ACKNOWLEDGEMENTS

I wish to thank particularly Dr. E.C.R. Reeve for his many kindnesses and his pleasant helpfulness.

I also wish to thank Dr. J.O. Bishop and Dr. J. Davidson for many helpful suggestions and discussions and many others, too numerous to mention, for their help.

I am also extremely grateful to Prof. H.J. Evans formerly of Aberdeen University for his interest in this work and also my many colleagues in Aberdeen for helpful discussion and advice on biochemical matters, particularly Dr. R.J. Ellis formerly of the Biochemistry Department. I must also mention the mammoth technical tasks painstakingly and accurately carried out by Mr. Robert MacKenzie formerly of the Genetics Department.

I am grateful to the Medical Research Council for a Training Award.
SUMMARY
Partial diploid strains of *Escherichia coli* have been constructed by finding recombinationless mutants in a suitable recipient strain. The behaviour of the partial diploids heterozygous at the streptomycin locus has been examined and it has been shown that only a very few partial diploids, heterozygous str-d/str-s, were capable of growing in the presence of streptomycin. In the absence of streptomycin growth was obviously much poorer with reassortment and outgrowths occurring very readily due to the residual level of recombination activity in the recipient. When the heterozygotes were selected in the absence of streptomycin it was found that growth was typically slow with once again clear evidence of genetic instability. Heterozygotes selected and grown on streptomycin medium plated with low efficiency on streptomycin free medium and vice versa. Growth in ethanol considerably improves the plating efficiencies.

A simple procedure is described for the recovery of mutants which behave as though streptomycin is bacteriostatic rather than bacteriocidal. The Sib locus appears to map close to the classical Str locus probably in the order AroD-Str-Sib-3pc. Sib$^-$ is shown to be dominant.

A method is developed for isolating spontaneous mutants in which the streptomycin - spectinomycin region is deleted. Some properties of these mutants are discussed and it is shown that they could perhaps have a role in fine structure deletion mapping of the region.

Partial diploids, deletion mutants and "cured" deletion mutants have been used in an unsuccessful attempt to map the ribosomal RNA locus leading to the conclusion that rRNA locus does not map in the Str-3pc region.
This is contrasted with work on *Proteus mirabilis* - *E. coli* hybrids which strongly indicate that rRNA is coded in the Mal - Spc region. The paradox is resolved by the use of *P. mirabilis* temperature sensitive mutants and a reinvestigation of the maltose locus involved in the *E. coli* to *P. mirabilis* transfer.
INTRODUCTION
The evidence for the role played by the ribosome in determining sensitivity or resistance of bacteria to the antibiotic streptomycin now seems well established. Early studies by Fitzgerald et al (1948) had shown that adaptive enzyme formation was inhibited by streptomycin. Later Anand and Davis (1960) and White and Flaks (1962) showed that protein synthesis in E. coli had virtually ceased within fifteen minutes of adding streptomycin. At the same time Dubin and Davis (1961) showed that the earliest observable effect of adding streptomycin was a rapid efflux of potassium ions from the cell, thus pointing to possible membrane damage. Dubin (1963) was then able to show that the loss in protein synthesizing activity of streptomycin treated cells was followed by a loss in cell viability although RNA synthesis continued unchecked.

In 1961 Spotts and Stanier (1961) advanced their hypothesis of ribosome involvement in streptomycin action on the basis of their studies with a streptomycin dependent mutant of E. coli. They suggested that the ribosome is the primary site of attack and speculated that messenger RNA attachment to the ribosome was inhibited by streptomycin in sensitive cells, was insensitive to the presence of the antibiotic in streptomycin resistant cells, and did not occur in streptomycin dependent cells unless the drug was present. Almost immediately Spyer et al (1962) and Flaks et al (1962) showed that streptomycin reduced polyphenylalanine production in the poly-u programmed protein synthesizing system devised by Nirenberg and Matthaei (1961).
It was shown that a much higher concentration of streptomycin was required to give a corresponding inhibition of incorporation when the cell-free system was prepared from a streptomycin resistant strain. Significantly, it was shown that the greater degree of insensitivity to the presence of streptomycin was a property of the ribosome component and not the supernatant. More persuasive evidence still was obtained by Cox et al. (1964) on the basis of mixing experiments. 70 s ribosomes were obtained from both streptomycin sensitive and resistant strains of E. coli and dissociated to their 50 and 30 s sub-units by lowering the magnesium concentration. The subunits were then mixed and the 70 s particle reconstituted such that it had a sensitive 30 s subunit together with a resistant 50 s subunit or vice versa. When the reconstituted ribosomes were tested in the poly-u system in the presence of streptomycin the results clearly indicated that only where the 30 s subunit was derived from the resistant strain did streptomycin fail to inhibit incorporation to the same degree as all other combinations. Recently, Stashelkin and Meselson (1966) have separated the 30 s subunit in the presence of CsCl into a meniscus fraction and a ribonucleoprotein core fraction, and by mixing experiments similar to those above have shown that resistance to streptomycin resides in the ribonucleoprotein core of the 30 s subunit. Hashimoto (1960) has shown by P1 mediated transduction that streptomycin resistance (str-r) and streptomycin dependence (str-d) are very closely linked and it is now generally assumed that they are allelic.
This implies that an extension of the results for resistance to dependence would be a simple task. Unfortunately attempts to show that the presence of streptomycin is required for ribosome function in the poly-u system have not been unequivocal. Cox et al (1964) showed that ribosomes from a str-d strain of E. coli did in fact behave as resistant in the in vitro poly-u test. It is claimed that after growth in streptomycin, the ribosomes of a streptomycin dependent mutant are sufficiently "charged" with streptomycin to function in the absence of added streptomycin. However, marginal stimulatory effects due to streptomycin have been observed by Likover and Kurland (1967) using Ca++ in the poly-u programmed system with ribosomes derived from a streptomycin dependent mutant.

