and Ref-IIB transductants were selected from a transduction-

TABLE 16

Linkage between serB and refIIB loci in Ref-II UV^r and Ref-II UV^s

P1 Donor	P1 Recipient	Selected Markers	Transductants per 10^6 P1	No. scored	Analysis of Transductants (scored as %)	
					$serB^+$	refIIB
ASH102	$4K\text{-}T^+$	serB	1.7	150	100	22
($serB^+$ refIIB)	($serB^-$ $refB^+$)	refIIB	1.8	150	21	100
ASH112	$4K\text{-}T^+$	serB	4.4	435	100	21.
($serB^+$ refIIB UV^s)	($serB^-$ $refB^+$)					

∴ compatible gene order and linkage

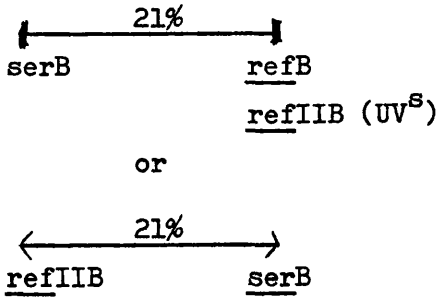
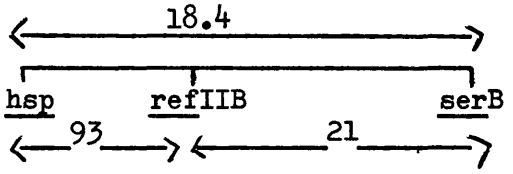


TABLE 17

Linkage between the hsp, refIIB and serB Loci

P1 Donor	P1 Recipient	Selected Markers	Transductants per 10^6 P1	No. scored	Analysis of Transductants (scored as %)		
					serB$^+$	*refIIB*	*hsp*$^+$
ASH102	4K-T$^+$	*serB*	3.2	250	100	20.8	18.4
(*hsp*$^+$ *serB*$^+$ *refIIB*)	(*hsp*$^-$ *serB*$^-$ *refB*$^+$)	*refIIB*	2.1	100	-	100	93

∴ indicated gene order and linkage



Transductional procedure was as described in General Methods; the proportion of the unselected *hsp*$^+$ and *refIIB* markers was determined as described in Methods, Section III.

al cross between the P1 donor strain ASH102 (*hsp*$^+$ *refIIB* *serB*$^+$) and the recipient, 4K-T$^+$ (*hsp*$^-$ *refB*$^+$ *serB*$^-$), and the proportions of all possible classes of unselected markers determined. Results are shown in Table 18.

Results obtained, however, were contradictory. In the selection of *serB*$^+$ transductants relative proportions of unselected markers indicated an order *hsp*-*refIIB*-*serB*, whereas the proportions of unselected markers arising from selection of E2-refractive transductants indicated the order *refIIB*-*hsp*-*serB*. These discrepancies arise from low numbers of the minority classes scored - in one case 3:1, in the other 8:1. Since there are technical difficulties in scoring the *hsp* marker, particularly evident in the judgement of inhibited growth of *hsp*$^-$ clones, such errors can occur. Nevertheless the results do indicate a close linkage (94%) between the *hsp* and *refIIB* loci. These loci nevertheless appear quite distinct. Both Ref-II UV^r and Ref-II UV^s mutants still showed host specific restriction whilst the *hsp*$^-$ mutant 4K, used in these studies, was still sensitive to colicin E2, a property similarly displayed by various other *hsp*$^-$ mutants tested by Holland (1967).

B. The Effect of the Ref-II Mutation upon Genetic Recombination

1. The rec$^-$ Character of Ref-II UV^s Mutants

Recombination frequencies obtained with the majority of Ref-II mutants of ASH10 at 37°C were similar to those obtained with ASH10 (Table 19). Moreover, in crosses carried out at 25°C where E2-

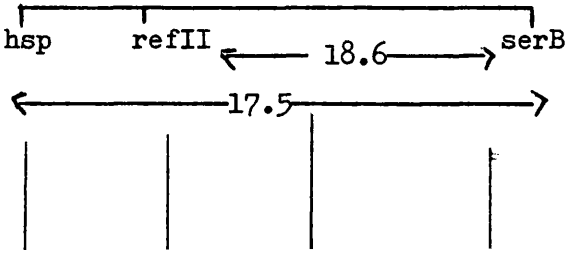
TABLE 18

Linkage and Orientation of the hsp, refIIB and serB Loci

P1 Donor	P1 Recipient	Selected Markers	Transductants per 10^6 P1	No. scored	Analysis of Transductants (scored as %)		
					serB$^+$	refIIB	*hsp*$^+$
ASH102 (*serB*$^+$ refIIB *hsp*$^+$)	4K-T$^+$ (*serB*$^-$ *refB*$^+$ *hsp*$^-$)	*ser*B	0.7	166	100	18.6	17.5

Possible classes	No. scored	%
serB$^+$ *leu*$^-$ *hsp*$^-$ *refB*$^+$	134	80.7
serB$^+$ *leu*$^-$ *hsp*$^-$ refIIB	3	1.8
serB$^+$ *leu*$^-$ *hsp*$^+$ refIIB	28	18.9
serB$^+$ *leu*$^-$ *hsp*$^+$ *refB*$^+$	1	0.6

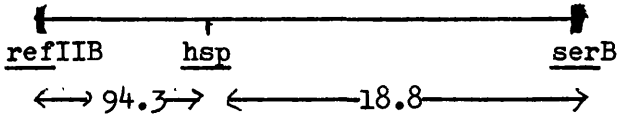
$\therefore$ indicated gene order and linkage



		refIIB	3.4	160	18.8	100	94.3

Possible classes	No.scored	%
$serB^+$ hsp^+ refIIB	30	18.8
$serB^+$ hsp^- refIIB	1	0.7
$serB^-$ hsp^- refIIB	8	5.0
$serB^-$ hsp^+ refIIB	121	76.4

∴ indicated gene order and linkage



Transductional procedure as described in General Methods; proportion of unselected refIIB and hsp markers determined as described in Methods, Section III.

TABLE 19

Reduced Recombination Frequencies with F^- strains carrying Ref-II UV^s Mutants[(a)]

Donor	Recipient	Recombinants as % of input male			Recombination Deficiency Index (RDI)[(a)] (UV^r/UV^s)
		leu$^+$ *str*^{r}	$T1^r$ *str*^{r}	*lac*$^+$ *str*^{r}	
HfrH	ASH10 (UV^r) *ref*$^+$	2.2	1.9	0.7	
HfrH	ASH112 (UV^s) Ref-II	0.33	0.17	0.09	7
HfrH	ASH113 (UV^s) Ref-II	0.005		0.002	4.4×10^2

Mating conditions were as described in Methods, Section III.

(a) Recombination Deficiency Index determined from frequency of *leu*$^+$ *str*^{r} recombinants in each cross relative to the parental control ASH10

refractivity is maximal, recombination frequencies were normal in the majority of strains. However at least two mutants, ASH112 and ASH113 were seen to be defective in some aspect of genetic recombination, and the recombination deficiency in these strains is shown in Table 19.

Previous physiological studies described (Section II) indicated that these particular strains were also UV sensitive, although similar Ref-II UV^S mutants, ASH115 and ASH116, gave no marked reduction in recombinant formation (Table 13). The recombination deficiencies obtained with ASH112 and ASH113 when crossed with HfrH, transferring the $\underline{ref}^+$ allele early, were 7 and 4.5×10^{-2} respectively (Table 19). In order to investigate the effect of late transference of the $\underline{ref}^+$ allele on the proficiency of recombination on these and other Ref-II UV^S mutants, including two male Ref-II mutants ASH112 and ASH114, it was first necessary to transduce the refII locus from these strains into the more genetically appropriate F^- strain ASH5. This was readily achieved by P1 transduction. The recombination proficiency of Ref-II derivatives of ASH5 derived from ASH111, 112, 113 and 114 was then determined in crosses with the male strain HfrP10 which transfers the $\underline{ref}^+$ allele as a distal marker (see footnote). As can be seen from Table 20, these Ref-II UV^S transductants were still UV sensitive and showed recombination deficiencies of at least 100-fold. In fact, the deficiency obtained with the trans-

Footnote. HfrP10 ($\underline{arg}^+$) used in these and subsequent crosses was a transductional derivative of HfrP10 ($\underline{arg}^-$).

Mating conditions were as described in Methods, Section III.

(a) Recombination Deficiency Index determined from frequency of *arg* *str*^{r} recombinants in each cross relative to the parental control, ASH5.

(1) Cross of Hfr P10 with ASH5 and 1, ASH5,Ref-II UV^{s} derivative of strains ASH111, 112, and 114 respectively

(2) Cross of HfrP10 with ASH5 and 4, ASH5, Ref-II UV^{s} derivatives of strain ASH113.

TABLE 20

Reduced Recombination Frequencies with F^- Strains Carrying Ref-II UV^s Mutations

Donor	Recipient	Recombinants as % Input Male			Recombination Deficiency Index(a) (UV^r/UV^s)
		arg str^r	his str^r	leu str^r	
(1) HfrP10	ASH5 (UV^r)	1.3	0.5	0.04	
	ASH5-112a (UV^s)	0.007	0.0014	0.0005	1.8×10^2
	ASH5-111a (UV^s)	0.012	0.002	0.0007	1.1×10^2
	ASH5-114a (UV^s)	0.006	0.004	0.0003	2.2×10^2
(2) HfrP10	ASH5 (UV^r)	2.6	1.1	0.02	
	ASH5-113a (UV^s)	0.004	0.003	0.00035	6.5×10^2
	ASH5-113b (UV^s)	0.007	0.005	0.0005	3.7×10^2
	ASH5-113c (UV^s)	0.014	0.006	0.0004	1.8×10^2
	ASH5-113d (UV^s)	0.006	0.0035	0.0003	4.3×10^2

ductional derivatives of ASH112 was little different to that found with the derivatives of ASH113. This result contrasts to those results obtained with the original Ref-II UV^S mutants in crosses with the male strains HfrH where there was a wide variation between the recombination deficiency of the two strains.

The UV sensitivity of Ref-II UV^S mutants therefore appeared to be correlated, in the majority of mutants investigated, with a reduced ability to form prototrophic recombinants in crosses with male strains. However it is important to notethat two Ref-II UV^S mutants, ASH115 and ASH116, were not deficient in recombination at least in crosses with HfrH.

Rec mutants previously isolated have also been reported to be recombination-deficient in P1 transductional crosses (Hertman and Luria, 1967). Therefore to compare Ref-II UV^S Rec^- mutants isolated in this study with other Rec mutants, the ability of these Ref-II UV^S Rec^- mutants to mother transductants was investigated. Thus two Ref-II UV^S Rec^- mutants and a Rec-II UV^r Rec^+ mutant were infected with P1 grown on the wild type donor strain, ASH3, and the number of recombinants obtained was determined (Table 21). The recombination frequency of these Ref-II UV^S Rec mutants was then compared to that of the <u>recB</u>$^-$ mutant JC4457, and the <u>recB</u>$^+$ parental strain AB1157 (Table 21).

The results obtained were in complete contrast to conjugation experiments, since transduction frequencies obtained with the Ref-II

TABLE 21

Transductional Recombination Frequencies of F^- Ref-II UV^s Rec^- Mutants and an F^- $recB^-$ Strain

P1 Donor	P1 Recipient	Transductants per 10^6 P1 Particles				
		thy	met	leu	his	arg
ASH3 (thy+ met+ leu+ his+ arg+)	ASH102 (Ref-II UV^r)	6.2	6.2	1.8	-	-
	ASH112 (Ref-II UV^s Rec^-)	4.9	-	1.2	-	-
	ASH113 (Ref-II UV^s Rec^-)	6.0	-	1.0	-	-
	AB1157 ($recB^+$)	-	-	2.1	5.5	4.4
	JC4457 ($recB^-$)	-	-	0.5	0.83	0.7

Transductional procedure was as described in General Methods; genotype of ASH strains (F^- thy$^-$ met$^-$ leu$^-$); genotype of AS1157 (F^- thr$^-$ leu$^-$ pro$^-$ his$^-$ arg$^-$ $recB^+$); JC4457 - $recB^-$ derivative of AB1157.

UV^S Rec^- mutants were not significantly different from those displayed by the Ref-II UV^r Rec^+ control, ASH102. Reduced transduction frequencies were however found with the RecB mutant tested, which displayed at least an 8-fold recombination deficiency. Previous results with RecA mutants (Hertman and Luria, 1967) and RecB mutants (Emmerson and Howard-Flanders, 1967) however indicated at least a 100 or a 20-fold reduction respectively, and although the recombination deficiency with the RecB mutant investigated in this study did not reach these levels, it was significantly greater than that displayed by the Ref-II UV^S mutants. The recombination deficiency of the Ref-II UV^S mutants obtained in Hfr-F^- matings is therefore for some reason not expressed in recombination involving P1-transduced DNA.

Further investigations into the nature of the recombination deficiency in these mutants is described in Section IV.

2. Effect of the Ref-II Mutation as a Distal Marker in Male Strains

It was not anticipated that Ref-II derivatives of male strains transferring the refII locus as an early marker would reduce the frequencies of recombinants in crosses with F^- strains, and as seen with the interrupted mating experiments, (Fig. 14 and 15) this expectation was fulfilled. Thus the frequencies of thr^+ leu^+ and lac^+ recombinants were not significantly reduced in crosses of the F^- strain ASH5 with the Ref-II males, ASH54 and ASH55.

Aberrant recombination frequencies were, however, sometimes obtained with Ref-II derivatives of ASH1, a male strain transferring the refIIB locus as a tail marker (Fig.2). The results of crosses of a Ref-II derivative of HfrH (ASH54) and two Ref-II male strains ASH101 and ASH122, derived from ASH1, are shown in Table 22.

The number of recombinants obtained in each case is presented as a percentage of that obtained in crosses with the corresponding E2-sensitive (ref$^+$) males. Somewhat variable results were obtained with ASH101, the frequency sometimes approaching that of the wild type, but ASH122 always gave the same low yield. The possibility was tested that the reduction in recombination frequency was due to e.g. restriction of "Ref-II DNA" in the ref$^+$ recipient. ASH122 was therefore crossed with a Ref-II derivative of the F$^-$ strain ASH5. The results (Table 22) showed that although both male and female strains carried a Ref-II mutation, the same marked reduction in recombinant formation occurred.

C. Genetic Analysis of Pleiotropic Characteristics Associated with the Mutation to E2-Refractivity

1. Mapping of the UVs Locus of a UV-Sensitive Ref-II Mutant by Interrupted Mating

In Section II it was reported that approximately 34% of the Ref-II mutants isolated were abnormally sensitive to UV irradiation. Such a high frequency of UV sensitive mutations made it unlikely that this

TABLE 22

Recombination Frequencies Obtained with Male (refII) Strains

Donor	Recipient	Recombinants as % of that obtained with wild-type (ref$^+$) males				
		leu str^r	lac str^r	gal str^r	try str^r	his str^r
ASH54 (HfrH refII)	ASH5 (ref$^+$)	100	100		100	100
ASH101 (HfrB11 refII)	ASH5 (ref$^+$)			5	8	13
ASH122 (HfrB11 refII)	ASH5 (ref$^+$)			4	4	2
ASH122 (HfrB11 refII)	ASH57 (refII)			4	4	2

Mating conditions as described in Methods, Section III.

character had arisen independently of the muation to E2 refractivity. To determine the relationship of E2 refractivity, UVsensitivity and the associated deficiency in recombination of some UV^s mutants, the position of the UV-sensitivity locus in one such mutant, ASH112, was determined. ASH112 was chosen for this study in preference to ASH113, as the Recombination Deficiency Index of ASH112 (Table 19) was much lower than the former, at least in crosses with HfrH male strains. Interrupted mating experiments were carried out with this strain in crosses with two UV resistant E2-sensitive male strains, HfrH and Hfr R4. The time of entry of UV resistance was determined in each case and the results showed (Fig. 16) that this UV^s locus, like the refII locus, was also located close to the thr marker.

When the UV resistant recombinants were replica plated to NB plates containing colicin E2, all colonies appeared to be E2-sensitive. In strain ASH112, therefore, the UVsensitivity locus and the refIIB locus were extremely closely linked, and were inseparable by interrupted mating techniques.

2. Isolation and Characterisation of UV^r Revertants of Ref-II UV^s Mutants

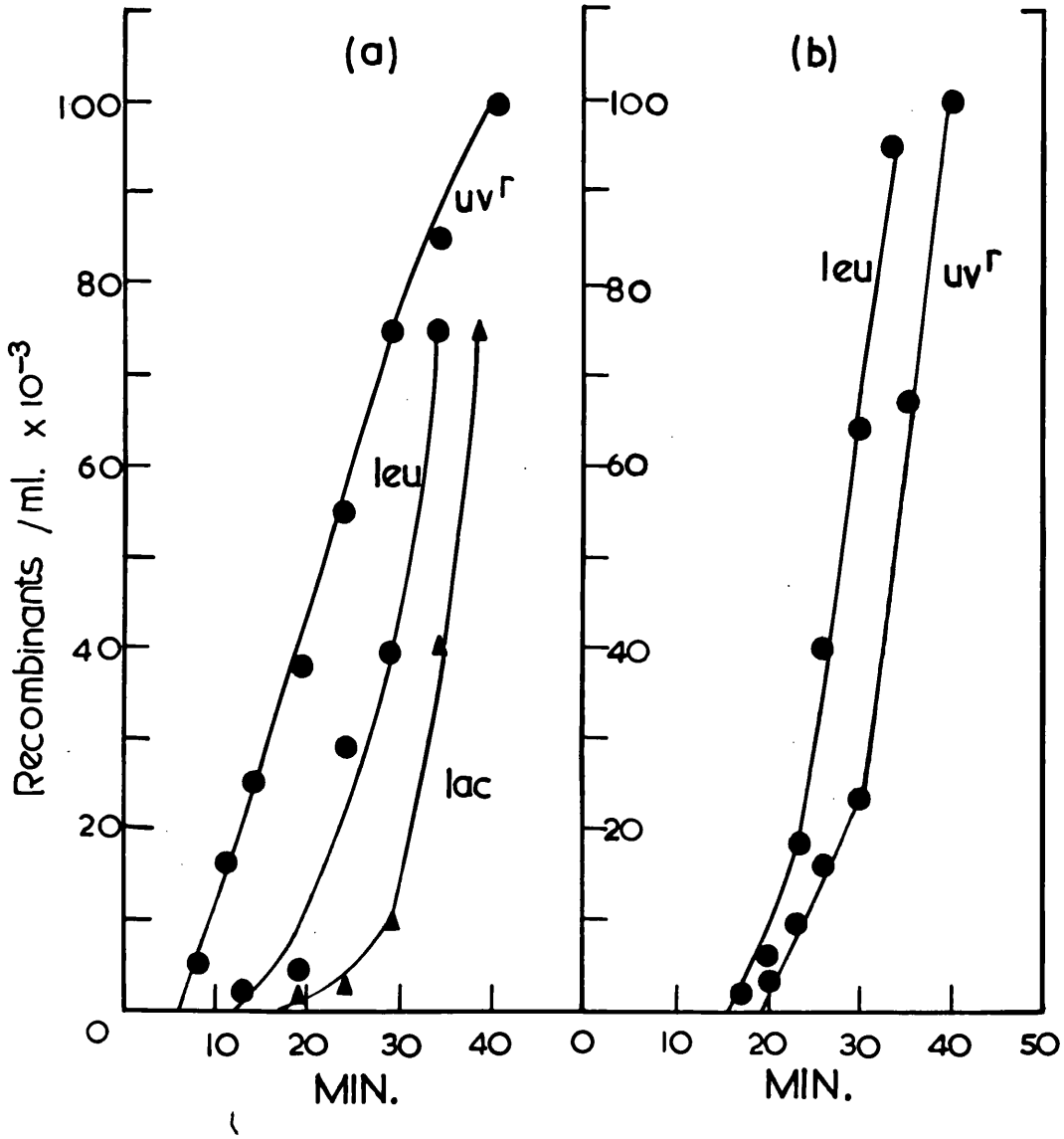
As Ref-II mutants had been selected by treatment with the base analogue 2-amino purine, it was not anticipated that the various pleiotropic characters associated with a small number of the mutants (see Table 12) was the result of a deletion covering several closely

Fig. 16 Time of Entry of the UV Marker in ASH112

(a) HfrH crossed with ASH112 (Ref-II UV^S)

(b) HfrR4 crossed with ASH112 (Ref-II UV^S)

The mating was carried out as described in Methods, Section III and samples removed at intervals, blended and plated for *leu*$^+$, *lac*$^+$ and UV^r recombinants.


(a)
(b)
Recombinants /ml. x 10⁻³
100
80
60
40
20
0
uvr
leu
lac
10
20
30
40
50
MIN.

linked genes. However to eliminate this possibility, the ability of Ref-II UV^s mutants to revert to UV resistance was tested. In fact, UV^r revertants of the Ref-II UV^s DOC *rec*$^-$ strains ASH112 and ASH113 were readily isolated by the technique described in Methods, Section III. The growth rates of such UV^r revertants, their response to colicin E2 and DOC, and their recombination proficiencies in crosses with an HfrH male were also examined. The results showed that the recombination proficiency of revertants isolated from both ASH112 and ASH113 (Table 23) was significantly increased over that of the parental Ref-II UV^s strains, although one revertant of ASH113 was still not as efficient as the wild-type *ref*$^+$ strain ASH10.