Davis et al (1964), Friedman and Jeinstein (1964) and Szer and Cechoa (1964), have shown that streptomycin has other interesting effects on the poly-u system. When ribosomes from sensitive cells are used in the presence of streptomycin the amino acids isoleucine and, to a lesser extent, serine are incorporated into the acid insoluble material. Ribosomes from resistant cells do not show this effect although they remain as sensitive as wild type to certain other conditions which result in coding ambiguity such as raised Mg++ concentrations, Szer and Cechoa (1964); polyamines, and organic solvents, So, Bodley and Davie (1964.) The capacity of streptomycin to introduce ambiguity may be related to the phenomenon of streptomycin dependence. Brock (1966) draws attention to the fact that some streptomycin dependent mutants will grow on certain other basic antibiotics such as neamine, catenulin and neomycin C, substances which are also known to induce misreading in vitro.
Even more surprising is the discovery by Gado and Horvath (1963) that a streptomycin dependent mutant of E. coli would grow in the absence of streptomycin provided acetone, methanol or ethanol was substituted; again these substances are known to induce misreading in vitro. Thus a possible explanation of streptomycin dependence may be that the substance is required to overcome an ambiguity.

There is a considerable body of evidence in support of streptomycin suppression of amber and ochre mutations; by both Sm-s and Sm-r although there is no evidence to suggest that this occurs by streptomycin induced misreading. In addition Davis (1966) states that all (21) of the streptomycin dependent mutants in six strains were able to suppress a T4 ochre mutant and a known lac^-z ochre to give $\frac{1}{10}$ the level of Sm c, although no Z amber were suppressed. One of his Str^-r mutants showed a similar pattern but with a higher level of suppression in the presence of streptomycin. All the Str^-d revertants retained their suppression pattern but only in the presence of the drug. Leboy et al (1964) showed that a ribosomal protein, the K12 protein is coded for by either the streptomycin locus itself or a gene closely linked to it by means of an interspecific cross, although the evidence presented by Mayuga et al (1968) makes it appear more likely that the K12 band is a ribosomal protein coded by a gene separable from the streptomycin locus. By a similar technique Sypherd et al, (unpublished) mentioned by Apirion, have evidence for several - at least three ribosomal proteins coded for genes in the immediate vicinity of the Str^g gene. It appears that several ribosomal proteins are coded in the region of the streptomycin locus. All to date are associated with the 30's subunit only.
In Bacillus subtilis Oishi and Sueoka (1965) and Dubnau et al (1965) have shown that 16s and 23s ribosomal RNA hybridizes with a restricted region of genome and that the closely linked markers str-2 and ery-2 are located in the same region. In E. coli, too, there is an indication that rDNA is located in the region of the str locus. Rudner et al (1965) showed that when two E. coli K12 Hfr cultures were pulse labelled with $^{32}$P orthophosphate during synchronized growth two bursts of RNA synthesizing activity occur during the cell cycle and that this RNA is in base composition similar to ribosomal RNA. On the assumption that the direction of replication of DNA is, in Hfr strains, the same as their direction of transfer, it is shown that one of these peaks coincides with the Str locus. In addition Cutler and Evans (1967) examined the ability of various segments of the E. coli genome, isolated by 5 bromouracil pulse labelling of synchronized cultures, to hybridize with rRNA. They too concluded that there exist two regions of the genome which hybridize with rRNA and one of those regions is located in the vicinity of the str-locus. However, Vermaelen and Atwood state that they have located only one region which hybridizes with rRNA and imply that it is located in the 75-95 minute region of the E. coli linkage map according to Taylor and Thomas (1965.) Unfortunately there is some doubt about whether Hfr strains of E. coli initiate vegetative DNA synthesis at the site of commencement of Hfr transfer and until the assumption is verified or refuted caution must be exercised in interpreting such results. It is clear, however, that if the site of initiation of replication and the direction were accurately known for any particular strain then the location of the rRNA cistrons could be readily determined.
Alternatively, if rRNA is coded in the str region, a partial diploid strain will, on a per cell basis, have an increased rRNA - DNA hybridizing capacity.

The production of partial diploids is normally achieved by selecting for a suitable terminal marker early in an Hfr x F- cross. Unfortunately, although a suitable Hfr may be available it is not possible to devise a selection system which specifically ensures the presence of drug markers on the exogenote. The reason for this is that streptomycin resistance is recessive to streptomycin sensitivity as was first shown by Lederberg (1951) on the basis of studies with transient heterozygotes. E. Goldschmidt (personal communication via C. Felkner) considers Lederberg's original observations to have been made on a recombinaseless strain of the type described by Clark and Margulies (1965). A puzzling feature of Lederberg's work was that his observations were confined to streptomycin resistance and sensitivity, no results being reported for streptomycin dependence. More recently Low (1963) has shown that most, or even all, of the low number of apparent recombinants which result from an Hfr x F- rec- mutant cross are in fact partial diploids and many of them are F's. Thus a suitable F' strain can be constructed much more easily by using an Hfr which has its origin reasonably close to the streptomycin locus and an F- which has a marker distal to the streptomycin locus.