In addition, as shown in Table 24, all revertants had regained normal DOC resistance, were sensitive to colicin E2 at 25°C as well as at 37°C and displayed normal growth rates in complex liquid medium.

From these results it appears unlikely that the various pleiotropic characters associated with the Ref-II UV^s mutants have arisen from a series of closely linked but independent multisite mutations. However it could not be overlooked that the mutagenic effect of the UV irradiation involved in the selection of UV^r revertants of the Ref-II UV^s mutants was sufficient to produce a mutation in a site unrelated to the Ref-II UV^s locus, but which somehow suppressed UV sensitivity, i.e. that these were not true revertants. In particular, if the *refIIA* gene does in fact potentiate the expression of UV sensitivity and DOC

TABLE 23

Recombination Frequencies Obtained with F^- Strains Carrying Ref-II UV^s Mutations, and with UV^r Revertants of these Strains[a]

Donor	Recipient	Recombinants as % Input Male *lac* str^r
ASH3 (HfrH)	ASH10 (*ref*$^+$)	1.5
ASH3 (HfrH)	ASH112 (UV^s)	0.18
ASH3 (HfrH)	ASH113 (UV^s)	0.025
ASH3 (HfrH)	ASH112-R1 (UV^r)[b]	1.7
ASH3 (HfrH)	ASH113-R1 (UV^r)[c]	1.0
ASH3 (HfrH)	ASH113-R2 (UV^r)[c]	0.25

(a) Mating conditions as described in Methods, Section III. All crosses at 37°C.

(b) UV^r revertant of ASH112

(c) UV^r revertant of ASH113.

TABLE 24

Certain Phenotypic Characteristics of F^- Strains Carrying Ref-II UV^s Mutations and of UV^r Revertants of these Strains

Strain	Origin	Phenotypic Characteristics			DOC Sensitivity (1%)(c)	Recombination Proficiency	Quantitative Response to UV(a)	Growth in Complex Medium(d)
		Response to UV (40 secs)(a)	Response to colicins(b)					
			E2 25°C	E3 25°C				
ASH10		R	S	S	R	rec$^+$	'normal'	'normal'
ASH112	AP(e) mutation of ASH10	S	R	S	S	rec$^-$	'sensitive'	'poor'
ASH112-R1	R$^-$revertant of ASH112	R	S	S	R	rec$^+$	'normal'	'normal'
ASH112-R2	ditto	R	S	S	R		'normal'	'normal'
ASH112-R3	ditto	R	S	S	R			
ASH112-R4	ditto	R	S	S	R			
ASH112-R5	ditto	R	S	S	R			
ASH112-R6	ditto	R	S	S	R			

ASH113	AP mutation of ASH10	S	R	S	S	$\underline{rec}^-$	'sensitive'	'poor'
ASH113-R1	R^-revertant of ASH113	R	S	S	R	$\underline{rec}^+$	'normal'	'normal'
ASH113-R2	ditto	R	S	S	R	$\underline{rec}^+$	'normal'	'normal'
ASH113-R3	ditto	R	S	S	R			
ASH113-R4	ditto	R	S	S	R			
ASH113-R5	ditto	R	S	S	R			
ASH113-R6	ditto	R	S	S	R			

(a) UV source and irradiation procedure as described in Methods, Section II

(b) Cultures of strains streaked across colicins E2 and E3 and incubated at 25°C

(c) Cultures of strains streaked in duplicate on NB plates containing 1% DOC

(d) Growth measured with Gilford microsample spectrophotometer, as described in Methods, Section II

(e) AP = 2-amino purine

(f) R = UV^r revertant

sensitivity, as well as E2-refractivity, then reversion of this gene would also produce UV^r phenotypes. In an attempt to clarify the site of the reversion mutations, *serB*$^+$ transductants were selected from a transductional cross between P1 grown on the UV^r revertant of ASH112, ASH112-R1, and the recipient *serB*$^-$ *refB*$^+$ strain, 4K-T$^+$. These transductants were replica plated to lawns of colicin E2 subsequently incubated at 25°C (Table 25). No *serB*$^+$ transductants selected were found to be colicin E2 refractive. This result indicates that the site of reversion at least in the various revertants tested, was at the original *refIIB* UV^s locus and not at some independent locus on the chromosome. However it does not necessarily follow that all UV^r revertants arising, but not tested, had reverted at the *refIIB* UV^s locus, and the only partial recovery in recombination proficiency of the UV^r revertant ASH113-R2 (Table 23) may be an example of an incomplete suppression of the Ref-II mutation, or of reversion at the *refIIA* locus.

3. Transductional Analysis of Pleiotropic Characters Associated with Ref-II UV^s Mutants

Ref-II and *serB*$^+$ transductants were obtained from the recipient strains 4K-T$^+$ and ASH5 after crosses with P1 grown through various Ref-II UV^s donor strains. These transductants were then tested for the different pleiotropic characters, to determine whether these characters were, in fact, subject to genetic separation by this technique. The results, covering a variety of experiments, are shown in Table 26.

TABLE 25

Transduction of serB from an F^- UV^r revertant of a Ref-II UV^s Mutant

Donor	Recipient	Transductants per 10^6 P1	No. scored	Transductants scored as%
				E2 refractive
ASH112-R1[(a)] ($serB^+$ refIIB)	4K-T^+ ($serB^-$ $refB^+$)	5.0	320	0

(a) UV^r revertant of the Ref-II UV^s mutant ASH112.

Transductional procedure was as described in General Methods; the proportion of the unselected $E2^r$ marker was determined by replica plating with subsequent incubation at 25°C for 24 hours.

Transductional and mating procedure, and conditions for screening for E2 refractivity, DOC sensitivity, filament formation and λ^r were as described in Methods, Section III.

TABLE 26

Transductional Analysis of Pleiotropic Characters Associated with Colicin E2 Refractivity

Pl Donor	Pl Recipient	Selected Marker	Transductants per 10^6 Pl	No. scored	No. of Transductants tested & scored as:					
					$E2^r$	DOC sens.	UV^s	rec^-	fil^+	λ^r
Expt. 1										
ASH112 ($E2^r$)	4K-T^+ ($E2^s$)	$E2^r$	0.3	19	19	19	19	19[b]	-	-
ASH113 ($E2^r$)	4K-T^+ ($E2^s$)	$E2^r$	0.4	38	38	12[a]	12[a]	38[b]	-	-
Expt. 2										
ASH112 ($\underline{serB}^+$ $E2^r$)	4K-T^+ ($\frac{\underline{serB}^-}{E2^s}$)	$\underline{serB}$	4.4	435	103	103	103	-	-	103
Expt. 3										
ASH112 ($E2^r$)	ASH5 ($E2^s$)	$E2^r$	3.2	14	14	14	14	1[c,d]	-	14
ASH113 ($E2^r$)	ASH5 ($E2^s$)	$E2^r$	1.9	4	4	4	4	4[d]	-	-
ASH114 ($E2^r$)	ASH5 ($E2^s$)	$E2^r$	2.1	14	14	14	14	1[c,d]	-	14
ASH111 ($E2^r$)	ASH5 ($E2^s$)	$E2^r$	2.3	14	14	14	14	1[c,d]	14[e]	-

(a) 12 out of 12 tested were UV^s and DOC^s. (b) $\underline{rec}^-$ character was scored from number of $\underline{leu}$ $\underline{str}^r$ recombinants in 60 minute mating with HfrH. (c) 1 out of 1 tested was $\underline{rec}^-$. (d) $\underline{rec}^-$ character scored from number of $\underline{arg}$ $\underline{str}^r$ recombinants in each cross with HfrP10. (e) 14 tested originally were $\underline{fil}^+$. After broth culture following plate storage for 14 days at 4^oC, 3 were $\underline{fil}^+$.

The pleiotropic characters, i.e. Rec$^-$,DOC^S, $\lambda^{"R"}$ and filament formation were, in fact, all found to be co-transducible with the refIIB locus, and furthermore showed 100% linkage to this locus. All these effects appear therefore to derive from extremely closely linked, if not identical genes. However, in the case of transduction of the fil$^+$ character from ASH111 into ASH5, 14 Ref-II transductants were initially selected and examined for filament formation and all were fil$^+$, but a storage of the "streaked-out" transductants on NB agar plates for 14 days at 4°C and subsequent NB subculture, on microscopic examination revealed that only 3 out of 14 had retained the fil$^+$ property. It seems likely, therefore, that the genetic environment provided by ASH5 modified the expression of this semi-lethal character.

Discussion and Conclusions

The position of the refIIB locus in mutants refractory to colicin E2 was determined first by linkage analysis, and then more precisely by interrupted mating using Ref-II male strains transferring the chromosome either in clockwise or anti-clockwise directions. These latter mapping studies were greatly facilitated by the proximal position of the refIIB locus on the HfrH chromosome and allowed a fairly precise position of 1-2 minutes to the left of thr to be assigned to it.

Further mapping studies with the transducing phage P1 have shown this locus to be co-transducible with, and to lie in a counter-clockwise position to serB. Furthermore, the refII locus is closely linked (93%)

to the hsp (host controlled modification) locus but the exact positional relationship of the refIIB locus to this marker was not conclusively established. When applying the formula derived by Wu (1966) of

$$\text{cotransduction frequency} = \left[1-\left(\frac{\text{distance between two markers}}{\text{length of transducing fragment}}\right)\right]^3$$

and taking the length of the transducing fragment to be 2/90 of the E.coli chromosome (Willetts, Clark and Low, 1969), the refIIB and serB loci are 0.8 minutes apart, and the refIIB and hsp loci are 0.04 minutes apart. Taking the average molecular weight of a protein to be 40,000 and assuming that 10^7 base pairs are present per DNA molecule, it can be calculated that the refIIB and hsp loci are 3-4 genes apart.

Colicin E2 refractory mutants have been found to fall into two distinct classes (Section II). The majority of mutants were refractory to colicin E2 with no associated pleiotropic characteristics, and a minority of mutants displayed a concurrent sensitivity to UV irradiation and low concentrations of the detergent sodium deoxycholate. The latter class contains mutants with a spectrum of additional properties. These include a number of mutants deficient in conjugal recombination (see Section IV). Others are unable to support the growth of bacteriophage λ. One has a pronounced tendency to grow in characteristic filaments, and one produces a mucoid capsule. This strain also appears unable either to receive or maintain episomal DNA (Section IV). Transductional analysis of the refIIB locus of one such Ref-II UV^S mutant, ASH112,

indicated a linkage to serB (20%) identical to that of the E2-refractivity marker of a conventional Ref-II UV^r mutant, ASH102. It is apparent, therefore, that if mutants of the Ref-II and Ref-II UV^s class do reflect different cistrons affecting E2-refractivity they must be extremely closely linked.

To determine whether the classes of E2-refractory mutants are a reflection of different cistrons, and if so, the number of cistrons involved, complementation analysis should be undertaken. For this purpose, it will be necessary to construct a heterogenote of the following genotype, assuming that in fact two cistrons are involved:

$$\frac{\text{refIIB} + \quad \text{refIIA}}{\text{refII } UV^s \quad \text{refIIA}}$$

Preliminary experiments were in fact undertaken to construct such a heterogenote. The P1-mediated transduction of the refII UV^r locus into the episome of the F' strain JM1 (F' thr^+ leu^+ refIIA/thr^- leu^-) was attempted, but experiments have so far been abortive. In addition, preliminary experiments which attempted the formation of refIIB +/refB refII UV^s zygotes from crosses between the Ref-II UV^r male strain ASH54 and the Ref-II UV^s F^- strain ASH112 were also unsuccessful, since no recombinants were obtained.

Genetic analysis of the UV^s locus by interrupted mating failed to separate the refIIB and UV^s loci in ASH112. This result was confirmed by P1 mediated transduction, when UV sensitivity and moreover the detergent sensitivity of the mutants was found to be 100% co-transducible with

the Ref-II gene. Similarly, somewhat limited analyses of associated phenotypic characters such as recombination deficiency, bacteriophage λ "resistance" and filament formation have also shown these to be 100% co-transducible with the Ref-II gene. These results are indicative of the derivation of these pleiotropic effects from mutations in very closely linked, if not identical genes. Further evidence for the close relationship of these genes was provided by the isolation of UV resistant revertants of the Ref-II UV^s strains ASH112 and ASH113, which were found to have simultaneously reverted to colicin E2 sensitivity, to be $\underline{rec}^+$ and to have regained normal growth characteristics and DOC resistance.

It must be emphasised, though that reversion in all pleiotropic characters may not be found in all UV^r revertants selected by this method. Indeed, the partial recovery in recombination proficiency displayed by the UV^r revertant ASH113-R2 (Table 23) may be an example of incomplete suppression of the refIIB UV^s mutation by a further, UV-induced, suppressor mutation. However the disappearance of E2-refractivity in all $\underline{ser}B^+$ transductants selected from one of the revertants does eliminate the possibility of a second, independent mutation influencing some aspect of UV repair, at least in this strain. This result also rules out the possibility that the reversion mutation occurred at the refIIA locus, if in fact the refIIA gene also controls the expression of pleiotropic characters associated with colicin E2-

refractivity. If reversion had taken place at the refIIA locus, then E2-refractivity would have been expressed in approximately 20% of the serB$^+$ transductants, following the transduction of the refIIB and serB genes into the refIIA serB$^-$ recipient strain, 4K-T$^+$

The isolation of UV^r E2^s revertants also makes it unlikely that the pleiotropic characters associated with E2-refractivity have arisen from a deletion covering several closely linked genes. All the genetic evidence suggests on the contrary that all the characteristics of Ref-II UV^s strains derive from a mutation or mutations in a single gene.

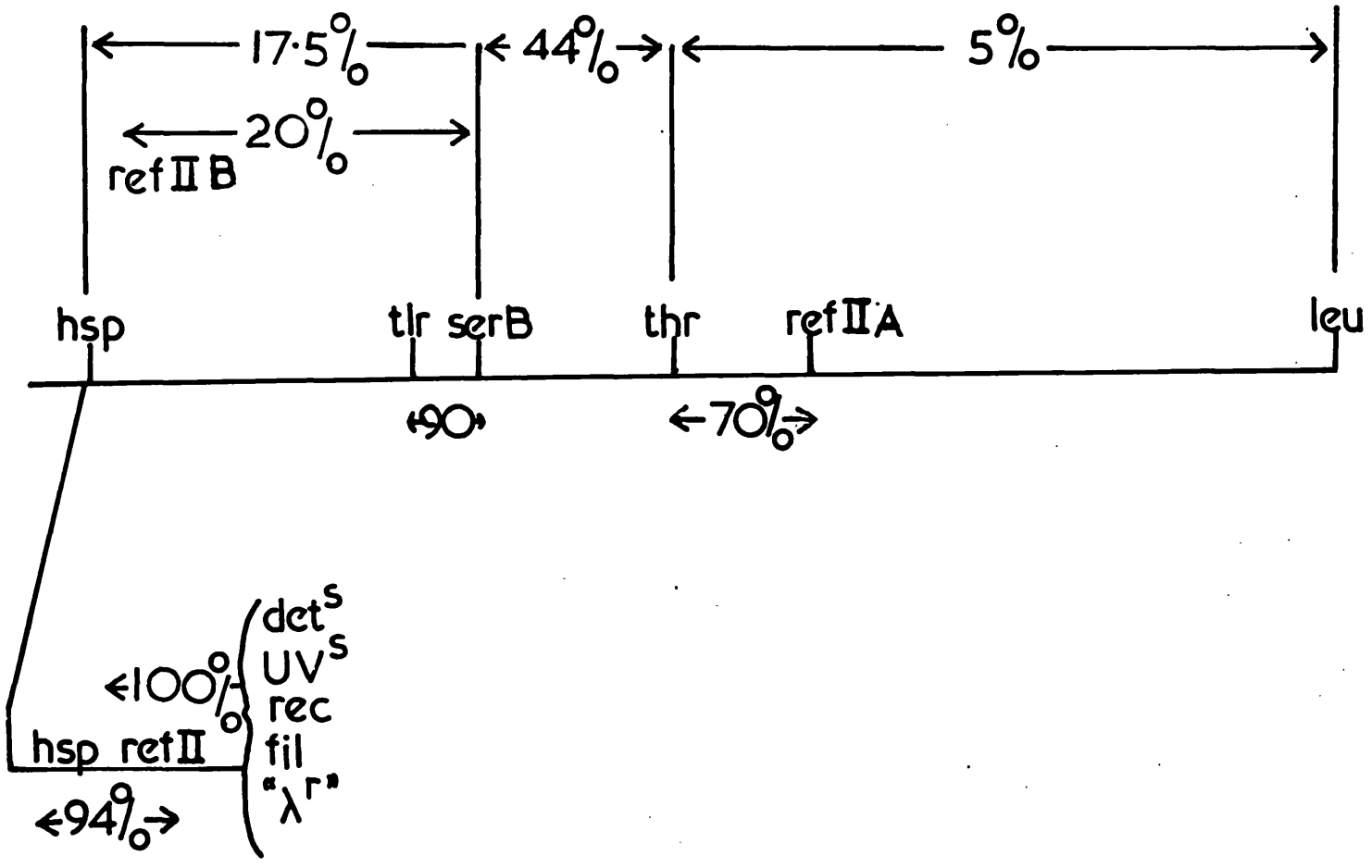
The actual chromosomal map position of the refIIB locus (Fig.17) is of some interest since several genes associated with various aspects of DNA metabolism are located in this limited area of the E.coli chromosome. Thus Ahmad and Pritchard (1969) have located four very closely linked genes involved in the catabolism of nucleosides and deoxynucleosides, for example the tlr gene, with 20% linkage to the hsp locus, and lying to the left of serB. In addition, the genes for the restriction and modification of foreign DNA (Lederberg, 1966; Wood, 1966; Arber 1968; Glover and Colson, 1969) also show very close linkage to the refIIB gene, and Meselson and Yuan (1968) have demonstrated that at least one such restriction enzyme is an endonuclease, possibly membrane bound, which degrades foreign DNA.

It was originally reported by Van de Putte, Zwenk and Rörsch,(1966) that one or more Rec loci were located in the serB-thr region. These

Fig. 17 Linkage of the hsp-refIIB-serB-thr-refIIA-leu genes.

Linkage data derived from transductional analysis (Section III).

P1 TRANSDUCTION MAP



Rec genes have however since been shown conclusively to map in the thy-cys region of the chromosome (Willetts, Clark and Low, 1969; Willetts, personal communication), and the anomalous results of Van de Putte et al have been ascribed to the probable presence of a genetic suppressor near thr-leu (Van de Putte, personal communication). In this respect it is interesting to speculate on the possible role of the E2-refractivity modifying gene, refIIA, as a genetic suppressor situated in this region of the chromosome. The ability of refIIA and refA$^+$ strains to support the growth of amber and ochre mutants of bacteriophage T4 may well assist in the clarification of the role of the refIIA gene. For example, if it was found that refIIA and not refA$^+$ strains were carrying a genetic suppressor, the position of the suppressor mutation could be mapped, and the correlation, if any, between this and the refIIA gene determined.

The Ref-II UV^S mutants, unlike RecA, B and C mutants investigated by other workers, were not recombination deficient as transductional recipients. Thus the recombination of phage carried homologous DNA is not impaired in these mutants. However it cannot be eliminated that this function is provided by superinfecting P1 particles, since P1 was used at a multiplicity of circa 10 in these experiments. In this respect, although no evidence is available at the present time relative to phage P1, it is interesting to consider certain results obtained by Franklin (1967) with phage λ. She isolated deletion mutants of λ which

had lost the ability to recombine genetically in Rec^- bacterial strains, yet which were capable of recombination in Rec^+ strains. Presumably then a bacterial enzyme or enzymes present in the Rec^+, but not the Rec^- host cell, are common to the recombination process in the infecting phage. If such a system is equally applicable to phage P1, it may be possible that superinfecting P1 can complement the Rec^- function of the host bacterium. For clarification of this possibility, there is scope for future experiments investigating the recombination deficiency of the Ref-II UV^S mutants under conditions of single infection of P1.

The 100% co-transducibility of the UV sensitivity and recombination deficiency with the Ref-II gene in Ref-II UV^S and Ref-II UV^S Rec^- mutants clearly distinguishes them from other UV sensitive mutants previously described by other workers. Thus the refII UV^S locus, linked to serB , is quite distinct to the sites, closely linked to thy, where RedA, B and C loci are located (Emmerson, and Howard-Flanders, 1967; Willetts, Clark and Low, 1969). Similarly this locus is distinct from the various uvr loci (Mattern, Zwenk and Rörsch, 1966), and from the lon^- and env loci (Normark, Boman and Matsson, 1969), located to the right of leu on the chromosome map.

With the exception of the UV^S mutants, the Ref-II mutation itself was found to have no effect upon recombination frequencies when present in the F^- strain and when crosses were carried out at 37°C and 25°C,

the latter temperature being that at which E2 refractivity is maximally expressed. Reduced recombination frequencies were however also sometimes obtained in crosses in which the refIIB allele was present in a male strain. This effect was restricted to Ref-II derivatives of ASH1, a male strain carrying the refIIB gene as a distal marker. This phenomenon was not due to an incompatibility between male and female DNA, comparable, for example, to that observed in inter-species restriction, since with both male and female strains carrying the refIIB gene, reduced recombination frequencies were still observed. This effect is still not understood, and should be investigated at least with different male strains.