Ideally, in a wide variety of situations, one would like a system which allows isolation of a particular region of the genome. The ideal can be approached by the discovery of Falkow et al (1964) that E. coli genetic information can be transferred to Proteus mirabilis.
The considerable differences between the E. coli guanine + cytosine (51%) and the Proteus mirabilis guanine + cytosine (39%) makes it possible to separate these DNAs in a suitable CsCl gradient. Whereas previously such gradients were formed in a swinging bucket with a low loading and resolution factor more recently Flamm et al (1962) have shown that fixed angle rotors will give a much higher resolution together with a greater capacity.

I decided that there would be some value in repeating Lederberg's original work on dominance and in attempting to extend the observations to include streptomycin dependence and its relationship to the other alleles at the streptomycin locus. The most promising approach seemed to be to seek for a suitable K12 recombinationless and streptomycin sensitive recipient which would allow easy construction of partial diploids and ready selection of both streptomycin resistant and dependent mutants.

At the same time I hoped to examine the prospects for constructing Proteus mirabilis - E. coli intergeneric hybrids with a view to investigating the effect of the E. coli streptomycin locus in the P. mirabilis cell. The ultimate aim of this part of the work was to extract the exogenous DNA from the hybrids and use it to determine whether ribosomal RNA was coded in the streptomycin region of the genome.

/Finally........
Finally a longer term project began to emerge as I accumulated more and more partial hybrids and developed some facility in DNA – RNA hybridization techniques.

If specific regions of the *E. coli* genome could be isolated then it ought to be a simple matter to determine the origin and direction of replication of the *E. coli* genome by DNA – RNA hybridization and pulse labelling of synchronised cultures. However, more recent work in this field has now reduced the latter ideas to historical interest only.
MATERIALS AND METHODS
MEDIA

M9 minimal medium as described by Adams (1959) was used throughout and supplemented as described in the text where required.

Nutrient broth was the product supplied by either Oxoid or Difco and prepared according to the manufacturer's specification. Nutrient agar was prepared by adding 1.5% agar powder, either Oxoid or Difco, to nutrient broth. For Proteus mirabilis the agar concentration was raised to 2% to inhibit swarming and the sodium chloride concentration, normally 0.5%, was raised to 3%.

Eosin methylene blue medium (EMB) was routinely employed to detect sugar fermenters. EMB contained 1% peptone, 0.5% NaCl, 0.2% K₂HPO₄, 0.04% Eosin Yellow, 0.0065% Methylene blue, 1.5% agar.

The eosin methylene blue and sugar solutions were added separately to the nutrient base. The final concentration of the sugar was 1%. The addition of potassium phosphate is said to improve the resolution of the medium.

Fermentation tests on Proteus mirabilis were usually performed on very dry EMB plates containing 2% agar.

Non-fermenting colonies were detected on Tetracolum agar. The medium consisted of 25% strength nutrient broth containing 1.5% agar, 1% sugar, 0.005% 2:3:5 tri-phenyltetrazoluim chloride.
A filter sterilized solution of the dye and the autoclaved sugar solution was added to the autoclaved nutrient agar base immediately before pouring the plates.
# LIST OF BACTERIAL AND PHAGE STOCKS

## E. coli K12

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Markers</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hfr H</td>
<td>met−</td>
<td>E.C.R. Reeve</td>
</tr>
<tr>
<td>Hfr R1</td>
<td>met−</td>
<td>&quot;</td>
</tr>
<tr>
<td>Hfr D11</td>
<td>met−</td>
<td>&quot;</td>
</tr>
<tr>
<td>Hfr C</td>
<td>met−</td>
<td>&quot;</td>
</tr>
<tr>
<td>Hfr J4</td>
<td>thr− leu− thi−</td>
<td>&quot;</td>
</tr>
<tr>
<td>Hfr AB312</td>
<td>thr− leu</td>
<td>&quot;</td>
</tr>
<tr>
<td>Hfr G6</td>
<td>his−</td>
<td>T. Matney</td>
</tr>
<tr>
<td>Hfr G6 thy−</td>
<td>Obtained from G6 by aminopterin selection</td>
<td></td>
</tr>
<tr>
<td>Hfr J1α-19*</td>
<td>trp− his− Cml A</td>
<td>D. Suttie</td>
</tr>
<tr>
<td>RE 26</td>
<td>F− proA− trp− his− str−s lac− gal−</td>
<td>E.C.R. Reeve</td>
</tr>
<tr>
<td>RE 103</td>
<td>F− proA− trp− his− str−r lac− cmlA</td>
<td>&quot;</td>
</tr>
<tr>
<td>20</td>
<td>gal− prototroph</td>
<td>J. Davison</td>
</tr>
<tr>
<td>20 mal−</td>
<td>Obtained from 20 by treatment with $\lambda^{857}$ at 40°C</td>
<td></td>
</tr>
<tr>
<td>AB 2347</td>
<td>mal A− Aro B− str−c</td>
<td>J. Pittard</td>
</tr>
<tr>
<td>AB 2347 thy−</td>
<td>Obtained from AB2347 by aminopterin selection</td>
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<tr>
<td>AB1157 rec A</td>
<td>thr− leu− thi− pro− arg− his−</td>
<td>R. Pritchard</td>
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<tr>
<td></td>
<td>rec A− lac− gal− mtl−</td>
<td>&quot;</td>
</tr>
<tr>
<td>E 34</td>
<td>his− ser/gly</td>
<td>J. Bishop</td>
</tr>
<tr>
<td>E 34 thy−</td>
<td>Obtained from E 34 by aminopterin selection</td>
<td></td>
</tr>
<tr>
<td>703</td>
<td>K 12 prototroph</td>
<td>R. Clowes</td>
</tr>
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</table>