The F^- strains ASH112 and ASH113 were defective in the capacity for the formation of prototrophic recombinants in crosses with male strains carrying the ref^+ gene as a proximal marker, by factors of 10 and 4.5×10^2 respectively. When the refIIB locus from these strains was transduced to a $refB^+$ refIIA female strain carrying additional auxotrophic markers and mated with a male strain carrying the $refB^+$ allele as a distal marker, the recombination deficiency with both mutants was about 100. The reason for the different behaviour of the mutants with different male strains is not clear. However Holland (personal communication) has found that ASH112 is completely refractive to colicin E2 in NB culture at 37°C as well as at 25°C, whereas ASH113 is completely sensitive at 37°C. On the other hand ASH112 has been

found (Section II) to display a much greater sensitivity to low concentrations of DOC than ASH113. When coupled with the recombination differences, the quantitative differences of these Ref-II UV^S mutants, both in E2-refractivity and DOC sensitivity, possibly suggests the presence of yet a third cistron associated with E2-refractivity in the hsp-serB region of the chromosome. However this cannot be investigated further without fine structure mapping and complementation analysis.

SECTION IV

FURTHER STUDIES INTO THE NATURE OF COLICIN E2 REFRACTIVITY, RECOMBINATION DEFICIENCY AND UV SENSITIVITY IN REF-II AND REF-II UV^S MUTANTS

Introduction

Colicin E2 refractivity, as discussed in the Introduction, may be imagined to arise by changes in several independent systems. For example, refractivity could result from a change in some cell membrane component thus preventing the transmission of the effect of the colicin at the extracellular receptor site to the chromosome and/or the cell division machinery. Alternatively, if it is assumed that the lethal effect of E2 is DNA breakdown, DNAases which presumably are at some stage activated by E2 may be defective in mutant strains. A third possibility, that modification of the DNA may render this immune to nuclease attack, must also be considered. With the first possibility, development of refractivity in zygotes after the transfer of the mutant allele into a sensitive cell might be expected to involve the resynthesis of structural cell components, and would presumably not be expressed for at least one generation. However if sensitivity was recessive to refractivity, and refractivity was a consequence of a defective enzyme, the expression of refractivity may be expected almost immediately on the entry of the mutant gene into a sensitive cell. To establish the genetical nature of the mutation to E2-refractivity, the kinetics of

the expression of refractivity after transfer of the refIIB allele into suitable sensitive strains was determined, and appropriate refB$^+$/refIIB heterogenotes were also constructed.

Secondly, studies initiated into the nature of the recombination deficiency of Ref-II UV^S mutants, and into the expression of UV resistance after formation of refB$^+$ UV^r/Ref-II UV^S heterogenotes are described in this Section.

Materials and Methods

Bacterial Strains. All ref$^+$ strains are shown in Table 1, and unless otherwise stated, all carried the refIIA allele. Ref-II mutants were isolated from ASH10 by 2-amino purine treatment.

Phage. The male specific RNA phage μ was initially obtained from R.C. Young.

1. Preparation of stocks of μ phage

Aliquots (0.1 ml) of μ phage and a NB grown stationary phase culture of the male strain ASH3 were mixed in equal proportions; after standing at 37°C for 5 min they were plated in 4 ml NB soft agar onto NB agar plates, and incubated at 37°C for 14 hours. Confluent lysis was observed and top layer of phage was then harvested, shaken in 2 ml buffer on a BTL rotory shaker for 15 min to release the phage, and the centrifuged supernatant retained. This was shaken with a few drops of chloroform, assayed on stationary phase ASH3 (HfrH) and retained.

2. Mating conditions

(a) Interrupted mating was carried out as described in Methods, Section III. For the direct selection of E2-refractive zygotes, samples from the mating mixture were blended in ice-cold NB plus streptomycin; these samples were then incubated at 37°C without shaking for 2 hours, and aliquots were removed at intervals, diluted through buffer and plated onto NB agar plates supplemented with 5×10^3 units/ml of colicin E2. Plates were incubated at 25°C for 24 hours and the E2-refractive colonies emerging were replica plated to plates containing colicin E2 and colicin E3 at 25°C or 40°C to confirm the Ref-II phenotype.

(b) F' transfer. F' and F^- strains were grown aerobically at 37°C in M9 supplemented with requisite amino acids to 5×10^7 and 5×10^8 cells/ml respectively; equal volumes were mixed together at 37°C with slow shaking for episome transfer to take place. After 15 minutes episome transfer was terminated by the addition of streptomycin and the mixture was diluted through buffer and plated on appropriate M9 agar plates for the selection of various classes of prototrophic merozygotes. Plates were incubated at 37°C for 36 hours unless otherwise indicated. For the direct selection of UV resistant heterogenotes, after streptomycin addition incubation was continued at 37°C and samples were removed at intervals over a three hour period; subsequent dilutions were plated onto TB agar and streptomycin plates and exposed to a 40 second UV dose

as previously described in Methods, Section II. Plates were then incubated in darkness for 14 hours at 37°C, and emergent colonies tested for episome status. To confirm the F^+ condition of heterogenotes, single colonies were resuspended in 0.5 ml buffer, and a small inoculum spotted with a wire loop onto NB agar plates. When this drop of culture had dried, a dilution of a suspension of the male specific μ phage was placed in such a way as to overlayer half of the drop of culture under test. After 8 hours incubation at 37°C, the plates were observed; F^+ cultures characteristically displayed a half-moon zone of inhbition of growth where in contact with the phage suspension, whereas F^- control cultures displayed normal confluent growth.

3. β-galactosidase formation, induction and assay

With certain modification, the procedure employed was based upon that described by Pardee, Jacob and Monod (1959).

(a) Single cultures

(i) Induction of β-galactosidase. As the zygote concentration after mating has to be increased 20 fold to give a sufficient number of cells to facilitate easy detection of β-galactosidase, it was necessary to increase the concentration of the single culture controls to equal that in the final mixture. 10 ml of NB was therefore inoculated with 0.5 ml of overnight cultures of appropriate strains. These were then grown with shaking at 37°C to a cell density of 2×10^8 cells/ml in the case of the male Hfr strain, and 4×10^9 cells/ml in the case of the various F^-

strains. For the induction of β-galactosidase, 0.5 ml of iso-propyl-thio-β-D- galactoside (IPTG) was then added to the respective cultures. 0.2 ml duplicate samples were immediately removed for enzyme determination; shaking was continued at 37°C and further samples were removed at 30, 60, 90 and 120 minute intervals. When the induction of β-galactosidase in the male strain in the presence of streptomycin was estimated, streptomycin was added to the culture 10 minutes prior to the addition of IPTG.

(ii) Release of β-galactosidase

0.2 aliquots of samples were pipetted into tubes containing 1.8 ml distilled water and 1 drop of toluene. The tubes were vigorously shaken on a "Whirlymixer" (Fisons Scientific Apparatus Ltd.) for 30 seconds and incubated at 37°C for 30 minutes to ensure enzyme release.

(iii) Assay of β-galactosidase

To the toluene treated samples 2.5 ml of 0.1 M Tris buffer, pH 8.0 was added and to initiate the reaction, 0.5 ml of O-nitrophenol-β-D-galactoside (ONPG) was then added. The mixture was incubated at 37°C for 30 minutes to allow the development of a sufficient colour intensity. The reaction was terminated by the rapid addition of 0.5 ml of M Na_2CO_3, after which samples were allowed to stand at room temperature for 30 minutes before OD determinations.

Optical density was measured with a Gilford 300 Microsample Spectrophotometer at 420 mμ against a reagent blank. The amount of enzyme present was calculated from a standard curve of μmoles of ONPG against spectrophotometric reading.

(b) Mixed cultures

(i) Mating conditions

Overnight NB cultures of the male strains and the respective F^- strains were inoculated into fresh NB and grown with aeration to approximately 5×10^8 cells/ml. Cultures were Coulter counted, and adjusted to cell densities of 2×10^7 cells/ml for the male strain and 4×10^8 cells/ml for the F^- strains. 20 ml of the Hfr strain and 20 ml of the F^- strains were then mixed and shaken slowly at 37°C for 30 minutes; streptomycin was added, the mixtured blended vigorously for 1 minute in the Whirlymixer, and shaken for a further 10 minutes at 37°C to complete the kill of the Hfr strain.

The mating mixture was then centrifuged and resuspended in 2ml of NB for β-galactosidase estimation as previously described.

4. Chemicals

Stocks of the chemicals ONPG and IPTG were obtained from Sigma Chemical Co., St Louis, U.S.A.

Results

A. Phenotypic Expression of Colicin E2 Refractivity in Ref-II Mutants

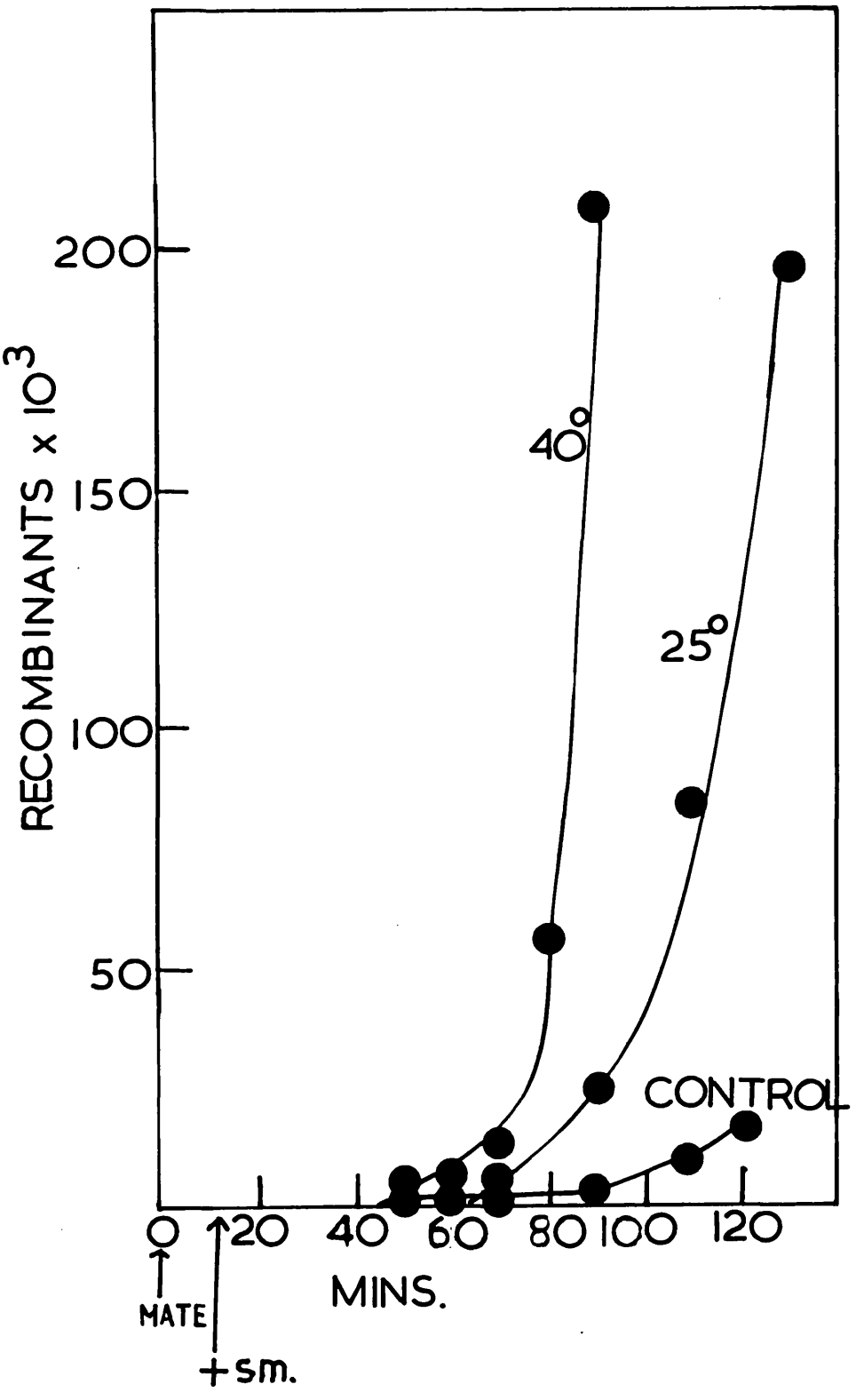
1. Kinetics of Expression

To analyse the kinetics of phenotypic expression of E2 refractivity, an E2 refractive male strain was mated with an E2 sensitive female strain and the time at which E2 refractivity became apparent in the zygotes was determined.

For this purpose ASH54, an E2 refractive derivative of the HfrH ref$^+$ strain ASH3, was mated with the ref$^+$ F$^-$ strain ASH5. After ten minutes, the mating was interrupted. Stationary incubation was continued at 25°C and 40°C in NB supplemented with streptomycin to prevent further growth and chromosome transfer by the streptomycin sensitive male strain and the expression of E2 refractivity followed. As a control, cultures of the Hfr and F$^-$ strains were mixed in the presence of streptomycin and incubated at 40°C. The spontaneous mutation of the F$^-$ strain to E2 resistance under these conditions was followed by plating the mixture on lawns of colicin E2 at intervals. The number of resultant colonies obtained was taken as an indication of the spontaneous rate of mutation to colicin E2 resistance under the conditions of the experiment. From the results, shown in Figure 18, it can be seen that following incubation at 40°C and 25°C, E2 refractivity was expressed in the zygotes after a time lag of 70 and 100 minutes respectively. This indicated that the mutation was either recessive to the wild type,

Fig. 18 Time of phenotypic expression of colicin E2 refractivity

ASH54 (Ref-II HfrH) crossed with ASH5 (ref$^+$ F$^-$). Mating terminated after 10 minutes, and incubation continued for 2 hours at 25° and 40°C; samples removed at 10 minute intervals and plated onto lawns of 5 x 10^3 units/ml colicin E2 for incubation at 25°C and 40°C. Control experiment to determine rate of spontaneous mutation to E2 under conditions of experiment.


RECOMBINANTS x 10^3
200
150
100
50
40°
25°
CONTROL
0
20
40
60
80
100
120
MINS.
MATE
+sm.

or if dominant, expression of refractivity requires resynthesis of, for example, structural cell constituents.

2. Formation of Heterogenotes

Heterogenotes were next constructed from strains carrying the ref$^+$-thr-leu region on extrachromosomal episomes.

For this purpose, two F' strains were initially used, KLF1 (refB$^+$ thr$^+$ leu$^+$/refB$^+$ thr$^-$ leu$^-$) and KLF4 (refB$^+$ thr$^+$ leu$^+$ pro$^+$/refB$^+$ thr$^-$ leu$^-$ pro$^-$). Cultures of each F' strain were mixed with cultures of the F$^-$ strain, ASH102 (refII leu$^-$) and after 15 minutes the mixture was diluted through buffer and plated on appropriate M9 agar plates for the selection of leu$^+$ heterogenotes. After incubation for 36 hours, 100 leu$^+$ colonies from each cross were taken, resuspended in 0.5 ml buffer, and tested for presence of the episome with the male specific μ phage as described in Methods, Section IV. By this procedure, 40 F' heterogenotes from each cross were obtained and tested for E2 refractivity, as shown in Table 27. Without exception, all refB$^+$/refIIB heterogenotes were colicin E2 sensitive. Any of the original leu$^+$ classes which were found to be refractive to colicin E2 were subsequently found to have lost the episome: clearly therefore, E2-refractivity is recessive to sensitivity in such heterogenotes.

To determine whether the apparent dominance of the refB$^+$ allele over the refIIB allele was in fact true, it was necessary also to establish the status of the refA gene on the episome. This could only

TABLE 27

Expression of Colicin E2 Refractivity in ref^+/refII Heterogenotes

Donor	Recipient	Frequency of Episome Transfer	No. of Heterogenotes tested	Response to colicin (25°C) E2	E3
KLF1 $\left\{\frac{refB^+\ thr^+\ refIIA\ leu^+}{refB^+\ thr^-\ refA^+\ leu^-}\right\}$	ASH102 (refIIB thr^+ refIIA leu^-)	1.3	40	S	S
KLF4 $\left\{\frac{refB^+\ thr^+\ refIIA\ leu^+\ pro^+}{refB^+\ thr^-\ refA^+\ leu^-\ pro^-}\right\}$	ASH102 (refIIB thr^+ refIIA leu^-)	0.9	40	S	S

Two F' strains ($\underline{ref}^+\ \underline{thr}^+\ \underline{leu}^+/\underline{ref}^+\ \underline{thr}^-\ \underline{leu}^-\ \underline{his}^-\ \underline{arg}^-$) were mated with ASH102, a Ref-II derivative of the F^- strain ASH10 ($\underline{leu}^-\ \underline{thy}^-\ \underline{met}^-$). Exponential cultures (2×10^8 cells/ml) of each strain were mixed; after 15 min selection was made for $\underline{leu}^+$ heterogenotes by the exclusion of his and arg from the selective medium. These were subsequently tested for colicin E2 refractivity by cross streaking over colicins E2 and E3 followed by incubation at 25°C for 24 hours.

be assumed to be refIIA, since the episome was derived by Low (1968) from an HfrH strain analogous to that from which Ref-II mutants were derived in this study. The refB$^+$/refIIB heterogenotes constructed from the cross between KLF1 (F' refB$^+$ thr$^+$ refIIA leu$^+$) x ASH102 (F$^-$ refIIB thr$^+$ refIIA leu$^-$) were therefore presumably homozygous for the refIIA allele. Thus the refB allele was truly dominant over the refIIB allele.

B. Studies into the Nature of Recombination Deficiency in Ref-II Mutants

As described in Section III, certain Ref-II UV^S strains were defective in genetic recombination following mating with suitable Hfr strains. However unlike RecA, B and C mutants which may actually be deficient in some recombination enzyme, the Ref-II UV^S mutants as recipients did not display any significant reduction in transduction frequencies.

For further insight into the nature of recombination deficiency in these strains, experiments were carried out to determine their capacity to receive genetic material from male strains.

1. Episome Transfer

(a) Construction of a streptomycin sensitive, F' thr$^+$ leu$^+$/thr$^-$ leu$^-$ heterogenote

For the ease of selection of thr$^+$ leu$^+$ heterogenotes without recourse to auxotrophic deficiencies and for the determination of the expression of UV resistance in Ref-II UV^S mutants, described later in

this Section, a *str*^{s} F' heterogenote with an extrachromosomal episome covering the *refB*$^{+}$-*thr*-*leu* region of the chromosome was constructed (Methods, Section IV). Thus several heterogenotes with the genotype *thr*$^{+}$ *leu*$^{+}$/ *thr*$^{-}$ *leu*$^{-}$ *his*$^{+}$ *arg*$^{+}$ *str*^{s} were selected after transfer of the episome from KLF1 (F' *thr*$^{+}$ *leu*$^{+}$/*thr*$^{-}$ *leu*$^{-}$ *his*$^{-}$ *arg*$^{-}$ *str*^{r}) into strain 158 (F^{-} *thr*$^{-}$ *leu*$^{-}$ *str*^{s}). One such strain, JM1, was then employed as the F' donor strain in subseqeunt crosses with Ref-II UV^{s} mutants.

(b) Ability of the Ref-II mutants to receive episomal DNA

For this purpose, cultures of the F' strain J.Co.1 (F' *lac*$^{+}$/*thr*$^{-}$ *leu*$^{-}$ *lac*$^{-}$ *thy*$^{-}$ *str*^{s}) and JM1 (F' *thr*$^{+}$ *leu*$^{+}$/*thr*$^{-}$ *leu*$^{-}$ *str*^{s}) were mixed with cultures of ASH10 (F^{-} *leu*$^{-}$ *lac*$^{-}$ *thy*$^{-}$ *met*$^{-}$ *str*^{r}) and Ref-II derivatives of ASH10. Selection was made after mating with J.Co.1 for *lac*$^{+}$ heterogenotes, and after mating with JM1 for *thr*$^{+}$ *leu*$^{+}$/*thr*$^{-}$ *leu*$^{-}$ heterogenotes (Table 28).