*Hfr J1α-19 is a donor of the D11 type obtained in a cross between D11 and RE 103 with selection for a terminal marker, lac, and proximal markers - pro and cmlA. Recombinants were checked for Hfr donor ability and one, J1α-19, retained.*

Continued..
### Proteus mirabilis

<table>
<thead>
<tr>
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<td>P2-1</td>
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<td>J. Bishop</td>
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<tr>
<td>P2-1 thy-</td>
<td>Obtained from P2-1 by aminopterin selection</td>
<td></td>
</tr>
<tr>
<td>P 6</td>
<td>nic- B, mal- str-s</td>
<td>H. Böhme</td>
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</table>

### Bacteriophages

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</thead>
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<tr>
<td>T1kc</td>
<td>K 12 transducing phage</td>
<td>E.C.R. Reeve</td>
</tr>
<tr>
<td>$\lambda$ &amp; $\psi$ &amp; $\nu$</td>
<td>Behaves like $\lambda$ above 39°C</td>
<td>J. Davison</td>
</tr>
<tr>
<td>P 1</td>
<td>Proteus mirabilis transducing phage</td>
<td>H. Böhme</td>
</tr>
</tbody>
</table>
The above map shows the genetic markers referred to in the text and the origin and direction of transfer of the *E. coli* Hfr strains employed.
METHODS

The bacterial strains used together with their origin are shown in the accompanying list.

Bacterial Methods

Hfr x F⁻ crosses

These were carried out in liquid medium usually nutrient broth (oxoid) but sometimes in M9 medium supplemented as required. The Hfr cells were taken from an overnight culture, diluted 1/50 into fresh prewarmed medium (usually 10 ml) and incubated at 37°C for a further ninety minutes or two hours. An overnight culture of the F⁻ strain was used. 1 ml of the F⁻ culture in 3 ml of medium in a 250 ml Erlenmeyer flask which was initially shaken to mix the cultures and then kept stationary in a water bath at 37°C. In some "time-of-entry" experiments the method of diluting the cells after effective pair formation was used as described by De Haan and Gross (1962). Mating cells were interrupted by making a suitable dilution into M9 buffer and blending in an MSE homogenizer at 2/3 maximum setting.

Crossoes involving Hfr and strains of Proteus mirabilis HM-1 or P6 were performed, for best results, by mixing 0.1 ml of an overnight culture of donor and recipient on a nutrient agar plate which was then incubated at 37°C overnight. The cells were then washed off with 10 ml of M9 buffer and suitable dilutions plated on selective media. The method results in 1 hybrid per $10^4 - 10^7$ Proteus mirabilis cells depending upon the history of the strain. Performing the cross in liquid gave a much poorer result.

/Transduction...
F1 transduction was performed according to the method used in Dr. Reeve's laboratory. Base agar consisted of Bacto tryptone 10 gms, yeast extract 5 gms, NaCl 10 gms, glucose 1 gm, 0.002 M Ca\(^{++}\) as CaCl\(_2\), Difco Bacto Agar 10 gms and water to 1 litre. Top agar consisted of oxoid tryptone 10 gms, NaCl 5 gms, Difco Noble Agar 7 gms and water to 1 litre.

Confluent lysis plates are prepared by adding 10\(^6\) F1 p.f.u.s. to 2.5 ml of top agar and then adding 0.2 ml of an overnight culture of the donor strain. The culture medium for the donor was broth with 1\% glucose made 0.002 M Ca\(^{++}\). The top agar is then added to a plate of bottom agar (not dried) and incubated overnight at 37\(^\circ\)C. The phage is harvested by scraping the top layer into a centrifuge tube and spinning at 4000 g for 15 minutes. A trace of chloroform is then added. Phage usually titres at around 10\(^{11}\) p.f.u.s/ml. The recipient is grown in phage broth containing 0.01 M Ca\(^{++}\) to approximately 5 \times 10^8 cells/ml, centrifuged and resuspended in \(\frac{1}{2}\) volume of fresh prewarmed broth. 0.1 ml of F1 phage is added to give a m.o.i. of 0.1 approximately. The mixture is allowed to stand for 90 minutes at room temperature after which the cells are harvested by centrifugation, resuspended in buffer + 0.5\% sodium citrate and plated on selective media containing 0.5\% sodium citrate. Assays were routinely performed on unadsorbed phage and cell viability before and after treatment.

/Mutation........
Mutation Treatment

Ethylemethane sulphonate (EMS) solution was prepared by adding aseptically 0.25 ml of EMS to 5 ml of M9 buffer. 1 ml of this solution was added to 1 ml of an overnight broth culture and incubated at 37°C for 30 minutes. The treated cells were washed on a membrane filter, resuspended in broth and allowed to grow for 4 hours for phenotypic expression. This procedure results in approximately 300 - 400 streptomycin resistant cells per ml. On testing, the resistent cells are predominantly found to be dependent.

Nitrosoguanidine mutation

Log phase cells were washed and resuspended in Tris-maleic acid buffer pH 5.5 with 0.1 mg/ml nitrosoguanidine (NG) and incubated at 37°C for 30 minutes. The treatment was stopped by transferring the cells on to a membrane filter and washing with TM buffer. The cells were grown in Nutrient broth for expression prior to plating as above.

Selection of thymine requiring mutants

Aminopterin was included in M9 agar to give a final concentration of 500 μg/ml containing glucose at 0.2% and thymine at 50 μg/ml together with any other nutritional requirement of the strain. Approximately 10^7 cells were spread per plate and incubated at 37°C until colonies appeared. The plates were then replicated on to medium lacking thymine and also in the case of Hfr strains on to a lawn of a suitable F⁻ recipient on selective plates in order to confirm mating ability.
**BIOCHEMICAL METHODS**

**Preparation of ribosomes**

Ribosomes were obtained from cells in the log phase of growth by centrifuging the cells and washing three times with Tris-HCl, $10^{-2}$M, pH 7.2 with $10^{-2}$M Mg++. The cell suspension was broken by passing through a French press at 10,000 p.s.i. The broken cell suspension was then centrifuged at 10,000 g for 15 minutes to remove cells and debris. The supernatant was centrifuged at 100,000 g for 120 minutes. The supernatant was discarded and the pellet taken up in Tris buffer pH 7.4, 0.01M Mg++. Another low speed-high speed centrifugation was performed to give a ribosomal pellet. The low speed spins were done in a Sorval RC2-B and the high speed runs in an MSE superspeed 65.

In some cases the cells were broken by grinding the washed cells with Alumina powder, 2 pts Alumina (w/w). The grinding was continued in a chilled mortar for a few minutes before extraction with Tris-HCl buffer. DNAse was added to give a final concentration of 10 mg/ml in order to reduce the viscosity.

**Extraction of RNA**

Freshly distilled phenol was equilibrated with 0.02 M phosphate buffer at pH 7.0 with 0.1% sodium lauryl sulphate (SLS). An equal volume of equilibrated phenol was added to the ribosome suspension and shaken vigorously in a wrist-action shaker for 30 minutes.
The aqueous (top) layer was removed and given two further phenol deproteinizations. The phenol was removed by extraction with ether (equal volume) and the ether removed by bubbling with nitrogen. The preparation was dialysed overnight at 4°C against 0.02 M phosphate buffer pH 7. The RNA preparation was then treated with 2 ug/ml DNase (RNase free) for 60 minutes and the DNase removed by 3 further phenol deproteinizations as described above. This treatment has the effect of reducing the blank values in DNA-RNA hybridization (J. Bishop personal communication).

DNA Preparation

Marmur's method was followed exactly in earlier experiments. In later work the cells were lysed with 0.2% SLS and only one chloroform-iscamyl alcohol deproteinization performed. The aqueous layer was made up to 1.71 M in cesium chloride by addition of the solid salt. The mixture was then centrifuged for 24 hours at 42,000 R.P.M. in the 10 x 10 ml rotor of an MSE superspeed 65. In fact, the 10 x 10 ml rotor is an MSE Superspeed 50 rotor and presents certain difficulties in controlling the temperature when used on the "65". After 24 hours the tubes were emptied by puncturing with an MSE tube piercer and pumping the contents through a UV-Cord II hooked to a Beckman 10" lin/log recorder. Twenty-five drop fractions were collected with a Radi-Rac fraction collector. The pump used was an LKB Perpex fitted with a gear-box to deliver approximately 40 ml per hour.

/Good.....
Good profiles were always obtained by this method provided care was taken to keep the contents under positive gravity. The tubes containing the satellite band were pooled and dialyzed against 0.1 x SSC for 24 hours with three changes followed by rebanding under exactly similar conditions.

**DNA-RNA hybridization**

DNA was denatured by treatment with 0.01 M KOH and loaded in 2 x SSC on to presoaked Schleicher and Schuell B-6 filters (27 mm). The filters were dried and baked at 70°C in a vacuum oven for 3 hours. The amount of DNA adsorbed to the filter was generally measured by counting the $^{3}H$ on the filter in a Beckman LS 100 scintillation counter.

The DNA filters were incubated for 6 hours in 5 ml of 2 x SSC containing the required concentration of RNA. The filters were washed on both sides with 50 ml 2 x SSC dried and counted for $^{32}P$ activity.

**Radioactive labelling**

$^{3}H$ labelled DNA was obtained by growing a suitable thymine requiring derivative of PM-1 or P65 in M9 medium containing 0.2% glucose, 0.05% yeast extract, 10 ug/ml nicotinic acid and 4 ug $^{3}H$ thymine /ml.
$^{32}P$ labelling of rRNA was accomplished by growing the cells overnight in Tris-buffered salts medium according to Hershey. The cells were harvested, washed and resuspended in 1 litre of Tris-buffered salts in which the $\text{KH}_2\text{PO}_4$ was reduced to $5 \times 10^{-3} \text{M}$ and which contained 10 mc of neutralized $^{32}P$. The culture was shaken at 37°C and after 4 hours $\text{KH}_2\text{PO}_4$ added to the original concentration. After a further 4 hours the culture was harvested and ribosomes extracted as above.