The results, shown in Table 28, indicate that, with the exception of one Ref-II UV^{s} mutant, ASH116, the ability of Ref-II UV^{s} mutants to receive and maintain episomes did not differ from that shown by parental controls or the Ref-II UV^{r} strain, ASH102. ASH116, although somehow defective in the maintenance or receipt of episomal DNA, was not deficient in recombination following conjugation with the HfrH strain (see Section III). It may be possible, however that the formation of the mucoid capsule particularly secreted by this mutant after growth on minimal agar (Section II) may in some way be correlated with the

TABLE 28

Ability of Strains of E.coli K12 Carrying Ref-II UV^S Mutations to Receive Episomal DNA

Donor	Recipient	Frequency of transfer (% input male)
J.Co.1 (F' lac^+/ thr^- leu^- lac^- thy^- str^S)	ASH10 (ref^+ leu^- lac^- thy^- met^- str^r)	1.6
J.Co.1	ASH102 (Ref-II UV^r)	2.2
J.Co.1	ASH112 (Ref-II UV^S)	2.1
J.Co.1	ASH113 (Ref-II UV^S)	1.0
J.Co.1	ASH115 (Ref-II UV^S)	1.9
J.Co.1	ASH116 (Ref-II UV^S)	0.06
JM1 (F' thr^+ leu^+/ thr^- leu^- str^S)	ASH102 (Ref-II UV^r)	3.7
JM1	ASH112 (Ref-II UV^S)	2.5
JM1	ASH113 (Ref-II UV^S)	1.8
JM1	ASH115 (Ref-II UV^S)	2.4
JM1	ASH116 (Ref-II UV^S)	0.1

Cultures of F' and F^- strains were mixed for 15 min at cell densities of 5×10^7 and 5×10^8 cells/ml respectively as described in Methods, Section IV. Selection was made for lac^+ heterogenotes in one case, and for thr^+ leu^+ heterogenotes in the other.

inability to form mating pairs under these conditions. In relation to the Ref-II UV^S Rec^- mutants tested, these results clearly demonstrated that the inability to form prototrophic recombinants after conjugation with suitable male strains does not arise from failure to form mating pairs.

2. β-galactosidase Induction in Merozygotes

It may be considered that although the ability of the recombination deficient Ref-II UV^S mutants to receive episomes was not significantly impaired, chromosomal entry from Hfr males may involve some different mechanism. To test this hypothesis, the transfer of *lac* genes from a male donor to the Ref-II UV^S *rec*$^-$ mutants ASH112 and ASH113 was studied directly by the determination of the ability of merozygotes to synthesise β-galactosidase after induction with isopropyl-thio-β-D-galactoside (IPTG).

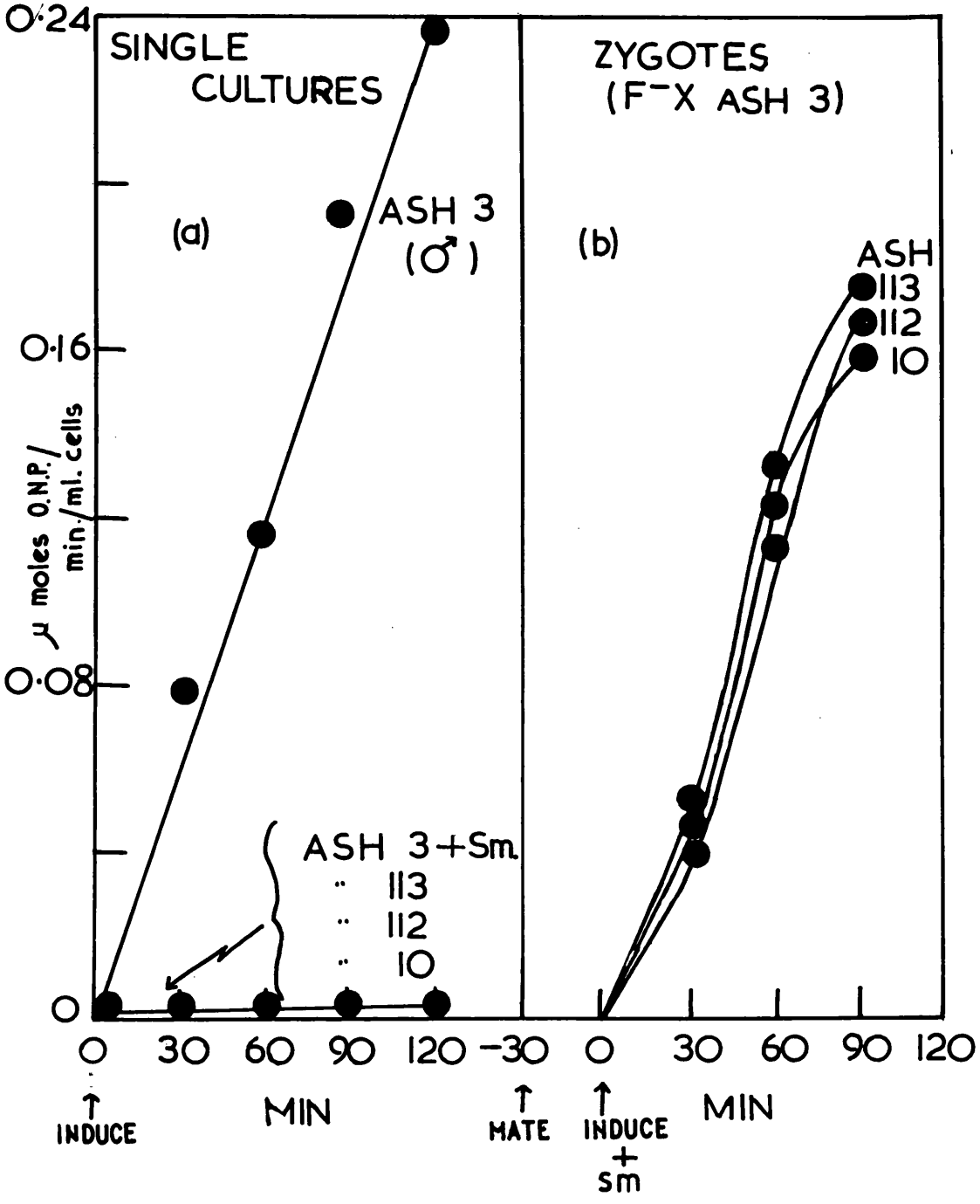
Initially, the synthesis of β-galactosidase after induction was followed in single cultures of the parental F^- strain ASH10 (*leu*$^-$ *lac*$^-$ *thy*$^-$ *met*$^-$ *str*^{r}), the Ref-II UV^S derivatives ASH112 and ASH113 and the donor HfrH strain ASH3 (*lac*$^+$ *str*^{s}), with and without streptomycin in the culture medium (Fig. 19).

The F^- strains were mated with the HfrH donor ASH3 for 30 minutes prior to interruption and the addition of streptomycin to kill the male strain, thus preventing further pair formation. The zygote concentration was first increased 20-fold by centrifugation and

Fig. 19 The Induction of β-galactosidase in merozygotes formed after crosses between a male donor strain and certain Ref-II UV^S mutants

(a) The F^- strains ASH10, ASH112 and ASH113 (leu⁻ lac⁻ thy⁻ met⁻ str^S) and the HfrH donor strain ASH3 (lac^+ str^S) were grown to densities of 4×10^9 and 2×10^8 cells/ml respectively. β-galactosidase was induced as described in Methods, Section IV, and followed by the removal of samples at 0, 30, 60, 90 and 120 minutes after induction. For the estimation of β-galactosidase in the male strain in the presence of streptomycin (8 mg/ml), the latter was added to the culture 10 minutes prior to IPTG addition. The assay of β-galactosidase is described in Methods, Section IV.

(b) 20 ml volumes of the F^- and Hfr strains at densities of 4×10^8 and 2×10^7 cells/ml respectively, were shaken slowly at 37°C for 30 minutes; streptomycin was added, the mating interrupted (Whirlymixer) and shaking continued for a further 30 minutes to complete the kill of the male strain. The mating mixture was centrifuged and concentrated x 20 before enzyme determination.


0·24
0·16
0·08
0
μ moles O.N.P./ min./ml. cells
SINGLE CULTURES
(a)
ASH 3 (♂)
ASH 3+Sm
" 113
" 112
" 10
ZYGOTES (F⁻ X ASH 3)
(b)
ASH 113
112
10
0 30 60 90 120
MIN
INDUCE
−30
MATE
0 30 60 90 120
MIN
INDUCE + sm

resuspension in a small volume before induced synthesis of β-galactosidase in the zygotes was determined. As shown in Figure 19(b) there was no difference either in the rate or the amount of β-galactosidase synthesised in the zygotes formed from the two Ref-II UV^S mutants ASH112 and ASH113, or the parental control strain ASH10. It may therefore be concluded that chromosomal entry into Ref-II UV^S strains is normal. In addition, the continued expression of the lac genes after transfer also shows that the failure to form recombinants does not apparently arise from rapid degradation of donor DNA.

C. Phenotypic Expression of UV Sensitivity in Ref-II UV^S Mutants

As described in Section II, Ref-II UV^S Rec^- mutants differed in several respects from conventional Rec^- mutants. Thus this class of refractory mutants, unlike the RecA, B and C mutants, were relatively insensitive to X-ray irradiation, were sensitive to low concentrations of detergent (sodium deoxycholate) in the growth medium, and in some cases were able to recover from the effects of UV irradiation on the inclusion of D-L-pantoyl lactone in the post-irradiation growth medium.

It was therefore of considerable importance to investigate further the nature of the concurrent UV sensitivity in these refractory mutants. For this purpose, heterogenotes were constructed with an extrachromosomal episome covering the refIIB UV^S-thr-leu region of the chromosome of Ref-II UV^S strains. The kinetics of the development of

UV resistance in such heterogenotes was then compared to that in a heterogenote constructed with an episome covering the thy-recB region of a RecB mutant.

1. Formation of Heterogenotes

Leu$^+$ heterogenotes were selected from a cross between the Ref-II UV^S strains ASH112 and ASH113 (refII UV^S leu$^-$ str^S) and the F' strain JM1 (F' ref$^+$ UV^r thr$^+$ leu$^+$/thr$^-$ leu$^-$ str^S). In all 10 leu$^+$ clones were obtained and the presence of the episome confirmed by testing with the male specific RNA phage μ. These were then tested for colicin E2 refractivity and UV sensitivity by screening methods previously described. Without exception, all heterogenotes of the type ref$^+$ UV^r leu$^+$ /refII UV^S leu$^-$ were both colicin E2 sensitive and UV resistant.

The recessive nature of UV sensitivity was anticipated from similar results found with E2 refractivity, and is also compatible with the recessive nature of UV^S in Rec$^-$ mutants (Willetts, personal communication). However this finding does not clarify the nature of the defect in the Ref-II UV^S mutants and with this objective in mind, the kinetics of the expression of UV resistance in newly formed heterogenotes was determined and compared to that in heterogenotes of a RecB mutant, JC4457.

2. Kinetics of Expression

Cultures of the Ref-II UV^S strains ASH112 and ASH113 (F$^-$ refII UV^S leu$^-$ str^r) were mixed for 15 minutes with the F' strain JM1

(F' ref$^+$ UV^r leu$^+$/ ref$^+$ UV^r leu$^-$ str^s) and episome transfer terminated by the addition of streptomycin. Incubation was then continued for a further 3 hours and samples were removed at intervals, diluted, plated and the number of UV^r heterogenotes determined. As a control the response to UV of single cultures of ASH112 and ASH113 over the period of post-irradiation incubation was also determined.

Similarly, heterogenotes of the type thy$^+$recB$^+$/thy$^+$recB$^-$ were constructed from a cross between JC4457 (F$^-$ recB$^-$ str^r) and an F' derivative of CR34 (F' thy$^+$ recB$^+$/thy$^-$ recB$^+$ str^s) and the expression time of UV resistance in the heterogenote also determined. Results are shown in Figure 20. It is clear from Figure 20(a) and (b) that in ref$^+$ UV^r/refII UV^s heterogenotes, UV resistance does not increase for at least 60 minutes after mating, despite rapid transfer of the episome. However with recB$^+$ UV^r/recB$^-$ UV^s heterogenotes, the expression of UV resistance commenced almost immediately after the receipt of the episome. This latter behaviour may be expected since the RecB mutants lack a specific exonuclease probably involved in recombination and repair (Buttin and Wright, 1969). In contrast, the large delay in the expression of wild type UV resistance in the Ref-II UV^s mutant corresponding approximately to 1 generation time is compatible with the replacement of some structural component of the cell prior to the efficient operation of a "UV-repair system".

A rather curious result was obtained, however, with heterogenotes formed from the Ref-II UV^s mutant, ASH112 (Figure 21). Initially this

Fig. 20 Time of phenotypic expression of UV resistance in Ref-II UV^s mutants and a conventional recB mutant

(a) JC4457 (recB$^-$ UV^s) crosses with CR34 (F' recB$^+$ UV^r)

(b) ASH113 (Ref-II UV^s) crossed with JM1 (F' ref$^+$ UV^r)

UV irradiation as described in Methods, Section II.

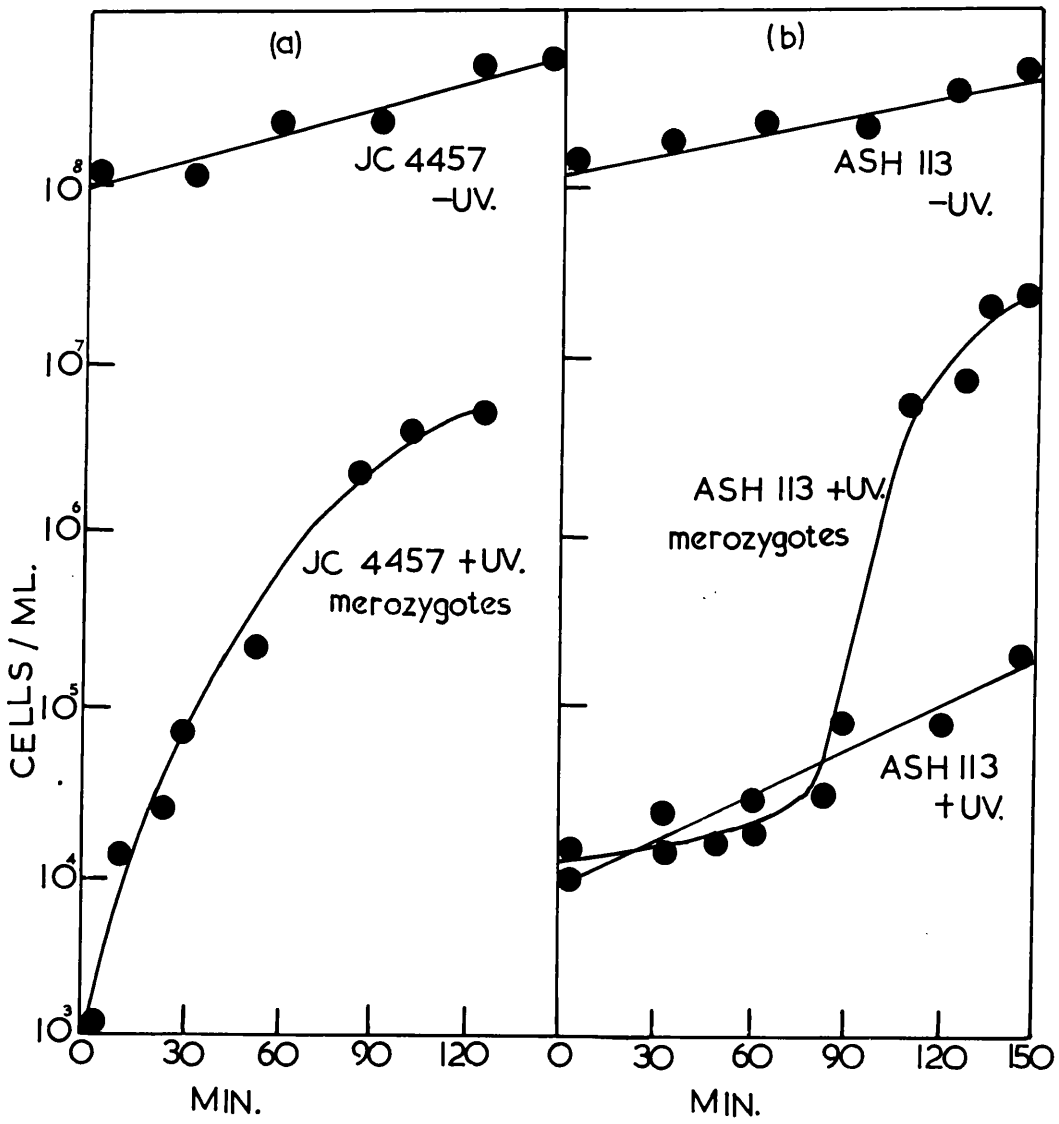
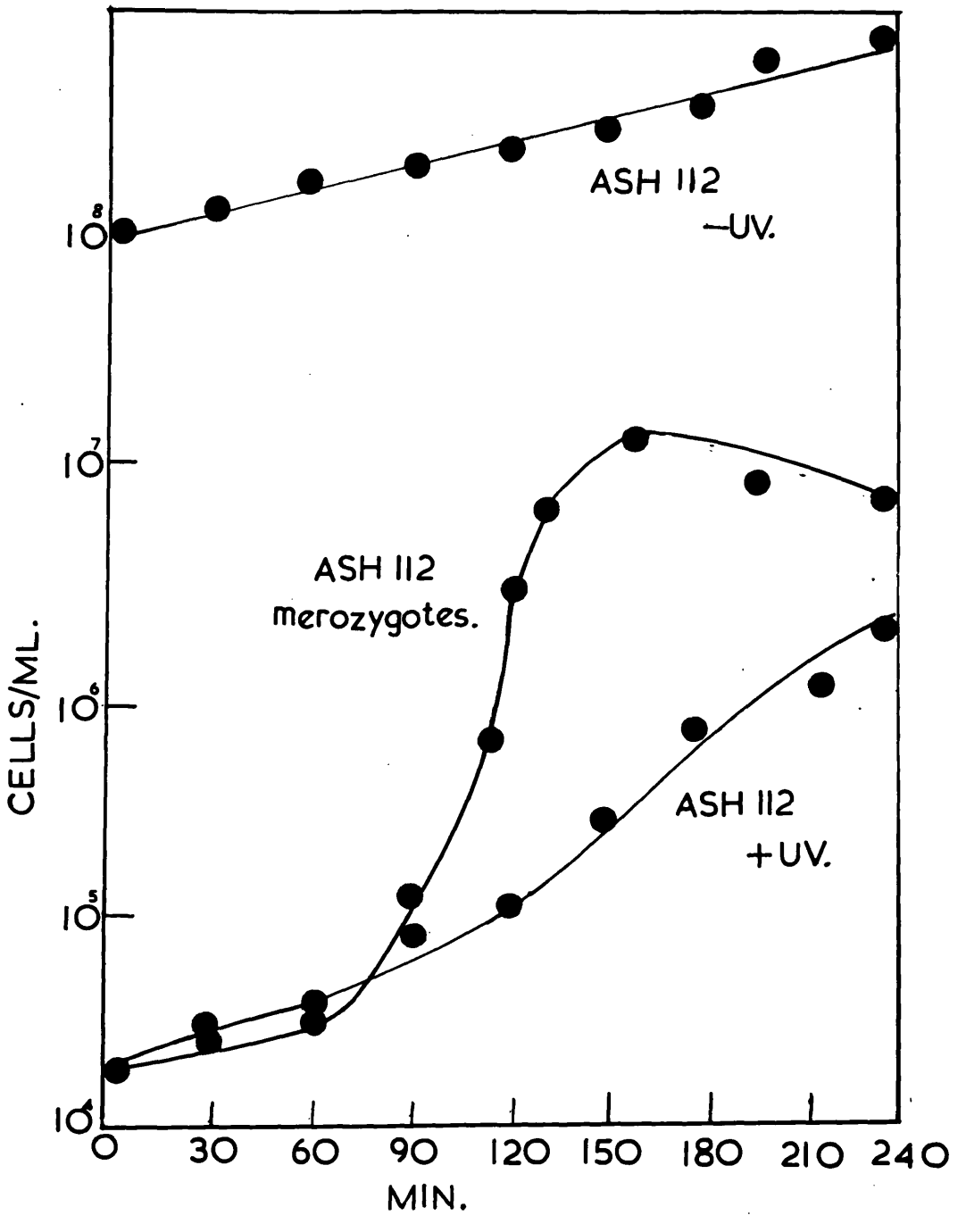

(a)
(b)
JC 4457
-UV.
ASH 113
-UV.
JC 4457 +UV.
merozygotes
ASH 113 +UV.
merozygotes
ASH 113
+UV.
CELLS / ML.
10^8
10^7
10^6
10^5
10^4
10^3
0
30
60
90
120
150
MIN.
MIN.

Fig. 21 Time of phenotypic expression of UV resistance in the Ref-II UV^s mutant, ASH112

ASH112 (Ref-II UV^s) crossed with JM1 (F' *ref*$^+$ UV^r). UV irradiation as described in Methods, Section II.


ASH 112
-UV.
ASH 112
merozygotes.
ASH 112
+UV.
CELLS/ML.
10^8
10^7
10^6
10^5
10^4
0
30
60
90
120
150
180
210
240
MIN.

strain behaved like ASH113 with UV resistance being finally expressed in all heterogenotes after circa one generation time. Confirmation of this is received from the number of leu$^+$ heterogenotes (2.5% - Table 28) obtained after such a cross which approximately corresponded to the maximum level of UV^r heterogenotes obtained. As shown in Figure 21, however, after 150 minutes the number of UV^r heterogenotes of ASH112 stopped increasing and possibly even declined, whereas the number of UV^r heterogenotes of ASH113, although not shown in Figure 20(b), continued to increase almost to the level of the unirradiated control. This effect in ASH112 may indicate that the episome is not replicating in many heterogenotes, or that this effect reflects death of the zygote corresponding to, for example, the lethal sectoring found with other UV^s Rec mutants (Haefner, 1968).