Filters were counted in a Beckman LS 100 spectrometer. The counting fluid consisted of PPO 7.0 gm/l, dimethyl POPOP 0.3 gm/l naphthalene in reagent dioxane 100 g/l.
RESULTS
THE BEHAVIOUR OF THE STREPTOMYCIN DEPENDENT ALLELE

IN PARTIAL DIPLOIDS

It is probable that the original strain used by Lederberg to demonstrate the dominance of streptomycin sensitivity over streptomycin resistance was in fact a naturally occurring recombination defective F"(Goldsmith, personal communication).

Any attempt to re-examine and extend Lederberg's work could usefully start, therefore, from the construction of appropriate diploids using a suitable recombinationless recipient. The most suitable recipient strain would appear to be streptomycin sensitive, thus allowing easy selection of streptomycin resistant and streptomycin dependent mutants, and Mal" at the malA locus which is closely linked to the str locus. Unfortunately all the available rec" strains were precluded because of their streptomycin resistance. For this reason it was decided to work with the streptomycin sensitive F" strain, RE 26, and following Margulies and Clark (1965), attempt to find a recombination deficient mutant.

Briefly, the method adopted by Margulies and Clark was to mutate a culture of a suitable F", grow single colonies on nutrient agar and replicate these on to a lawn of a suitable Hfr which is counter-selected nutritionally. My early unsuccessful attempts followed this procedure exactly. Repeat failures caused me to adapt the above method slightly.

An overnight culture of RE26 was grown up in broth, diluted into fresh broth and mutagenized with nitrosoguanidine as described in the Materials and Methods section. The mutated cells were...
allowed to grow in broth for four hours and plated on M9 agar containing 0.2% glucose, 50 ug/ml histidine, 0.2 ug/ml each of proline and tryptophan. When the plating density is approximately 1000 cells per plate only small colonies are obtained. The plates were incubated overnight at 37°C and were then overlaid with soft M9 agar supplemented with 0.2% glucose, 50 ug/ml histidine; and a mixture of washed, broth grown log phase cells of the donor strains Hfr H and Hfr C at approximately $2 \times 10^7$ of each per plate. The volume of top agar is 2.5 ml. The plates were returned to the incubator and examined after 3 days. 120 plates were examined in this way and 171 small colonies obtained. These were first stabbed out and purified twice on M9 agar complete with 0.2% glucose and proline, tryptophan and histidine at a final concentration of 50 ug/ml. The selection is based on the observation that the F$^-$ colonies grow only to pin head size on the low amino acid supplemented plates unless the form met$^+$ pro$^+$ trp$^+$ recombinants with either Hfr H or Hfr C. These recombinants then produce normal sized colonies which are easily distinguished from the small unmated colonies.

The colonies which were able to grow on the above medium, 128/171, were stabbed into bacteriological tubes containing broth, grown overnight and tested by spotting onto freshly spread lawns of once-washed broth grown cells of Hfr H and Hfr C on M9 glucose histidine agar. The majority of the spots gave near confluent growth but a number gave patterns which ranged from many isolated colonies to very few colonies per spot. Three strains which gave only a few colonies per spot were retained and tested by the conventional liquid mating technique. These mutants RE 26 - 39, 26-44 and 26-70 were all obtained from the same mutagenized culture of RE26 and are possibly allelic although this was not tested further.

/Their........
Their inability to form recombinants is shown in Table I.

<table>
<thead>
<tr>
<th>Strain</th>
<th>% gal(^+) recombinants</th>
<th>gal(^+) segregating gal(^-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RE 26</td>
<td>4.4</td>
<td>0/40</td>
</tr>
<tr>
<td>RE 26-39</td>
<td>0.0028</td>
<td>6/80</td>
</tr>
<tr>
<td>RE 26-44</td>
<td>0.0051</td>
<td>11/80</td>
</tr>
<tr>
<td>RE 26-70</td>
<td>0.0047</td>
<td>5/80</td>
</tr>
</tbody>
</table>

Table I Mating ability of RE 26 and its rec\(^-\) derivatives. The table shows the percentage of gal\(^+\) recombinants obtained in a 30 minute interrupted cross between Hfr E11 and RE 26 and the three rec\(^-\) derivatives. The number of gal\(^+\) colonies segregating gal\(^-\) was obtained by streaking on Tetrazolium galactose agar followed by a further verification of those cases which appeared to be segregating.

The original intention had been to select malA mutants in these rec\(^-\) strains because of the proximity of the malA locus to the streptomycin locus. It was, therefore, with some disappointment that I discovered RE 26 and its derivatives to be \(\lambda^R\) thus making the easy selection of Mal\(^-\) by treatment with \(\lambda^V\) impossible. However, in view of the difficulty experienced in obtaining the rec mutants I decided to continue working with them and attempt to obtain Mal\(^-\) mutants by nitrosoguanidine mutagenesis.
In initial experiments the survival dropped from 20-30% to less than 0.1% but by halving the time of exposure the survivors were brought up to 3-4%. This helps to confirm that the strains were truly rec- since such mutants are known to have a reduced ability to recover from any treatment which results in DNA damage.

Selection for Mal- mutants was performed on tetrazolium maltose agar plates at approximately 700-800 cells per plate. Maltose negative mutants appeared at one per 12-13 plates.

The maltose negative colonies were tested for their ability to revert to Mal+ and a low frequency of formation of Mal+ recombinants with a malA \( ^{R} \) mutant of Hfr G6 thy- obtained by spotting onto an EMB maltose plate spread with G6 thy- and incubating at 42°C. As a final verification the putative F- rec- malA mutants were crossed with Hfr J4, a known malB mutant. The first Mal- mutant obtained which answered the above criteria namely a low reversion frequency, low ability to form recombinants with a malA \( ^{R} \) Hfr and increased ability to form recombinants with a known malB Hfr was RE 26-70-7.

The results are presented in Table 2.

<table>
<thead>
<tr>
<th>Cross</th>
<th>N°. of mal+</th>
<th>N°. of gal+</th>
</tr>
</thead>
<tbody>
<tr>
<td>J4 x RE 26-70-7</td>
<td>181</td>
<td>617</td>
</tr>
<tr>
<td>G639 x RE 26-70-7</td>
<td>7</td>
<td>292</td>
</tr>
</tbody>
</table>

Table 2 0.1 ml of a log phase broth culture of the donor strain was plated together with 0.1 ml of an overnight culture of RE 26-70-7, on M9 agar supplemented with tryptophan, proline and histidine, each at a final concentration of 50 \( \mu \)g/ml. The carbon source was either maltose or galactose at a final concentration of 0.2%.

The donor strain G639 is G6 thy- malA(\( \lambda^{R} \)) str-r.
Selection of streptomycin dependent mutants in RE 70-7

In the initial experiments designed to obtain Str-d mutations in RE 26-70-7 the mutagen nitrosoguanidine was used. Killing was always rather high and most of the streptomycin dependent mutants obtained by plating the mutated culture on nutrient agar + 200 μg/ml streptomycin sulphate failed to grow when tested on M9 glucose streptomycin medium supplemented with the required amino acid, proline, tryptophan and histidine. Those which did grow on the M9 plates did so rather poorly. Many of the primary isolates when restreaked on nutrient agar with streptomycin gave large colonies and large numbers of small translucent colonies which on restreaking again gave rise to large and small translucent colonies. When the large colonies were restreaked several times many continued to produce small translucent colonies. These observations, although of considerable potential interest, were not pursued further and it was decided to seek spontaneously arising mutants.

Cultures of RE 26-70-7 were grown overnight in nutrient broth supplemented with yeast extract and 0.2% glucose, centrifuged and resuspended in 1/10 volume of M9 buffer and 0.1 ml aliquots plated on M9 glucose containing 200 μg/ml streptomycin with proline, tryptophan and histidine each at 50 μg/ml. By this means, RE 26-70-7 str-d1, d2 and d7 were shown to be streptomycin dependent mutants which arose independently and were capable of growing on M9 glucose, proline, tryptophan and histidine when supplemented with streptomycin.
Effect of streptomycin deprivation on the limited recipient ability of RE 26-70-7 Str-dl

Since it was envisaged that the recipient cells would under certain conditions, be deprived of streptomycin possibly before a partial zygote had an opportunity to become established it was considered important to determine the effect of streptomycin deprivation on the capacity of RE-26-70-7 str-dl to form recombinants and partial diploids.

RE 26-70-7 str-dl was grown overnight in nutrient broth + 200 μg/ml streptomycin. The cells were washed on a membrane filter and resuspended in fresh broth at 37°C without streptomycin. 1 ml samples were withdrawn every thirty minutes over a three hour period and immediately mated with a log phase culture of Hfr B11 for 20 minutes. The matings were stopped by blending and the mixture of cells was washed on a membrane filter, resuspended in M9 buffer and 0.1 ml samples plated on M9 agar with 0.2% galactose, proline, tryptophan and histidine each at a final concentration of 50 μg/ml and 200 μg/ml streptomycin. A series of dilutions was also made to determine the viable counts of Hfr B11 and RE 26-70-7 str-dl.

The number of gal+ colonies which segregated gal- i.e. the diploid colonies, was determined by streaking on Tetrazolium Galactose agar where the gal- segregants appear red.

The results are presented in Table 3 where it appears that there is a decline in the number of gal+ colonies obtained together with a slight increase in the number of partial diploids after 90 minutes of streptomycin deprivation.
It would perhaps be worthwhile repeating this work to obtain more extensive data in order to study the processes involved in zygote formation and recombination.

<table>
<thead>
<tr>
<th>Time of streptomycin deprivation in minutes</th>
<th>% of gal$^+$</th>
<th>No. of gal$^+$ segregating gal$^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0066</td>
<td>5/120</td>
</tr>
<tr>
<td>30</td>
<td>0.0070</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>0.0054</td>
<td>8/120</td>
</tr>
<tr>
<td>90</td>
<td>0.0068</td>
<td>-</td>
</tr>
<tr>
<td>120</td>
<td>0.0031</td>
<td>17/120</td>
</tr>
<tr>
<td>150</td>
<td>0.0018</td>
<td>-</td>
</tr>
<tr>
<td>180</td>
<td>0.0022</td>
<td>15/120</td>
</tr>
</tbody>
</table>

Table 3 The effect of streptomycin deprivation on the recipient ability of RE 26-70-7 str-dl. The procedure is described in the text.