Discussion and Conclusions

The expression of colicim E2 refractivity in zygotes formed from a mating between a Ref-II male and E2-sensitive female strains took place after incubation for 70 and 100 minutes at 40° and 25°C respectively. This result implied that E2-sensitivity was dominant, and the refIIB allele was recessive to the corresponding refB$^+$ allele. This was indeed confirmed by the sensitivity of refB$^+$ refIIA/refIIB refIIA heterogenotes to colicin E2. Furthermore it was found that UV sensitivity in heterogenotes formed from Ref-II UV^s mutants, like colicin E2-refractivity, was recessive to UV resistance, and in this

respect was similar to the UV sensitivity of Rec$^-$ mutants (Willetts, personal communication).

However when the kinetics of the expression of UV resistance in ref$^+$ UV^r/refII UV^s heterogenotes, formed after the transfer of a suitable episome, was determined and compared to a similar UV^r/UV^s heterogenote constructed from a RecB mutant, a long delay in the expression of the wild type character, UV resistance, was noted. Now similar patterns of expression have been observed in the formation of resistance to many bacteriophages, and have been ascribed to a replacement of the phage receptor on the bacterial cells surface (Hayes, 1968(b). The long delay in the expression of UV resistance in Ref-II UV^s/ref$^+$ UV^r heterogenotes is also compatible with the formation of a structural component, which presumably has to be replaced before some aspect of UV-damage can be overcome. The synthesis of a new repair enzyme in Ref-II UV^s/ref$^+$ UV^r heterogenotes, which probably occurs in the RecB heterogenote employed, is unlikely. Indeed, the delayed expression of E2-refractivity in zygotes may also be ascribed to the formation of structural components in the cell in addition to the replacement of the dominant refB$^+$ allele.

The nature of the recombination deficiency of the Ref-II UV^s rec$^-$ mutants ASH112 and ASH113 was investigated by determining the capacity of these strains to receive genetic material from male strains, and the results indicated that both chromosomal and episomal DNA transfer

to the mutants was normal. The failure of these strains to form prototrophic recombinants, therefore, is not due to some defect in pairing with male strains, and, as lac genes continue to be expressed after chromosome transfer, cannnot apparently be ascribed to a rapid degradation of donor DNA. Moreover, as a basic difference between these Ref-II UV^S Rec^- strains and conventional Rec mutants in their ability to recombine with P1 transmitted, homologous DNA (Hertman and Luria, 1967) has already been established and commented on in Section III, it is suggested that these mutants therefore constitute a new class of Rec mutants.

DISCUSSION

The chain of components in a bacterial cell permitting the specific and successful action of an extracellular colicin very likely includes cell surface adsorption sites, a transmission system presumably located within the cytoplasmic membrane, and a final biochemical target. The colicin molecules neither appear to penetrate the cell surface layer, nor cause gross cell membrane change (Nomura, 1967) and interaction with the cell must therefore be of a highly specific and unique nature.

The studies described here have been concerned with the basis of sensitivity to colicin E2. This colicin, one of the E group of colicins is characterised by its induction of a rapid degradation of DNA (Nomura, 1963) and the inhibition of cell division in sensitive bacteria (Holland, 1968). To facilitate this study, mutants refractory to colicin E2 were isolated from certain strains of E.coli K12. These refractory mutants, although still adsorbing the colicin molecule to specific E group receptors in the cell surface, fail to induce degradation of the intracellular DNA, and the cells survive. The mutants have presumably become blocked at some late stage in the transmission of the effect of the extracellular colicin to the intracellular target. Alternatively these mutants may have undergone some change in the final target, for example, the DNA,preventing DNA breakdown and so rendering the cells refractory to colicin E2. If, in

particular, these mutants are of the first type, it is probable that refractivity is a direct result of some change in the cell membrane. It was hoped therefore that a genetical and physiological study of mutants refractory to colicin E2 would provide information on the mechanism of action of colicin E2, and moreover yield evidence of the functional organisation of the cell membrane in bacteria.

Mutants refractory to colicin E2 (designated Ref-II) were initially isolated by 2-amino purine treatment from a female strain of E.coli K12 ASH10, and subsequently from several other strains of E.coli K12. The position of the refIIB locus was determined first by linkage analysis and then more precisely by interrupted mating, using various Ref-II male strains. From these results, the refII locus was positioned 2-3 minutes to the left of thr on the bacterial chromosome. Further mapping studies with the transducing phage P1 have shown this locus to be co-transducible with serB (20%) and closely linked (93%) to the hsp locus. Thus the refIIB locus can be clearly distinguished from multi-refractory loci previously mapped near gal (De Zwaig,and Luria, 1967; Nomura and Witten, 1967; Hill and Holland, 1967 - see Table a) and from a locus near thyA controlling specifically refractivity to colicin E1 (Clowes, 1965; De Zwaig and Luria, 1967; Nomura and Witten, 1967; Hill and Holland, 1967).

Although the exact positional relationships of the refIIB and hsp loci were not conclusively established in this study, these loci

are however quite distinct. Thus several Hsp$^-$ mutants tested by Holland (1967) were not refractory to colicin E2, and likewise Ref-II mutants tested in this study displayed normal host specific restriction. However the very close linkage of a gene determining sensitivity to colicin E2 to a locus known to determine the formation of a DNAase, probably surface bound, (Meselson and Yuan, 1968) may not be fortuitous since the primary action of colicin E2 may be specific degradation of the cell DNA. Further study of the relationship between these genes and between the enzymes determined by these genes should be rewarding.

Considerable difficulties were encountered in the transductional analysis by the lack of expression, or lethality of the refIIB gene in the particular serB$^-$ thr$^-$ recipient strain initially employed for this purpose. Furthermore, it had previously proved impossible to isolate Ref-II mutants from several strains of E.coli. Such results suggested the involvement of a second gene, either affecting the phenotypic expression or the otherwise lethal effects of E2 refractivity. Subsequent transductional studies in fact indicated that a second gene affecting E2 refractivity was closely linked (70%), and probably to the right of thr on the bacterial chromosome. The transduction of this gene from a strain from which Ref-II mutants may be derived to one in which they cannot, for example the serB$^-$ transductional recipient strain 4K, then allowed the expression of E2-refractivity and was also contingent with the isolation of Ref-II mutants from such

a strain. The role of this second gene, tentatively designated refIIA in colicin E2 action is not yet known. This gene may determine the formation of a further membrane component, or an enzyme involved in the synthesis of some membrane component which is essential for the transmission of the colicin effect. However if the refIIA gene does in fact affect some component in the cytoplasmic membrane, this is not reflected by any significant increase in the sensitivity of Ref-IIA strains to the detergent sodium deoxycholate (DOC), although this does not eliminate any subtle changes in membrane structure. Alternatively the second gene may be acting as a genetic suppressor which can, for example, suppress an otherwise lethal effect of the Ref-II mutation. Indeed, if one accepts that the temperature dependent expression of E2-refractivity in all Ref-II mutants derives from mutation at a limited number of mutational sites, and that only such mutations can be modified by a second gene, then it can be postulated that all other mutations in the refB gene cannot be modified and are lethal. The frequency of Ref-II mutants isolated from refB^{+} refIIA strains is considerably lower than the frequency of other refractory mutants and it may be further postulated that even the conditional refractivity of Ref-II mutants to colicin E2 is a partially lethal character. In the case of the Ref-II UV^{s} mutants, which display significantly reduced growth rates and plating efficiencies when compared to wild type, and RefII UV^{r} control strains, this is clearly

the case.

Ref-II mutants may be divided into two distinct phenotypic groups on the basis of sensitivity of the mutants to UV irradiation, and to the detergent DOC. The majority of the mutants showed no increased sensitivity to UV or DOC, but approximately 34% of those tested were found to display increased sensitivity to both these agents. It may be postulated that these two phenotypic classes of Ref-II mutants reflect mutation in at least two separate cistrons, mutations in one cistron producing Ref-II UV^r DOC^r phenotypes, and in the other Ref-II UV^s DOC^s phenotypes. However transductional analysis revealed that if two Ref-II cistrons do exist, they are both 20-21% co-transducible with serB. It is therefore apparent that if mutation in two distinct cistrons can indeed produce colicin E2 refractivity, then these cistrons must be extremely closely linked, and probably are adjacent on the chromosome. If more than one cistron is present, then intergenic complementation between these is theoretically possible. For this purpose, the construction of a strain carrying either the refII UV^r or the refII UV^s DOC^s mutation on an extrachromosomal episome was attempted in this study. Regretably this attempt was abortive, but future investigation should concentrate on the formation of this class of heterogenote for the determination of the precise number of Ref-II cistrons in the hsp-serB area of the chromosome, and their relationship with other genes

in this area. As will be discussed later, this is now a crucial task in further clarification of the genetical nature of colicin E2-refractivity and the associated pleiotropic characters.

The majority of the Ref-II UV^s class of colicin E2-refractory mutants were found to possess additional pleiotropic characters not shown by the Ref-II UV^r mutants. For example, the majority showed poor growth rates in complex media and also displayed morphological cell surface abnormalites, such as the extrusion of almost free spheroplasts, when examined microscopically under phase contrast. Out of six strains examined, four were also deficient in some aspect of genetic recombination. Furthermore one such Rec^- mutant in particular was also characterised by a filamentous growth pattern, and three more mutants had a reduced capacity to support the growth of bacteriophage λ. Another mutant, although not recombination deficient in crosses with at least one male strain showed a reduced capacity to receive (or replicate) episomal DNA, and also produced a mucoid capsule when grown on minimal agar. Only the UV sensitivity, recombination deficiency and DOC sensitivity of these Ref-II UV^s mutants was examined at both 25°C and 40°C, but it was apparent that these pleiotropic characters at least, unlike E2-refractivity, were not temperature dependent. At the same time, it proved impossible to sub-divide the Ref-II UV^s mutants in any simple way into discrete phenotypic groups on the basis of their different pleiotropic

characters.

To account for the complex pleiotropy of the Ref-II UV^s mutants, including UV and deoxycholate sensitivity, three genetic models may be postulated.

(1) Pleiotropic characters may have arisen through deletions covering several closely linked genes, concerned with, for example, UV irradiation repair, recombination events and the replication of phage λ in the cell in addition to colicin E2-refractivity.

(2) Secondly, pleiotropy may be the result of a series of closely linked, multisite mutations induced by the mutagen, 2-amino purine.

(3) Finally, pleiotropy may derive from different mutations in a single gene.

Each possibility will now be discussed in turn.

1. Are Ref-II UV^s Strains Deletion Mutants?

To test the first hypothesis, the isolation of UV resistant revertants of the two Ref-II UV^s mutants ASH112 and ASH113 was attempted. Such revertants were in fact readily selected, and moreover were found to be colicin E2-sensitive, and to have regained normal recombination proficiency and DOC sensitivity. However it could not be overlooked that UV^r revertants of the Ref-II UV^s mutants could have arisen through the suppression of the refIIB UV^s mutation by a further UV induced mutation. An equally applicable hypothesis, if the refIIA gene does in fact potentiate the expression of UV sensitivity in

addition to E2 refractivity, is that reversion or suppression of the refIIA gene could give rise to UV^r revertants. Although the site of the reversion mutation was not carefully mapped in these studies it was found that E2-refractivity no longer segregated with serB after the transduction of $serB^+$ from at least one UV^r revertant strain into a $serB^-$ E2-sensitive recipient. In this strain, therefore, it appears most probable that the reversion mutation occurred in the refIIB gene and not in the refIIA gene or at any external suppressor locus. This result thus indicates that Ref-II UV^s mutants do not arise as a result of deletions spanning independent refractivity and UV sensitivity loci.

At this point it is interesting to note how further studies on UV^r revertants of Ref-II UV^s mutants could clarify the role of the refIIA gene. For example, if the refIIA gene merely potentiates the expression of E2-refractivity and UV sensitivity, then some UV^r revertants should arise through reversion at the refIIA locus. Re-introduction by transduction of the refIIA allele should then restore E2-refractivity. Alternatively, if the refIIB gene is lethal to any particular strain in the absence of the refIIA gene then it should be impossible to select UV^r revertants arising from reversion to $refA^+$. Therefore, by screening a large number of revertants to determine the status of the refIIA allele, the role of the refIIA gene in either the expression of E2-refractivity or in the masking of lethality of the refIIB gene should be apparent.

If the mutation in the Ref-II UV^s strains did produce an alteration in a membrane protein which impaired the ability of a UV repair enzyme to bind to the cell membrane, then a second, UV induced mutation may produce enzymes now capable of correct binding to the mutated membrane site. In this event, it may be expected that UV^r revertants would be E2-refractive, but this was not observed in any of the UV^r revertants selected and tested.

Further, albeit negative, evidence against the likelihood of the pleiotropic characters arising through a deletion covering several genes is the failure to isolate any Ref-II mutants which are also hsp$^-$. Since these loci are only 3-4 genes apart, deletions covering the refIIB gene might be expected to extend into the hsp genes. In addition, although several tlr$^-$ loci have been mapped by Ahmad and Pritchard (1969) tothe left of, and 20% co-transducible with serB, of several Ref-II UV^s mutants examined by Beacham (personal communication), all were Tlr^+.

Finally, the temperature sensitivity of all the Ref-II mutants strongly suggests an actual refIIB gene product with a functional conformation at high, but not low temperatures. This fact alone cannot be explained in terms of a deletion covering the refIIB gene.

2. Are Ref-II UV^s Strains Multisite Mutants?

The second possibility, that the pleiotropic characters have arisen through a series of multisite mutations in closely linked

genes is unlikely in view of the mutagen employed in the isolation of Ref-II mutants in this study. Thus unlike nitrosoguanadine (NMG) which has been shown to induce multiple, but closely linked mutations in E.coli (Cerda-Olmedo, Hanawalt and Guerola, 1969) there is no evidence that the base analogue 2-amino purine induces anything other than single point mutations (Hayes, 1969(a)). Direct evidence to this effect comes from genetical analysis of the association of the pleiotropic characters with E2-refractivity. Initial interrupted mating experiments indicated a very close linkage between UV sensitivity and E2 refractivity, and a later more comprehensive transductional analysis failed to separate E2 refractivity, UV sensitivity and DOC sensitivity. Similarly, although analyses were rather limited, the recombination deficiency, bacteriophage λ "resistance" and filament formation found in certain Ref-II UV^S mutants were also 100% co-transducible with E2-refractivity.

All available evidence therefore suggests that the Ref-II UV^S mutants carry single point mutations, and the complex pleiotropy of the mutants can best be reconciled on the basis of the following two models described below.

3. Are Ref-II UV^S Strains Point Mutants?

The first model assumes that the refB gene constitutes part of an operon concerned specifically with chromosomal metabolism and cell

surface formation. Mutations in this gene may then have strong polar effects upon adjacent genes (Ames and Martin, 1964; Yanofsky and Ito, 1966). As an example of this it was found by Whitefield, Martin and Ames (1966) that mutants of the aminotransferase (C gene) in the histidine operon of Salmonella, induced by 2-amino purine, were almost all either amber or ochre mutants. Although it was thought by these authors that missence mutations were also induced by 2-amino purine but escaped detection, this result does indicate the inducibility of amber and ochre mutants by 2-amino purine. However, if it is postulated that the refIIB mutation is in fact a chain terminating mutation, it would be difficult to reconcile this with the conditional nature of E2-refractivity, unless it was further postulated that the primary mutation is in fact partially suppressed in E2-refractory strains. The closely associated refIIA gene may well be thought to fulfill this role, functioning as an amber or ochre suppressor of the refIIB gene. Indirect evidence in support of the suppression of the refIIB gene by some further gene may be derived from the conditional nature of E2-refractivity in all Ref-II mutants, since a similar preponderance of temperature-sensitive phenotypes have been reported by Eggerton and Adelberg (1965), following the suppression of ochre and amber mutants of the ilv gene of E.coli. Further genetic analysis, however, is now necessary to determine whether in fact genetic polarity is operating in this system.

The second model to explain the pleiotropy of Ref-II UV^S mutants assumes that the refB$^+$ gene determines the synthesis of a single membrane component, the alteration of which affects a multitude of different cellular functions. This at first sight appears difficult, since although deoxycholate sensitivity, cell surface defects, and in once case excessive polysaccharide formation all suggest a defective membrane component concerned with cell surface integrity and metabolism, UV sensitivity and recombination deficiency suggest a loss of function concerned with some aspect of DNA metabolism.

Before attempting to reconcile all the pleiotropic characters in these mutants with one single mutational change, one should first consider the different ways in which each character might in fact arise.

(a) Deoxycholate Sensitivity. Changes in at least two systems may produce an increased sensitivity of the Ref-II UV^S mutants to deoxycholate. For example, sensitivity may have directly arisen from an alteration in some cell surface or membrane constituent normally participating in the transmission of the colicin E2 effect from the extracellular receptor site to the intracellular target. Deoxycholate sensitivity could also be the result of general disturbance of cell surface metabolism in consequence of defective DNA metabolism arising from the mutation to E2-refractivity. In an extreme case, this may

also be manifest by disturbance in polysaccharide synthesis, as is in fact found to take place in the mutant ASH116 when grown on minimal agar. At the moment it is difficult to evaluate the increased sensitivity of the Ref-II UV^S mutants to deoxycholate in terms of specific membrane alteration wihtout further knowledge of the precise mode of action of this detergent. Fractionation of the membrane of the Ref-II UV^S mutants, by, for example, the technique of Nagata, Shibuya and Morris (1967) is now required for the identification of the altered membrane protein in these mutants, and this may help clarify the accompanying increased detergent sensitivity.

Increased deoxycholate sensitivity is only evident in Ref-II UV^S mutants. This characteristic was not evident in UV^S Rec^- strains tested, nor has it been reported in other Uvr^- and Rec^- strains, or in temperature sensitive mutants of DNA synthesis in E.coli. Detergent sensitivity is therefore unlikely to result from a general disturbance of DNA metabolism.

(b) UV Sensitivity. To consider how UV sensitivity could have arisen in the Ref-II UV^S mutants, it is first necessary to discuss the properties of previously isolated, UV sensitive mutants and to compare them with UV^S strains isolated in this study. Thus UV sensitive mutants which no longer have the ability to repair UV damaged DNA fall into two broad but distinct categories. These are based upon the ability of different UV sensitive strains to reactivate irradiated

phage, or to form recombinants in genetic crosses. A first category incapable of the reactivation of irradiated phage (Uvr^-) in fact fail to excise pyrimidine dimers from UV damaged DNA (Howard-Flanders, Boyce and Theriot, 1966; Howard-Flanders and Boyce, 1966). These strains are sensitive to even very low doses of UV, yet are not affected by X-irradiation. The second category includes mutants deficient in the ability to form viable recombinants in genetic crosses with male strains. Such Rec^- mutants are sensitive to both UV and X-irradiation, yet unlike the Uvr^- mutants, are generally capable of host cell reactivation, i.e. they are Uvr^+ (Howard-Flanders and Theriot, 1966(a); Clark and Margulies, 1965; Clark, 1967; Van de Putte, Zwenk and Rörsch, 1966). It has been suggested (Howard-Flanders and Boyce, 1966) that both Uvr^- and Rec^- mutants are deficient in an enzyme or enzymes concerned with the repair of irradiated DNA. Subsequently in fact Grossman, Kaplan, Kushner and Mahler (1968) have found that an endonuclease, producing single-stranded breaks in UV-irradiated DNA, was lacking in certain UV sensitive mutants of Micrococcus lysodeikticus. This endonuclease appears to require Mg^{++} ions for activation, and in this respect is distinct from another endonuclease isolated from M. lysodeikticus by Takagi, Sekiguchi, Okubo, Nakayama, Shimada, Yasuda, Nishimoto and Yoshihara (1968) which is also specific for irradiated DNA. Takagi et al however found no

evidence for any UV sensitive mutant lacking the magnesium independent enzyme. In Rec^- mutants of E.coli, there is as yet no direct correlation between UV sensitivity and enzyme deficiency. However Buttin and Wright (1968) have shown that a nucleotide triphosphate dependent DNAase is apparently defective or absent in a RecB mutant, rec21.

A further class of UV sensitive mutants, although apparently able to carry out normal repair of UV-damaged DNA, are unable to divide after UV irradiation. These Lon^- and Fil^+ mutants are characterised by post-irradiation filament formation, interpreted by Walker and Pardee (1968) as an imbalance between DNA synthesis and either membrane or cell wall biosynthesis, leading to a failure of normal septum formation. Septum formation, and hence cell survival, can be increased by post-irradiation growth on media supplemented with pantoyl lactone.

Thus the UV sensitivity of the Ref-II UV^S class of Ref-II mutants may be envisaged to have occurred either through the loss of, for example, a repair enzyme. Alternatively, UV sensitivity may have arisen through alteration of a membrane component either affecting the control or activity of repair enzymes, if such enzymes are in fact membrane bound, or , as in Lon^- mutants, disturbing the cell division process. Since Ref-II UV^S mutants, like conventional Rec^- mutants, are in fact capable of the reactivation of irradiated phage (Holland, 1967), they appear capable of normal dimer excision. On theother hand, the delayed expression of UV resistance in $+ UV^r$/refII UV^S

heterogenotes suggests a defect in some structural component, presumably membrane, affecting UV sensitivity. Indeed, the UV sensitivity of the Ref-II UV^S mutants was similar to that reported for Lon^- mutants (Donch and Greenberg, 1968), and furthermore one Ref-II UV^S mutant out of three tested resembled Lon^- mutants by its post-irradiation, pantoyl lactone recovery. It may well be that not all Ref-II UV^S mutants can respond to pantoyl lactone, indicative that different Ref-II UV^S mutants are not necessarily sensitive for the same reason. Nevertheless, Ref-II UV^S mutants as discussed elsewhere (Section II), are quite distinct from Lon^- mutants.

Although evidence indicates that the UV sensitivity of Ref-II UV^S mutants derives from a defective structural component, this does not rule out the possibility that defects in other UV^S strains may also be altered in these mutants. For example, enzymes participating in the repair and recombination of DNA, and which are specified by the various Rec and Uvr loci, may normally be attached to the altered component in Ref-II UV^S mutants. Construction of Ref-II UV^S,Uvr^- and Ref-II UV^S Rec^- double mutants will prove critical in confirmation of this hypothesis.

(c) Recombination Deficiency. The recombination deficiency and UV sensitivity of Ref-II UV^S mutants may be completely independent defects, or alternatively be manifestations of the same pleiotropic effect. Before these possibilities can be discussed, it will be necessary to

outline the properties of Rec$^-$ mutants previously isolated, and how a recombination deficiency may be envisaged to arise.

Now Rec$^-$ mutants isolated by workers in other laboratories may be divided into two groups on the basis of recombination deficiency, UV sensitivity, inducibility of bacteriophage λ, and the DNA breakdown pattern after UV irradiation. Thus RecA mutants completely fail to form recombinants, they are very sensitive to UV and phage λ cannot be induced in lysogenic derivatives. When mutants of this group are irradiated, DNA breakdown is excessive or "reckless". In contrast, mutants of the second group, RecB and RecC mutants, display approximately a hundred to a thousand-fold reduction in recombinant formation, and are not as sensitive as the RecA mutants to UV. When irradiated there is less DNA breakdown in RecB and C mutants than in wild-type controls, i.e. they are said to be "cautious" in this respect, and prophage λ can be induced in lysogenic derivatives of these mutants. Although possessing similar properties, RecB and C mutants may, however, be distinguished genetically by transduction and complementation.

Several groups, for example Clark and Margulies (1965), Van de Putte, Zwenk and Rörsch (1966) and Clark (1967), have postulated that RecA, B and C classes of mutants are defective in an enzyme or enzymes which participate in the recombination process. However although an ATP dependent nuclease appears to be lacking in RecB and C mutants (Buttin and Wright,1968; Clark, personal communication), the nature

of the defective step in recombination in any Rec mutants has not yet been identified.

Although all Rec^- mutants that have so far been studied appear defective in some recombination enzyme, it is likely that recombination deficiency could arise in several different ways. Thus, for example, defective pairing of male and female cells, or the "restriction" of incoming DNA could produce recombination deficiency. Alternatively, the presence of a defective membrane component could also be envisaged to prevent the correct pairing of the female and incoming Hfr chromosomes by a variety of mechanisms. For example, current ideas indicate that in mating single-stranded DNA is transferred from the Hfr cell, and the complementary strand is rapidly synthesised in the recipient cell (Ohki and Tomizawa, 1968). Then Rec^- mutants could arise if either the transfer or replication of the incoming, single-stranded DNA is modified, or chromosomal pairing is prevented. It is easy to envisage that all these processes may involve a membrane component in some way.

The recombination defect in the Ref-II UV^S mutants could therefore have arisen in a number of ways, either involving some recombination enzyme directly, through failure of cells to pair, or through alteration of a membrane component. Results in this study indicated that defective cell pairing and restriction of incoming DNA appeared not to be the basis of the recombination deficiency, since lac genes were transferred,

and continued to be expressed in Ref-II UV^S mutants. It is also unlikely, for several reasons already discussed, that the defect in these mutants was similar to the enzyme defects in RecA, B and C mutants. In particular, Ref-II UV^S mutants, unlike Rec^- mutants, were relatively insensitive to X-irradiation, and were not recombination deficient in transductional crosses.

The discrepancy between conjugal and P1-mediated recombination in the Ref-II UV^S mutants is particularly intriguing, and the elucidation of this may contribute to an understanding of the recombination deficiency in Rec^- mutants. If this is in fact a real discrepancy, and is not the result of, for example, complementation by enzymes produced by superinfecting phage DNA, then two alternatives may be postulated. For example, the recombination of small phage-carried DNA fragments and the bacterial chromosome on the one hand, and recombination between larger chromosome pieces on the other, may be different processes. In this event a mutation in the "chromosome recombination pathway" may not necessarily affect the recombination of small fragments of phage carried DNA with the bacterial chromosome. An altered membrane component in theRef-II UV^S mutants may, for example, affect chromosomal pairing, but not episome transfer or infection, and hence recombination, by P1-transducing particles. An alternative, but relatively trivial explanation, is that zygosis in Ref-II UV^S mutants is lethal. For example, normal mating in Ref-II UV^S mutants may put

the cells in some imbalance, from which they cannot recover. In this event, both the recombination deficiency and the UV-sensitivity could be a manifestation of the same pleiotropic effect, and recombination proficiency, like the UV sensitivity of at least ASH113, may prove to be retrieved on the addition of pantoyl-lactone to the recombination and subsequent selective media. Before these possibilities may be resolved, it is first essential to determine the degree of the recombination deficiency in the Ref-II UV^S mutants. This can be achieved by actual measurement of formation of the enzyme β-galactosidase after crosses between lac$^-$ Ref-II UV^S mutants and control lac$^-$ mutants with a suitable lac$^-$ male strain.

(d) Bacteriophage λ "resistance". Bacteriophage λ "resistance" in Ref-II UV^S mutants might also be the result of an independent mutation. Thus λ "resistance" may be the result of some change in the activity of a restriction enzyme. This is unlikely, since Hsp$^-$ mutants tested were not refractory to colicin E2, nor did Ref-II mutants fail to restrict phage λ as do "conventional" Hsp$^-$ mutants. This possibility is made further unlikely, as in these studies λ "resistance" was found to be 100% co-transducible with E2 refractivity. Alternatively, λ "resistance" could be a further manifestation of an altered membrane protein, which directly or indirectly also promotes E2 refractivity, UV sensitivity, deoxycholate sensitivity, filament formation, and possibly recombination deficiency. The reduced ability of phageλ to

replicate in Ref-II UV^S mutants may then be analogous to the abortive replication of ØX174 DNA in Rep^- mutants (Denhardt, Dressler and Hathaway, 1967), or the failure of P2 to lysogenise lyd mutants of E.coli C (Sironi, 1969). These characteristics have been ascribed to an inability of the DNA of the respective phages to bind to altered membrane components in the mutants.

It is interesting to note that although ASH112 is "resistant" to λgv, the induction of λ prophage in this strain is normal. This result suggests some host-controlled difference in the properties of the DNA of the λ genome from different sources. Further investigations, for example, into the physico-chemical properties of DNA extracted from λ wild type and from λ,induced from a Ref-II UV^S mutant or other Rec mutants, may well confirm this observation, and help characterise the modification in the λ DNA.

All the various pleiotropic characters associated with colicin E2 refractivity may therefore have arisen directly through changes in different systems. However it appears more likely that all characteristic changes in the properties of the Ref-II UV^S mutants derive from a change in the integrity of, for example, a membrane protein. This protein, in addition to being specific for the transmission of the colicin E2 effect, may be concerned directly in the cell division process, and in the replication and recombination of both phage and bacterial DNA. Thus different mutations in the refIIB UV^S gene could

provoke different conformational alterations in this membrane protein, giving rise to UV-sensitivity, recombination deficiency, filament formation and λ "resistance", as has been outlined, in addition to E2-refractivity and DOC sensitivity.

An equally applicable hypothesis is that the refB gene product could be a structural membrane protein in a functional aggregate of proteins analogous to the arom cluster of enzymes, controlling steps two to six in the polyaromatic pathway prior to chorismic acid in Neurospora crassa (Giles, Case, Partridge and Ahmed, 1967), or the his enzyme cluster, catalysing the biosynthesis of histidine in yeast (Fink, 1966). Different mutations in the refB gene could then be envisaged to alter the association of different proteins bound to the refB gene product. If these proteins are in fact themselves variously concerned with recombination, UV repair, or the cell division process, then their dissociation or conformational alteration might be reflected in just the pleiotropic effects that are observed in the Ref-II UV^S mutants. If this model is correct, then it is possible that the products of some rec and uvr genes specified previously by other workers may well be affected by the refIIB UV^S gene. As indicated above, the properties of double mutants containing rec or uvr loci and the refIIB UV^S locus and the study of different UV resistant revertants of Ref-II UV^S mutants, may help resolve these possibilities.

Since at least two different phenotypes of Ref-II mutants were found in this study, Ref-II UV^r and Ref-II UV^s, it is possible that these have arisen through mutations in different cistrons. There is no evidence as yet that different cistrons do control E2 sensitivity, but if so, then they must be adjacent. However the refIIB UV^s and refIIB UV^r gene products may not be identical. Thus although UV resistance, and presumably the associated E2 refractivity in Ref-II UV^s mutants, was not expressed until after the renewal of a structural cell component, Ref-II UV^r mutants may produce, for example, a temperature-sensitive DNAase normally specific to colicin E2. This is unlikely, since without exception, Ref-II mutants were sensitive to colicin E2 when tested on minimal agar at both 25 and 40°C. E2 refractivity in Ref-II UV^r mutants, like Ref-II UV^s mutants, is more probably produced from conformational alteration of a membrane component.

In conclusion, the isolation of mutants refractory to colicin E2, but not to colicin E3, clearly indicated the participation of a cellular component specific to interaction with colicin E2 in sensitive bacteria. Support that this component is unique to the successful action of colicin E2 is also derived from the physiological properties of Ref-II mutants, by which they may readily be distinguished from other classes of refractory mutants. It may therefore be implied that

colicin action does indeed involve a multistep pathway, containing components specific for the lethal action of different colicins.

Mutants refractory to colicin E2 were frequently characterised by poor growth rates and cell surface abnormalities. These results are indicative of the participation of the altered cellular component in normal cellular metabolism. Moreover, since the primary observable effects of colicin E2 on sensitive cells are in fact DNA degradation and inhibition of cell division, the disturbed DNA metabolism and cell division abnormalities of at least some Ref-II mutants indicate an alteration late in the multistep pathway of colicin E2 action in these mutants.

Results in this study also indicate that UV sensitivity, and probably also E2 refractivity, in Ref-II UV^S mutants, is more likely to have arisen from alteration in a membrane, or membrane bound, component, than from, for example, loss of a soluble exonuclease. Indeed, the variety of pleiotropic characters displayed by Ref-II UV^S mutants is compatible with the theory that at least the refIIB UV^S gene product is a membrane protein. The association of E2-refractivity with a variety of functions concerned with DNA metabolism and cell division further suggests that the altered membrane protein in Ref-II UV^S mutants may well be one of an aggregate of proteins, possibly including the DNA attachment site (Tremblay, Daniels and Schaechter, 1969) concerned specifically with chromosomal metabolism and cell division.

REFERENCES

ABRAMS, A.,NIELSEN, L., & THAEMENT, J. (1964) Biochim. Biophys. Acta, 80, 325

AHMAD, S.I. & PRITCHARD, R.H. Molec.Gen.Genetics, 104, 351.

ANDERSON, H. (1946) Proc. Natl. Acad. Sci. U.S., 32,120.

AMES, B.N. & MARTIN, R.G. (1964) Ann.Rev. Biochem., 33, 235.

ARBER, W. (1969) XVIII Symp. Soc. Gen. Microbiol. p.295.

BARRY, G.T., EVERHART, D.L., ABBOT, V. & GRAHAM, M.G. (1965) Zentbl. Bakt. ParasitKde. (Abt.1) 196, 248.

BARRY, G.T., EVERHART, D.L. & GRAHAM, M.G. (1965) Nature (Lond), 198, 211.

BAZARAL, M. & HELINSKI, D.R. (1968) J.Mol.Biol., 36, 185.

BEN-GURION, R. (1963) J. Gen. Microbiol., 30, 173.

BEPPU, T. & ARIMA, K. (1967) J.Bacteriol., 93, 30.

BEPPU, T. & ARIMA, K. (1967) 7th Int. Congr. Biochem., Tokyo Abs. C10.

BORDET, P. (1948) C.R. Seanc. Soc. Biol., 142, 257.

BORDET, P. & BEUMER, J. (1948) C.R. Seanc.Soc. Biol., 142, 259.

BOYCE, R.P. & HOWARD-FLANDERS, P. (1964) Z.Vererbungslere 95, 334.

BRADLEY, D.E. (1967) Bacteriol. Rev., 31, 230.

BRADLEY, D.E. & DEWAR, C.A. (1965) J.Gen. Microbiol., 45, 399.

BUTTIN, G. & WRIGHT, M. (1968) Cold Spring Harbor Symp. Quant. Biol., 33, 259.

CAVARD, D. & BARBU, E. (1966) C.r. hebd. Seanc. Acad. Sci. Paris, 262, 1089.

CERDA-OLMEDO, E., HANAWALT, P.C. & GUEROLA, N. (1968). J. Mol. Biol., 33, 705.

CHANGEUX, J.P. & THIERY, J. (1967) J.Theoret. Biol., 17, 315.

CLARK, A.J., CHAMBERLIN, M., BOYCE, R.P. & HOWARD-FLANDERS, P. (1966) J. Mol. Biol., 19, 442.

CLARK, A.J. & MARGULIES, A.D. (1965) Proc. Natl. Acad.Sci., U.S. 53, 451.

CLOWES, R.C. (1963) Genet. Res., Camb. 4, 162.

CLOWES, R.C. (1965) Zentbl. Bakt. ParasitKde. Abt. 1. 196, 152.

COHEN, G.N. & MONOD, J. (1957) Bacteriol. Rev., 21, 169.

DENHARDT, D.T. & BURGESS, A.B. (1968) Cold Spring Harbor Symp. Quant. Biol., 33, 449.

DENHARDT, D.T., DRESSLER, D.H. & HATHAWAY, N. (1967) Proc. Natl. Acad. Sci., 57, 813.

DE ZWAIG, N., ANTON, D.N. & PUIG, J. (1962) J. Gen. Microbiol., 29, 473.

DE ZWAIG, N. & LURIA, S.E. (1967) J.Bacteriol., 94, 1112.

DE ZWAIG, N. & PUIG, J. (1964) J.Gen. Microbiol., 36, 311.

DONCH, J., GREEN, H.L. & GREENBERG, J. (1968) J.Bacteriol., 96,1704.

DONCH, J. & GREENBERG, J. (1968) Molec. Gen. Genetics, 103, 105.

EARHART, C.F., TREMBLAY, G.Y., DANIELS, M.J. & SCHAECHTER, M. (1968) Cold Spring Harbor Symp. Quant. Biol., 33, 707.

EGGETSSON, G. & ADELBERG, E.A. (1965) Genetics, 52, 2, pt.1, 319.

EMMERSON, P.T. & HOWARD-FLANDERS, P. (1967) J.Bacteriol., 93, 1729.

FIELDS, K.L. & LURIA, S.E. (1969) J. Bacteriol., 97, 57.

FINK, G.R. (1963) Genetics, 53, 445.

FRANKLIN, N.C. (1967) Genetics, 57, 301.

FREDERICQ, P. (1946a) C.r. Seanc. Soc. Biol., 140, 1033.

FREDERICQ, P. (1946b) C.r. Seanc. Soc. Biol., 140, 1057.

FREDERICQ, P. (1948a) Rev. Belge. Path. med. exptl. 19, suppl.4 1.

FREDERICQ, P. (1948b) C.r. Seanc. Soc. Biol., 142, 543.

FREDERICQ, P. (1948c) C.r.Seanc. Soc. Biol., 142, 853.

FREDERICQ, P. (1950) C.r. Seanc. Soc, Biol., 144, 297.

FREDERICQ, P. (1951) Antonie van Leeuwenhoek, 16, 41.

FREDERICQ, P. (1952a) C.r. Seanc. Soc. Biol., 146, 1295.

FREDERICQ, P. (1952b) C.r. Seanc. Soc. Biol., 146, 298.

FREDERICQ, P. (1953) C.r. Seanc. Soc. Biol., 147, 357.

FREDERICQ, P. (1956) C.r. Seanc. Soc. Biol., 150, 1514.

FREDERICQ, P. (1958) Symp. Soc. exp. Biol., 12, 104.

FREDERICQ, P. (1965) Zentbl. Bakt. ParasitKde. (Abt.1) 196, 140.

FREDERICQ, P. & BETZ-BAREAU, M. (1951) C.r. Seanc. Soc. Biol., 144, 1427.

FREDERICQ, P. & BETZ-BAREAU, M. (1952) Ann. Inst. Pasteur, Paris 83, 283.

FREDERICQ, P. & DELCOUR, G. (1953) C.r Seanc. Soc. Biol., 147, 1310.

FRIEDBERG, E.C. & GOLDTHWAIT,D.A. (1968) Cold Spring Harbor Symp. Quant. Biol., 33, 261.

GANESAN, A.T. & LEDERBERG, J. (1965) Biochem. Biophys. Res. Commun. 18, 824.

GANESAN, A.K. & SMITH, K.C. (1968) J. Bacteriol., 96, 365.

GILBY, A.R. & FEW, A.B. (1960) J. Gen. Microbiol., 23, 19.

GILES, N.H., CASE, M.E., PARTRIDGE, C.W.H. & AHMED, S.I. (1967) Proc. Natl. Acad. Sci., 58, 1453.

GLOVER, S.W. (1962) Genet. Res., 3, 448.

GLOVER, S.W. & COLSON, C. (1969) Genet. Res., 13, 227.

GOEBEL, W.F. (1962) Proc. Natl. Acad. Sci. U.S.,48, 214.

GOEBEL, W.F. & BARRY, G.T. (1958) J. Exp. Med. 107, 185.

GOEBEL, W.F., BARRY, G.T. & AMANO, T. (1957) Science, N.Y. 126, 1231.

GOEBEL, W.F., BARRY, G.T., JESAITIS, A.A. & MILLER, E.M. (1958) Nature, Lond. 176, 700.

GOEBEL, W.F., BARRY, G.T. & SHEDLOUSKY, T. (1956) J. Exp. Med., 103, 577.

GRATIA, A. (1925) C.r. Secanc. Soc. Biol., 93, 1040.

GRATIA, A. (1932) Ann. Inst. Pasteur, Paris, 48, 413.

GRATIA, J.P. (1966) Biken J. 9, 77.

GROSSMAN, L., KAPLAN, J. KUSHNER, S., & MAHLER, I. (1968) Cold Spring Harbor Symp. Quant. Biol., 33, 229.

GRULA, E.A. & GRULA, M.M. (1962) J.Bacteriol., 83, 981.

HAEFNER, K. (1968) J.Bacteriol., 96, 652.

HAMON, Y. & PERON, Y. (1960) C.r. hebd. Seanc. Acad.Sci. Paris, 251, 1840.

HAMON, Y. & PERON, Y. (1965) C.r. hebd. Seanc. Acad. Sci. Paris, 260, 5401.

HAMON, Y. & PERSON, Y. (1966) Zentbl. Bakt. ParasitKde. (Abt. 1) 200, 375.

HAMON, Y. & PERON, Y. (1967) C.r. hebd. Seanc. Acad. Sci., Paris, 265, 1433.

HAYES, W. (1968a) In Genetics of Bacteria and their Viruses p.303 2nd Edition, Blackwell Scientific Publications.

HAYES, W. (1968b) In Genetics of Bacteria and their Viruses pp.700-705 2nd Edition, Blackwell Scientific Publications.

HELINSKI, D.R. & HERSCHMAN, H.R. (1967) J.Bacteriol., 94, 700.

HELMSTETTER, C.E. & PIERUCCI, O. (1968) J. Bacteriol.., 95, 1627.

HERSCHMAN, H.R. & HELINSKI, D.R. (1967a) J. Bacteriol., 94, 691.

HERSCHMAN, H.R. & HELINSKI, D.R. (1967b) J.Biol. Chem., 242, 5360.

HERTMAN, I. & LURIA, S.E. (1967) J.Mol.Biol., 23, 117.

HILDEBRAND, J. & LOW, J. (1964) Biochem. Biophys. Res. Commun., 5, 378.

HILL, C. & HOLLAND, I.B. (1967) J.Bacteriol., 94, 677.

HINDSHILL, R.D. & GOEBEL, I.F. (1966) J. Exp. Med. 123, 881.

HOLLAND, I.B. (1961) Biochem. J., 78, 641.

HOLLAND, I.B. (1962) J. Gen. Microbiol., 29, 603

HOLLAND, I.B. (1965) J. Mol. Biol., 12, 429.

HOLLAND, I.B. (1967) Molec. Gen. Genetics, 100, 242.

HOLLAND, I.B. (1968) J. Molec. Biol., 31, 267.

HOLLAND, I.B. & THRELFALL, E.J. (1969) J.Bacteriol., 97,91.

HOWARD-FLANDERS, P. & BOYCE, R.P. (1966) Radiation Res. Suppl.6 156.

HOWARD-FLANDERS, P. & THERIOT, L. (1966a) Genetics, 53, 1119.

HOWARD-FLANDERS, P. & THERIOT, L. (1966b) Genetics, 53, 1137.

HUTTON, J.J. & GOEBEL, W.F. (1961) Proc. Natl. Acad. Sci., US 47, 1498.

HUTTON, J.J. & GOEBEL, W.F. (1962) J. gen. Physiol., 45, 125.

IIJIMA, T. (1962) Biken J. 5, 1.

ISHII, S-I., NISHI, Y.& EGAMI, F. (1965) J. Molec. Biol., 13, 428.

ITERSON, W. von & LEENE, W. (1964) J. Cell Biol., 20, 361.

ITO, J. & CRAWFORD, I.P. (1965) Genetics, 52, 1303.

JACOB, F., BRENNER, S. & CUZIN, F. (1963) Cold Spring Harbor Symp. Quant. Biol., 28, 329.

JACOB, F., LWOFF, A., SIMINOVITCH, L. & WOLLMAN, E. (1951) Ann. Inst. Pasteur, Paris, 84, 222.

JACOB, F., SIMINOVITCH, L. & WOLLMAN, E. (1951) C.r. hebd. Seanc. Acad. Sci. Paris, 233, 1500.

JACOB, F., SIMINOVITCH, L. & WOLLMAN, E. (1952) Ann. Inst. Pasteur Paris, 83, 295.

JACOB, F., SIMINOVITCH, L. & WOLLMAN, E. (1953) Ann. Inst. Pasteur Paris, 84, 313.

JENKIN, C.R. & ROWLEY, D. (1955) Nature, Lond., 175, 779.

KABACK, H.R. (1968) J. Biol. Chem., 243, 3711

KAGEYAMA, M. (1964) J.Biochem (Tokyo) 55, 49.

KANFER, J. & KENNEDY, E. (1964) J.Biol. Chem., 239,1720

KAWATA, T. & INOVE, T. (1965) Japan. J. Microbiol., 11, 115.

KEENE,J.H. (1966) Canad. J.Microbiol., 12, 425.

KELLENBERGER, E. & RYTER, A. (1958) J.Biophys.Biochem. Cytol., 4, 323.

KONINSKY, J. & NOMURA, M. (1967) J.Molec.Biol., 26, 181.

KUNDIG, W., GHOSH, S. & ROSEMAN, S. (1964) Proc. Natl. Acad. Sci., 52, 1067.

KNIPPERS, R. & SINSHEIMER, R.L. (1968) J.Mol. Biol., 34, 17.

LAW, J., ZALKUN, & KAVESHINO, T. (1963) Biochem. Biophys. Acta, 70, 143.

LEDERBERG, S. (1966) J.Bacteriol., 91, 1029.

LEVISOHN, R., KONINSKY, J. & NOMURA, M. (1967) J.Bacteriol., 96, 811

LEWIS, M.J. & STOKER, B.A.D. (1965) Zentbl. Bakt. ParasitKde (Abt.1) 196, 173.

LOW, B. (1965) Genet. Res., 6, 469.

LOW, B. (1966) Proc. Natl. Acad. Sci. U.S., 60, 160.

LURIA, S.E. (1964) Ann. Inst. Pasteur, Paris, 107, suppl.5, 67.

LURIA, S.E. (1969) J. Bacteriol. (In press).

LUZZATI, P. & CHEVALLIER, M.R. (1964) Ann. Inst. Pasteur, Paris, 107, suppl.5, 152.

MAEDA, A. & NOMURA, M. (1966) J.Bacteriol., 91, 685.

MALAMY, M.H. & HORECKER, B.L. Biochem. J. 3, 188. (1965)

MARR, A.G. (1960) In The Bacteria ed. Gunsalus, I.C. & Stanier, R.Y. Vol.1 Academic Press, New York. pp.443-468.

MARTIN, H.H. & FRANK, H. (1962) Z. Naturf. 17b, 190.

MATTERN, I.E., ZWENK, H. & RÖRSCH, A. (1966) Mutation Res., 3, 374.

MAYR-HARTING, A. (1964) J. Path. Bacteriol. 87, 255.

MESELSON, M. & YUAN, R. (1968) Nature (Lond.) 217, 110.

MUNOZ, E., FREER, J.M., ELLAR, D.J. & SALTON, M.R.J. (1968) Biochim. Biophys. Acta, 150, 531.

NAGATA, Y., SHIBUYA, I. & MARUS, B. (1967) J.Biochem., 61, 623.

NAKAMURA, H. (1968) Mem. Konan Univ. Sci. Ser. 11, Art.57

NOMURA, M. (1963) Cold Spring Harbor Symp. Quant. Biol., 28, 315.

NOMURA, M. (1964) Proc. Natl. Acad. Sci. U.S., 52, 1514.

NOMURA, M. (1967) Ann. Rev. Microbiol., 21, 257.

NOMURA, M. & MAEDA, A. (1965) Zentbl. Bact. ParasitKde. (Abt.1) 196, 216.

NOMURA, M. & NAKAMURA, M. (1962) Biochem. Biophys. Res.Commun., 7, 306.

NOMURA, M. & WITTEN, C. (1967) J.Bacteriol., 94, 1093.

NORMARK, S., BOMAN, H.G. & MATSSON, E. (1969) J.Bacteriol., 97, 1334.

NOSE, K., MIZUNO, D. & OZEKI, H. (1966) Biochim. Biophys. Acta, 119, 636.

NOSSAL, N.G. & HEPPEL, L.A. (1966) J.Biol. Chem., 241, 13.

O'LEARY, W.M. (1965) In Transmethylation and Methionine Biosynthesis ed. Shapiro, S.K. & Schlenk, F. University of Chicago Press, pp.94-106.

OBDRZALEK, U., SMARDA, J., CECH, O. & ADLER, J. (1969) Sep. Exper. (Basel) 25, 331.

OBINATA, M. & MIZUNO, D.I. (1968) Biochim. Biophys. Acta, 155, 98.

OHKI, M. & TOMIZAWA, J.I. (1968) Cold Spring Harbor Symp. Quant. Biol., 33, 651.

OZAKI, M., HIGASHI, Y., SATIO, H., AN, T. & AMONO, T. (1966) Biken J., 9, 201.

OZEKI, H., STOKER, B.A.D. & DE MARGERIE, H. (1959) Nature, Lond. 184, 337.

PARDEE, A.B., JACOB, F. & MONOD, J. (1959) J.Mol. Biol., 1, 165.

PERSIEL, I. (1965) Zentbl. Bakt. ParasitKde. (Abt.1) 196, 275.

PUIG, J., AZOULAY, E. & PICHINOTY, F. (1967) C.r. Seac. Soc. Biol. 264, 1507.

REEVES, P.R. (1963) Austral. J. exp. Biol. med. Sci. 41, 163.

REEVES, P.R. (1965) Bact. Rev., 29, 24.

REEVES, P. (1966) Austral. J. exp. Biol. med. Sci., 44, 301.

REYNOLDS, B.L. & REEVES, P.R. (1963) Biochem. Biophys. Res. Commun., 11, 140.

RYTER, A. (1968) Bacteriol.,Rev. 32, 1, 39.

RYTER, A., HIROTA, Y. & JACOB, F. (1968) Cold Spring Harbor Symp. Quant. Biol., 33, 669.

RYTER,A. & JACOB, F. (1964) Ann. Inst. Pasteur Paris, 107, 384.

RYTER, A. & JACOB, F. (1966) Ann. Inst. Pastuer, Paris, 110, 801.

RYTER, A. & JACOB, F. (1967) C.r. Seanc. Soc. Biol., 264, 2254.

RYTER, A. & LANDMAN, O.E. (1964) J.Bacteriol., 88, 467.

ROSENBERG, B.H. & CAVALIERI, L.F. (1968) Cold Spring Harbor Symp. Quant. Biol., 33, 65.

SALIVAR, W.O. & SINSHEIMER, R.L. (1969) J. Mol.Biol., 41, 39.

SANDOVAL, H.K., REILLY, H.C. & TANDLER, B. (1965) Nature, Lond., 205, 522.

SCHLESSINGER, D. (1963) J.Mol.Biol., 7, 569.

SICARD, N. & DEVORET, R. (1962) C.r. Seanc. Acad. Sci., 255, 1417.

SICCARDI, A.G. (1966) Genet. Res. Camb. 8, 219.

SILVER, S. & LEVINE, E. (1968) J.Bacteriol., 96, 338.

SILVER, S. & OZEKI, H. (1962) Nature, Lond., 195, 873.

SIRONI, G. (1969) Virol., 37, 163.

SMARDA, J. (1967) Nature, Lond., 213, 576.

SMARDA, J., KOUDELKA, J. & KLEINWACHTER, U. (1964) Experientia, 20, 500.

SMARDA, J. & TAUBENECK, U. (1968) J. Gen. Microbiol., 52, 161.

STEED, P. & MURRAY, R.G.E. (1966) Canad. J. Microbiol., 12, 263.

STOKER, B.A.D. (1965) Microbiol. Genet. Bull., 19, 9.

STOKER, B.A.D. (1966) Hereditary, 21, 166.

STOURHAMER, A.H. & TIEZE, G.A. (1966) Antonie von Leeuwenhoek, 32, 171.

SUEOKA, N. & QUINN, W.G. (1968) Cold Spring Harbor Symp. Quant. Biol., 33, 695.

TAKAGI, Y., SEKIGUCHI, M., OKUBO, S., NAKAYAMA, H., SHIMADA, K., YASUDA, S., MISHIMOTO, T. & YOSHIHARA, H. (1968) Cold Spring Harbor Symp. Quant. Biol., 33, 219.

TARVLOV, A.R. & KENNEDY, E.P. (1965) J.Biol. Chem., 240, 49.

TAYLOR, A.L. & TROTTER, C.D. (1967) Bacteriol.Rev. 31, 332.

TREMBLAY, G.Y., DANIELS, M.J. & SCHAECHTER, M. (1969). J.Mol.Biol., 40, 65.

VAN DE PUTTE, P. WESTENBROEK, C. & RÖRSCH, A. (1963) Biochim. Biochim. Biophys. Acta, 76, 247.

VAN DE PUTTE, P., ZWENK, H. & RÖRSCH, A. (1966) Mutation Res., 3, 381.

WALKER, J.R. & PARDEE, A.B. (1967) J.Bacteriol., 93, 107.

WALKER, J.R. & PARDEE, A.B. (1968) J.Bacteriol., 95, 123.

WEIDEL, W., FRANK, H. & MARTIN, H.H. (1960) J.Gen.Microbiol., 22, 158.

WEIDEL, W. & PRIMOSIGH, J. (1958) J.Gen.Microbiol., 18, 513.

WHITEFIELD ,M.J., MARTIN, R.G. & AMES, B.N. (1966) ~~Proc.Natl.Acad. Sci.~~ J. Mol. Biol., 21, 335.

WILLETTS, N.S., CLARK, A.J. & LOW, B. (1969) J.Bacteriol., 97, 244.

WINKLER, H.H. (1968) Biocheim. Biophys. Acta, 117, 231.

WOOD, W.B. (1966) J.Mol.Biol., 16, 118.

WU, T.T. (1966) Genetics, 54, 405.

YANOFSKY, C. & ITO, J. (1966) J.Mol.Biol., 21, 313.

YOSHIKAWA, N. & SUEOKA, H. (1963) Genetics, 52, 747.

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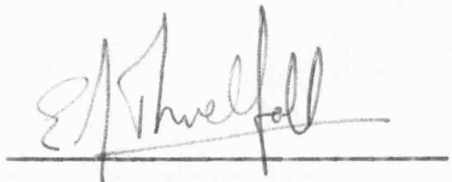
This is to certify that the Thesis I have submitted in fulfilment of the requirements governing candidates for the degree of Doctor of Philosophy in the University of Leicester, entitled "Genetical and Physiological Studies into the Basis of Colicin E2 Susceptibility in E.coli K12" is the result of work done mainly by me during the period of registration for the above degree.



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Identification of Closely Linked Loci Controlling Ultraviolet Sensitivity and Refractivity to Colicin E2 in *Escherichia coli*

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Mutants (phenotypic symbol Ref-II) refractory to colicin E2 have been isolated in several strains of *Escherichia coli* K-12, and a *ref*II locus has been mapped 1 to 2 min counter clockwise to *thr*. A small number of Ref-II mutants are also ultraviolet (UV)-sensitive and the uv^s locus in one such strain has been mapped close to the *ref*II locus near *thr*. The Ref-II mutation alone does not affect recombinant formation in F^- strains, but the Ref-II, UV^s strains behave in many respects like Rec^- mutants, giving reduced recombination frequencies in crosses with male strains. It is suggested that the *ref*II and uv^s loci correspond to closely linked if not identical genes, concerned in some way in the activity of one or more deoxyribonucleases, and that the Ref-II, UV^s mutants arise as the pleiotropic expression of a single gene or of a deletion or polar mutation affecting linked genes.

The adsorption of colicin to specific receptors in the cell surface of sensitive bacteria is followed by characteristic intracellular changes and ultimately by cell death (18). The colicin molecules which apparently fail to act in cell-free systems (11, 17) seemingly promote their specific effects in vivo from the extracellular attachment site. Although colicin receptors probably reside in the bacterial cell wall (3, 4), the attached colicin, in order to act, must presumably interact in some specific way with the cytoplasmic membrane of the affected cell. Nevertheless, gross physical changes in the properties of the cell membrane do not accompany colicin action (14, 16). The presence of colicin at the cell surface therefore introduces some subtle change in the cell membrane, triggering a series of events eventually leading to a specific and lethal intracellular change. This series of steps may be envisaged as the "colicin pathway" connecting the extracellular colicin to its intracellular target, fixation to the colicin receptor being the first step in this process. In the case of colicins E2 and E3, this and possibly some subsequent steps in the pathway are shared (6), although ultimately two quite distinct targets are affected. Thus, colicin E2 induces deoxyribonucleic acid (DNA) degradation, whereas E3 induces a specific inhibition of protein synthesis (16).

Resistant mutants lacking the colicin E receptor have been isolated, and a genetic locus controlling in some way receptor formation has been mapped between *met* and *thi* (10). Mutants resistant to colicin E, which nevertheless still retain the colicin E receptor, have also been isolated. Such mutants which are presumably blocked at some post-fixation point in the "colicin pathway" are designated refractory to colicin E (6). Mutants refractive (or tolerant) to colicin E have been isolated by several workers (2, 6, 15, 19, 20) and fall into two main groups. The major group includes multirefractory mutants, refractory to colicin E2 and E3 or to colicin E1, E2, and E3. Several classes of this type of mutant, which are often also refractory to other unrelated colicins, have been mapped at one of at least three distinct sites closely linked to *gal*. The second group of refractory mutants includes strains refractory to only a single colicin. Two such classes are known: E1-refractory mutants, first isolated by Clowes and Moody (2) and designated by us Ref-I mutants, and E2 refractory mutants (Ref-II) (6). All Ref-I strains appear to map at a single locus linked to *thy* (6), whereas (as described in this paper) refractivity of the Ref-II type is probably controlled by a single locus closely linked to *thr*.

Ref-II mutants are conditional mutants refractory to colicin E2 at low temperature but largely sensitive at high temperature. Detailed properties of these mutants, in which DNA breakdown, following E2 adsorption, takes place

at 40 C but not at 30 C, have been described elsewhere (8). A small number of Ref-II mutants, in addition to E2 refractivity, also show an increased sensitivity to ultraviolet (UV)-irradiation, although this effect is not temperature-dependent (7). The genetic properties and the location of the UV sensitivity locus of one such strain are also described in this paper.

MATERIALS AND METHODS

Organisms. The following E2-sensitive strains were used in this study: *Escherichia coli* HfrH ($T1^r$ str^s λ^-); HfrR4 (Col-I^r str^s met^-) and F^-, strain 203 (*thi*$^-$ *met*$^-$ *arg*$^-$ *thr*$^-$ *leu*$^-$ *gal*$^-$ *trp*$^-$ *his*$^-$ str^r) (6); ASH10 (F^-, *thi*$^-$ *met*$^-$ *thy*$^-$ *lac*$^-$ *leu*$^-$ str^r) and ASH1 (Hfr, *thi*$^-$ *met*$^-$ *thy*$^-$ *leu*$^-$ str^r) (7). The source of colicin E2 in these studies was *Salmonella typhimurium* LT2, 906 carrying the E2 (P9) factor, and the culture conditions, production, and assay of colicin were all as reported previously (6). The origins of the Hfr strains used are shown in Fig. 1.

Mating conditions. Linkage analysis and selection for different recombinant classes was carried out as described previously (6). In interrupted mating experiments, male and female strains were first grown in nutrient broth (NB) to 5×10^7 cells/ml and 5×10^8 cells/ml, respectively; equal volumes were mixed and incubated at 37 C with gentle shaking for 5 min. The culture was then diluted 10-fold in NB to reduce further pairing; 0.5-ml samples were removed at intervals, diluted 10-fold in ice-cold buffer, and blended for 1 min with a flask shaker (B.T.L. Laboratory Centre, Birmingham, England) before plating for recombinants. For the direct selection of E2-refractive recombinants, samples from the mating mixture were blended in ice-cold NB plus streptomycin; these samples were then incubated at 37 C for 90 min without shaking, to allow expression of E2 refractivity before plating on NB plates plus 10^4 units of colicin E2. Plates were incubated at 25 C, and the E2-refractive colonies were then replica-plated to plates containing colicin E2 and colicin E3 at 25 and 40 C to confirm the Ref-II phenotype. For the direct selection of UV-resistant recombinants, blended samples were also allowed 90 min of expression time before plating, in this case at 37 C, on PEP plates [1% peptone (Oxoid), 0.1% Beef Extract (Difco), 1% sodium chloride], plus streptomycin. The plates were then irradiated with a UV dose of 260 ergs with a low-pressure mercury lamp (Hanovia Ltd) set at 46 cm. The presence of streptomycin in samples during incubation for expression of the UV^r and E2-refractivity markers prevented further growth and initiation of transfer by the str^s male strain. For the selection of $T1^r$ recombinants, samples were plated (after blending) directly onto NB plates previously spread with 10^7 T1 phage particles.

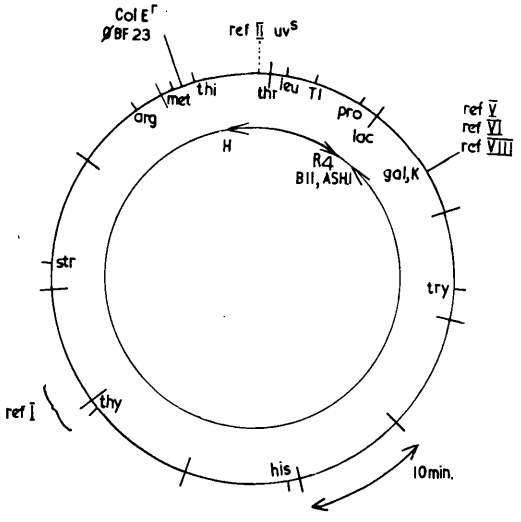

FIG. 1. *Linkage map of E. coli K-12 showing the origin of the Hfr strains used and the location of colicin E-refractory loci.*

RESULTS

Isolation of mutants refractory to colicin E2. Ref-II mutants refractory to E2 at 30 C but sensitive at 40 C were readily isolated from strain ASH10 (6). However, Ref-II mutants were not previously obtainable from other strains. In the present work, further attempts were made to isolate Ref-II mutants in additional strains, and it was observed that, if selection for, and sensitivity tests with, E2 refractory mutants were carried out at 25 C rather than 30 C, such mutants did appear in several strains (Table 1). The frequency of Ref-II mutants was, however, usually lower than with ASH10, and some strains still failed to produce any such mutants.

Linkage analysis of the Ref-II mutation. Several independently isolated Ref-II mutants of strain ASH10 were crossed with an HfrH male strain. Selection was made for *leu*, $T1^r$, and *lac* recombinants and, in each case, the recombinants were replica-plated to colicin E2 and E3 lawns at 25 and 40 C to determine the proportion that were still resistant to E2 at 25 C. The distribution of the E2-sensitivity marker among selected markers in a typical cross is shown in Table 2. The results indicated that the *ref*II locus was linked to *leu*; this was confirmed in crosses where selection was made for recombinants for the distal marker, *lac*. In all, 26 Ref-II, F^- strains were crossed with the HfrH strain, and the proportion of the unselected markers, *leu* $T1^r$ and E2-sensitivity, among *lac* recombinants was determined by replica plating. The results (Table 2) clearly indicated the presence of a single *ref*II locus closely linked with and to the left of the *leu* locus (Fig. 1).

Mapping of the refII locus by interrupted mating. Confirmation of the position of the *ref*II locus was also sought from interrupted

mating experiments. Mutants refractory to colicin E2 (Ref-II) were isolated in an HfrH strain (ASH54) and in HfrR4 (ASH55). The E2-refractory mutants were crossed with the F^-, colicin E-sensitive strain 203, and the time of entry of the *ref*II locus was determined as described above. As anticipated, colicin E2 refractivity enters close to the *thr* and *leu* loci (Fig. 2). The results with both male strains indicated a map position 1 to 2 min counterclockwise to the *thr* locus (*see* Fig. 1). The different scales used in Fig. 2 should be noted; in several experiments using ASH54, reduced numbers of Ref-II recombinants relative to other classes were obtained. Low (13) reported such an effect previously for markers very near the origin of the transferred chromosome.

Mapping studies with a UV-sensitive Ref-II mutant. It was reported previously (7) that, out of 20 Ref-II mutants examined, three strains were found to be UV-sensitive in addition to being refractive to colicin E2. Attempts were

TABLE 1. *Characteristics of some E. coli K-12 Ref-II mutants*

Strain no.	Origin	Sex	Response to UV	Other markers												
				met	*thi*	*arg*	*thy*	*his*	*try*	*gal*	*lac*	*leu*	*thr*	T1	*str*	λ
ASH50	AP[a] mutation from ASH10	F^-	R	−	−	+	−	+	+	+	−	−	+	S	R	+
ASH51	AP mutation from ASH10	F^-	R	−	−	+	−	+	+	+	−	−	+	S	R	+
ASH52	AP mutation from ASH10	F^-	R	−	−	+	−	+	+	+	−	−	+	S	R	+
ASH53	AP mutation from ASH10	F^-	R	−	−	+	−	+	+	+	−	−	+	S	R	+
ASH54	AP mutation from HfrH	Hfr	R	+	+	+	+	+	+	+	+	+	+	R	S	−
ASH55	AP mutation from HfrR4	Hfr	R	−	+	+	+	+	+	+	+	+	+	S	S	+
ASH57	AP mutation from 203	F^-	R	−	−	−	+	−	−	−	−	−	−	S	R	+
ASH101	AP mutation from ASH1	Hfr	R	−	−	+	−	+	+	+	+	−	+	S	R	−
ASH102	AP mutation from ASH1	Hfr	R	−	−	+	−	+	+	+	+	−	+	S	R	−
ASH112	AP mutation from ASH10	F^-	S	−	−	+	−	+	−	+	−	−	+	S	R	+
ASH113	AP mutation from ASH10	F^-	S	−	−	+	−	+	+	+	−	−	+	S	R	+

[a] 2-Amino purine.

TABLE 2. *Linkage analysis of Ref-II mutants*[a]

Ref-II mutants tested (21)	Recombinants			$E2^s$	Type strains tested (24)	Selected marker	No. tested	Proportion of unselected markers		
	Selected marker	Input male	No. tested					$T1^r$ *str*^{r}	*leu str*^{r}	$E2^s$
		%		%				%	%	%
(e.g., ASH50)	*leu str*^{r}	2.7	199	72	(e.g., ASH52)	*lac str*^{r}	333	86	79	54
	$T1^r$ *str*^{r}	1.2	96	56						
	lac str^r	0.8	228	34						
(e.g., ASH51)	*leu str*^{r}	2.0	200	74	(e.g., ASH53)	*lac str*^{r}	224	83	72	56
	$T1^r$ *str*^{r}	1.2	124	49						
	lac str^r	0.9	98	33						

[a] An HfrH strain ($E2^s$ $T1^r$ λ^- *str*^{s}) was crossed with several Ref-II derivatives of the F^- strain ASH10 (*met*$^-$ *thi*$^-$ *leu*$^-$ *lac*$^-$ *thy*$^-$ λ^+ Col-I^r *str*^{r}). Exponential cultures (10^8 cells/ml) of each strain were mixed; after 90 min at 37 C, selection was made for various recombinant classes; subsequently, the proportion of the unselected markers, including E2 sensitivity, was determined by replica-plating.

therefore made to map the UV-sensitivity locus in one such Ref-II mutant, ASH112. Interrupted mating experiments were carried out with this strain in crosses with two UV-resistant E2-sensitive male strains, HfrH and HfrR4. The time of entry of UV resistance was determined in each case, and the results showed (Fig. 3) that this UV-sensitivity locus is also located close to the *thr* marker. When the UV-resistant recombinants were replica-plated to NB plates containing colicin E2, all colonies appeared to be E2-sensitive. In strain ASH112, therefore, the UV-sensitivity locus and the *ref*II locus are extremely closely linked.

The rec⁻ character of Ref-II, UV^s mutants. Previous studies (7) indicated that the UV sensitivity of the Ref-II, UV^s mutants was of the Rec type. The recombination frequencies obtained with ASH112 and another UV^s Ref-II mutant, ASH113, in crosses with an HfrH strain were therefore determined (Table 3). Although the UV marker was transferred early in these crosses, markedly reduced recombination frequencies were obtained. Significantly different recombination-deficiency indices were obtained with the two mutants; the possible significance of this will be discussed.

Effect of the Ref-II mutation upon genetic recombination. Since the majority of Ref-II mutants, unlike ASH112, are not UV-sensitive, it was not anticipated that mutations at the *ref*II locus would affect recombination frequencies. As indicated in Table 2, this expectation was fulfilled; F⁻ strains carrying the Ref-II mutation all gave normal recombination frequencies. Moreover, when crosses were carried out at 25 C (where E2 refractivity is maximally expressed), similar results were obtained.

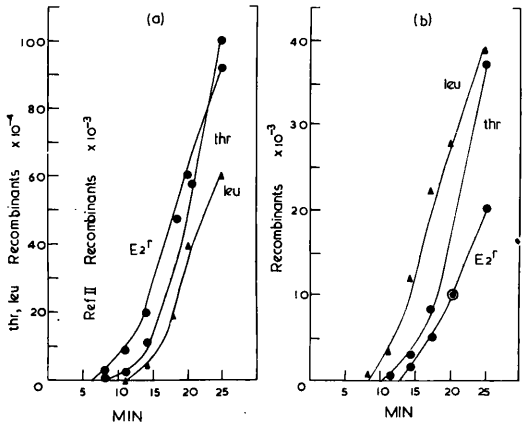

FIG. 2. *Time of entry of the colicin E2-refractory locus. (a) ASH54 (HfrH) crossed with E2-sensitive F⁻ strain 203; (b) ASH55 (HfrR4) crossed with E2-sensitive 203.*

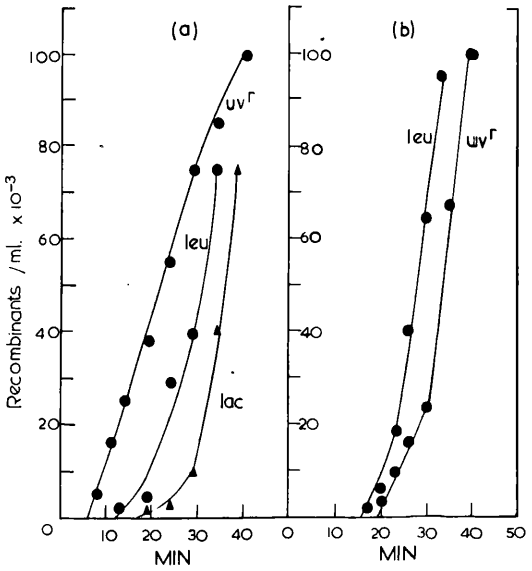

FIG. 3. *Time of entry of the UV^s marker in ASH112. (a) HfrH crossed with ASH112; (b) HfrR4 crossed with ASH112.*

Aberrant recombination frequencies were, however, sometimes obtained with Ref-II derivatives of ASH1, a male strain transferring the *ref*II locus as a tail marker (see Fig. 1). Table 4 shows a comparison of the results of crosses of a Ref-II derivative of HfrH (ASH54) and two Ref-II male strains (ASH101 and ASH102) derived from ASH1. The number of recombinants obtained in each case is presented as a percentage of that obtained in crosses with the corresponding E2-sensitive (*ref*$^+$) males. Somewhat variable results were obtained with ASH101, the frequency of recombinants sometimes approaching that of the wild type, but ASH102 always gave the same low yield. The possibility was tested that the reduction in recombination frequency was due to, e.g., restriction of "Ref-II DNA" in the *ref*$^+$ recipient. ASH102 was therefore crossed with a Ref-II derivative (ASH57) of the F⁻ strain 203. The results (Table 4) showed that, although both male and female strains carried a Ref-II mutation, the same marked reduction in recombinant formation occurred.

DISCUSSION

Mutation to E2 refractivity may be envisaged as a change in one of two main types of system. The Ref-II mutants at low temperature may fail to (i) transmit the effect of the adsorbed colicin through the cytoplasmic membrane, (ii) initiate

TABLE 3. *Reduced recombination frequencies with F⁻ strains carrying Ref-II UV^s mutations*[a]

Donor	Recipient	Recombinants as percentage of input male			Recombination-deficiency index (UV^r/UV^s)
		leu str^r	T1^r *str*^{r}	*lac str*^{r}	
HfrH	ASH10 ($UV)^r$	2.2	1.9	0.7	
HfrH	ASH113 (UV^s)	0.005		0.002	4.5×10^2
HfrH	ASH112 (UV^s)	0.33	0.17	0.09	7

[a] Mating conditions were as described in Table 2. The recombination deficiency index (1) was determined from the frequency of *leu str* recombinants in each cross.

TABLE 4. *Recombination frequencies obtained with male (Ref-II) strains*[a]

Donor	Recipient	Recombinants as percentage of that obtained with wild-type (*ref*$^+$) males				
		leu str^r	*lac str*^{r}	*gal str*^{r}	*try str*^{r}	*his str*^{r}
HfrH (Ref-II)	203 (*ref*$^+$)	100	100		100	
ASH101 (Ref-II)	203 (*ref*$^+$)			5	8	13
ASH102 (Ref-II)	203 (*ref*$^+$)			4	4	2
ASH102 (Ref-II)	ASH57 (Ref-II)			4	4	2

[a] Crosses carried out at 37 C as described in Table 2.

nuclease attack. Some specific change in the cell membrane could account for the former possibility, whereas, lack of a deoxyribonuclease, inhibition of its activity, or possibly even modification of its substrate DNA could account for the latter. Previous reports (6, 19) indicated that some early steps in the colicin E pathway were in fact common to both E2 and E3. Moreover, it has also been shown (5a) that the two colicins probably contain regions of similar structure. The specific refractivity of Ref-II mutants to E2, therefore, suggests that these mutants are blocked in some distal step in the pathway, e.g., perhaps some step affecting a deoxyribonuclease. This hypothesis gained some support from the finding (7) that several Ref-II mutants also showed an increased sensitivity to UV irradiation, a situation that has been presumed to arise after the loss of, or a change in, the activity of deoxyribonucleases or other repair enzymes (9). Genetic analysis of Ref-II mutants has now provided further indications that the *ref*II gene product may in some way be concerned with the activity of a deoxyribonuclease.

The mapping studies in this work were greatly facilitated by the proximal position of the *ref*II locus on the HfrH chromosome. This has allowed a fairly precise position, 1 to 2 min counterclockwise to *thr*, to be assigned to it. The position of the UV-sensitivity locus in the Ref-II mutant ASH112 was found to be extremely closely linked to the *ref*II locus. This finding, together with the high incidence of mutants like ASH112 among Ref-II strains, makes it improbable that E2 refractivity and UV sensitivity arose in these strains by two independent mutations. It appears more likely that the concommitant UV sensitivity of certain Ref-II mutants may be explained as the pleiotropic effects of a single gene or a polar mutation or deletion affecting closely linked genes. Fine-structure genetic analysis with bacteriophage P1 and reversion studies with the UV-sensitive Ref-II mutants should provide the answer to these alternatives.

Previous physiological studies (7) showed that the UV-sensitive Ref-II mutants resembled in several respects Rec$^-$ rather than UVR$^-$ mutants. Furthermore, it was observed in the present experiments that reduced recombination frequencies were obtained with ASH112 and ASH113 as recipients in crosses with male strains. Moreover, experiments (*unpublished*) have shown that the entry of the β-galactosidase gene, whether present on the chromosome or on a sex factor, from male donors is normal in these mutants. Such strains appear, therefore, to fulfill all the requirements of Rec mutants, and selection for E2 refractivity could be a convient method of isolating this particular group of *rec*$^-$ mutants. The two strains ASH112 and ASH113 were significantly different in their capacity for recombinant formation. Clark (1) has observed that strains with low-deficiency indices show "cautious" UV-induced DNA breakdown, whereas those mutants with high-deficiency indices show "reckless" breakdown.

Preliminary results (*unpublished*) indicate that both ASH112 and ASH113 are rather cautious in this respect, and alternative reasons for their markedly different deficiency indices must be sought.

The Ref-II mutation itself was found to have no effect upon recombination frequencies when present in the F^- and when crosses were carried out at 37 C or at 25 C, the latter temperature being that at which E2 refractivity is maximally expressed. In contrast, markedly reduced recombination frequencies were sometimes obtained in crosses in which the *ref*II locus was present in the male strain. This effect has been observed only with Ref-II derivatives of ASH1, a male strain carrying the *ref*II gene as a tail marker. The phenomenon is not due to an incompatibility between male and female DNA, comparable for example to that observed in interspecies restriction, since, if both male and female strains carry the *ref*II gene, reduced recombination frequencies are still observed. Experiments are now in progress to determine whether this effect is found with other male strains, especially those carrying a distal *ref*II locus, and also to identify the defective step in the conjugation process in these mutants.

The function of the *ref*II gene product in E2 action is still unclear, although it seems probable that it is associated with some late step in the pathway and possibly with the activity of a deoxyribonuclease. It seems unlikely, however, that the *ref*II gene determines the synthesis of a specific deoxyribonuclease. An alternative hypothesis that is now being considered is that the *ref*II gene determines the formation of a component of the cell membrane which may bind, and hence regulate, the activity of one or more deoxyribonucleases. In this connection, the map position of the *ref*II locus and the closely linked uv^s locus is particularly intriguing. Previous workers reported the location of rec^- (21) and r^-m^- (restriction-modification; 12, 22), genes in this relatively small region of the chromosome delineated by the origin of HfrH and *thr*. It is tempting to think that all these genes may constitute one or more operons concerned with the formation and regulation of various deoxyribonucleases and repair enzymes. Further studies will be directed toward determining the relationship of all these genes to each other.

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LITERATURE CITED

1. Clark, A. J. 1967. The beginning of a genetic analysis of recombination proficiency. J. Cell. Physiol. **70**:(Suppl. 1) 165–180.
2. Clowes, R. C., and E. E. M. Moody. 1965. Mutational immunity to a colicin. J. Gen. Microbiol. **39**:XI.
3. Fredericq, P. 1949. Sur la résistance croisée entre colicin E et bactériophage. Compt. Rend. **143**:1011.
4. Fredericq, P. 1950. Recherches sur les colicines du groupe E. Compt. Rend. **144**:297.
5. Hayes, W. 1964. The genetics of bacteria and their viruses, p. 568. Blackwells, Oxford.
5a. Herschman, H. R., and D. R. Helinski. 1967. J. Biol. Chem. **242**:5360–5368.
6. Hill, C., and I. B. Holland. 1967. Genetic basis of colicin E sensitivity in *Escherichia coli*. I. Isolation and properties of refractory mutants and the preliminary mapping of their mutations. J. Bacteriol. **94**:677–686.
7. Holland, I. B. 1967. The properties of UV sensitive mutants of *Escherichia coli* K12 which are also refractory to colicin E2. Mol. Gen. Genet. **100**:242–251.
8. Holland, I. B. 1968. Properties of *Escherichia coli* K12 mutants which show conditional refractivity to colicin E2. J. Mol. Biol. **31**:267–275.
9. Howard-Flanders, P., and R. P. Boyce. 1966. DNA repair and genetic recombination: Studies on mutants of *Escherichia coli* defective in these processes. Radiation Res. **6**(Suppl.):156–184.
10. Jenkin, C. R., and D. Rowley. 1955. Resistance to colicin E as a genetic marker in *E. coli* K12. Nature **175**:779.
11. Konisky, J., and M. Nomura. 1967. Interaction of colicins with bacterial cells. II. Specific alteration of *Escherichia coli* ribosomes induced by colicin E3 *in vivo*. J. Mol. Biol. **26**:181–195.
12. Lederberg, S. 1966. Genetics of host-controlled restriction and modification of deoxyribonucleic acid in *Escherichia coli*. J. Bacteriol. **91**:1029–1036.
13. Low, B. 1965. Low recombination frequency for markers very near the origin in conjugation in *E. coli*. Genet. Res. **6**:469–473.
14. Luria, S. E. 1964. On the mechanism of action of colicins. Ann. Inst. Pasteur **107**:67–73.
15. Nagel de Zwaig, R., and S. E. Luria. 1967. Genetics and physiology of colicin-tolerant mutants of *Escherichia coli*. J. Bacteriol. **94**:1112–1123.
16. Nomura, M. 1963. Mode of action of colicines. Cold Spring Harbor Symp. Quant. Biol. **28**:315–324.
17. Nomura, M. 1964. Mechanism of action of colicines. Proc. Natl. Acad. Sci. U.S. **52**:1514–1521.
18. Nomura, M. 1967. Colicins and related bacteriocins. Ann. Rev. Microbiol. **21**:257–284.
19. Nomura, M., and C. Witten. 1967. Interaction of colicins with bacterial cells. III. Colicin-tolerant mutations in *Escherichia coli*. J. Bacteriol. **94**:1093–1111.
20. Reeves, P. 1966. Mutants resistant to colicin CA42-E2: cross resistance and genetic mapping of a special class of mutants. Australian J. Expt. Biol. Med. Sci. **44**:301–316.
21. Van de Putte, P. H., H. Zwenk, and A. Rörsch. 1966. Properties of four mutants of *Escherichia coli* defective in genetic recombination. Mutation Res. **3**:381–392.
22. Wood, W. B. 1966. Host specificity of DNA produced by *Escherichia coli*. Bacterial mutation affecting the restriction and modification of DNA. J. Mol. Biol. **16**:118–133.

SUMMARY

In recent years a significant amount of biological research has been focussed on the cytoplasmic membrane of the cell. This has emerged as a complex structure, participating in cell biosynthetic activities in addition to osmoregulation. However the functional organisation of the membrane is little understood, and in this may lie the key to several biological problems. An approach to the study of the coordination of biosynthetic processes by the cell membrane may be through the use of agents interacting specifically with the membrane. One such class of compounds may well be the natural protein antibiotics, colicins.

Colicins may be distinguished from conventional antibiotics by their unique mode of action with sensitive cells, whereby lethality is promoted from specific fixation sites in the bacterial cell wall. The colicin molecule neither penetrates the cell surface layer, nor causes gross cell membrane damage, and it has been postulated that interaction of the colicin protein with proteins of the cell membrane lead eventually to cell death.

The studies described in this Thesis have been concerned in particular with the interaction of colicin E2, one of the E-group of colicins, with the bacterial cell, and the mode of action of this colicin. Colicin E2 is characterised by the induction of a rapid degradation of cellular DNA, and the inhibition of cell division in sensitive bacteria. It was hoped that the genetical and physiological

characterisation of bacterial mutants specifically refractive to colicin E2 would indicate the nature of the altered component, normally participating in the transmission of the effect of the extracellular colicin to the intracellular DNA, and/or the cell division machinery.

Mutants refractive to colicin E2, designated Ref-II, were therefore isolated from a number of strains of E.coli K12. These mutants were systematically examined, and it was found that although the majority were relatively insensitive to UV irradiation, approximately 33% did display significantly increased sensitivity. Furthermore, these Ref-II UV^S strains were sensitive to the detergent, Sodium Deoxycholate, and grew slowly in complex medium, properties indicative in the first instance of an altered membrane component in this class of mutant, and in the second, of a normal role of the component in some aspect of cellular metabolism. These Ref-II UV^S mutants could also be distinguished from Ref-II UV^r mutants by possession of various other pleiotropic characters. For example, the majority of Ref-II UV^S mutants were defective in recombination, some were "resistant" to bacteriophage lambda, one grew in long filaments, and one produced excessive polysaccharide particularly when cultured on minimal agar, properties which may all be related to defective DNA metabolism in the mutants.

Genetic analysis revealed that pleiotropic characteristics shown by the Ref-II UV^S mutants were 100% co-transducible with the refII

locus. Moreover, if two or more cistrons were present, mutations in these producing either Ref-II or Ref-II UV^S mutants respectively, they must be adjacent on the chromosome. The refII locus was mapped and found to be co-transducible with serB, and to lie within four genes of the hsp locus on the bacterial chromosome. Reversion studies confirmed that the complex pleiotropy displayed by Ref-II UV^S mutants did in fact arise from mutation in a single gene, and not from a series of closely linked, multisite mutations arising from the action of the mutagen employed. Moreover, the site of the reversion mutation proved to be at the refII locus, and not at a second locus, mapped and found to be closely linked to thr, potentiating the expression of colicin E2-refractivity.

Different hypotheses may account for the occurrence of each individual pleiotropic character of the Ref-II UV^S mutants. However, collectively, it becomes apparent that the altered component in this class of mutants normally participates in DNA metabolism in the cell, and either (a) the enzymes participating in the repair and recombination of bacterial DNA, and possibly even the DNA molecule itself, may be bound to the altered component; or (b) the altered component may be one of an aggregate of proteins concerned specifically with chromosomal metabolism. Direct evidence that the altered component, at least in Ref-II UV^S mutants, is in fact structural, and presumably membrane, is received from the delayed expression of UV resistance after the receipt

of the UV^r gene in Ref-II UV^r/UV^s heterogenotes. Future experiments involving the isolation and comparison of membrane fractions from mutant and non-mutant strains will now be critical in the confirmation of this hypothesis.