Construction of Partial Dioloids for the Mal-Str region

The only Hfr strain available which is streptomycin sensitive and which will transfer the streptomycin-maltose region early is Hfr G6. A Thy$^-$ mutation was selected in this male strain by the aminopterin method as described in the Materials and Methods Section. The Hfr strain AB312 appears to have the same origin as G6; it differs, however, in being streptomycin resistant.
RE 26-70-7 str-d1 was grown overnight in nutrient broth + 200 ug/ml streptomycin. The cells were washed on membrane filters, resuspended in fresh broth and mated with Hfr G6 thy-. Transfer was interrupted by blending after 30 minutes. 0.1 ml samples were then plated on L9 agar with 0.2% maltose with proline, tryptophan and histidine at 50 ug/ml either with or without streptomycin. The colonies which appeared on both types of plate were tested on Tetrazolium Maltose + streptomycin plates for ability to segregate mal- and subsequently for the ability to segregate the streptomycin allele. In the experiment, 47 Mal+ colonies were obtained in the presence of streptomycin. When these 47 colonies were restreaked on Tetrazolium Maltose agar + streptomycin only 3 colonies gave red Mal- colonies. The Mal- colonies obtained in the presence of streptomycin remained streptomycin dependent (10 out of 10 tested) and gave rise to streptomycin non-requiring mutants at a rate comparable with the reversion rate of the Str-d1 mutation i.e. only a few colonies appear in the well with a heavy loopful. This indicates that 3 colonies out of 47 are heterozygous at the Mal A locus and homozygous or hemizygous at the Str locus.

However, when the 47 Mal+ colonies were tested on Tetrazolium Maltose agar without streptomycin the same three colonies as before gave rise to fast growing Mal+ colonies, fast growing Mal- colonies and a large number of slow growing colonies which remained pale, i.e. Mal+. By five days of incubation most small slow growing colonies had produced rapidly growing sectors and papillae, many of which became deep red indicating that the Mal- allele was segregating.
All categories of colonies when removed from the Tetrazolium Maltose plate and restreaked on streptomycin nutrient agar failed to grow with the exception of a few slow growers, about 10%, which gave rise to a few colonies i.e. less than 20 per plate. When tested, these colonies gave a similar pattern to that described above.

The results indicate that the Str-d allele is still present in the three colonies which segregate Mal'. The various classes being readily accounted for in the following way.

Str-dl is partially dominant over Str-s hence the strains utilize maltose and require streptomycin. As they grow the low residual recombination ability gives Mal' Str-dl and Mal' Str-s and Mal+ str-s recombinants. Elimination of the exogenomic fragment gives the original RE-26-70-7. During growth on streptomycin all the Str-s classes will be eliminated. During growth which is obviously poorer in the absence of streptomycin only the Str-dl class will be eliminated and sectors of faster growing haploid recombinants will appear.

The poor plating efficiencies from streptomycin onto antibiotic free plates and vice versa present a serious problem to the simple picture presented above. I have, however, subsequently shown that there is an explanation although the explanation itself only poses deeper problems.
Before discussing this new evidence I will continue with the analysis of the three colonies segregating Mal\(^-\).

The original isolates were designated CR 1, 2, 3. Two fast growing Mal\(^+\) mutants obtained from fast growing sectors of CR 1 when plated on Tetrazolium Maltose agar lacking streptomycin were also studied. The CR 1 stocks were kept on streptomycin nutrient agar slopes and the modified stocks CR 1-M 1, CR 1-M 2 were held on nutrient agar.

In an attempt to demonstrate the presence of both str alleles in the isolates, P 1 phage was grown on all five and used to transduce a streptomycin dependent mutant (obtained by EMS mutagenesis) of strain 20.

CR 1, 2, and 3 were grown in the presence of streptomycin and CR 1 - M 1 and CR 1 - M 2 were grown without streptomycin.

Only the modified mutants derived from CR 1 were able to transduce the streptomycin dependent strain 20 and the frequency of transduction corresponds to that observed with a donor which possesses the str-s allele. The results are shown in Table 4.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>20-dl</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR 1</td>
<td>*512</td>
<td>2830</td>
<td></td>
</tr>
<tr>
<td>CR 2</td>
<td>*589</td>
<td>3400</td>
<td></td>
</tr>
<tr>
<td>CR 3</td>
<td>*488</td>
<td>2360</td>
<td></td>
</tr>
<tr>
<td>CR 1 - M 1</td>
<td>2270</td>
<td>NIL</td>
<td></td>
</tr>
<tr>
<td>CR 1 - M 2</td>
<td>1620</td>
<td>NIL</td>
<td></td>
</tr>
</tbody>
</table>

* Indistinguishable from spontaneous reversion.

Table 4 Transduction of Strain 20 and 20 str-dl, obtained as described above, with transducing lysates of P1 prepared on the putative partial diploids CR-1, CR-2, CR-3 and two fast growing Mal\(^+\) mutants derived from CR-1. Selection was for streptomycin resistance in the case of 20 and for streptomycin independence in the case of 20 str-dl.

/Surprisingly.....
Surprisingly then, the transduction test indicates that the Str-s allele derived from Hfr G6 is not present in CR 1, 2 or 3, yet two fast growing sectors derived from the slow growers obtained when CR 1 is spread on tetrazolium agar lacking streptomycin obviously contain only the Str-s allele.

It is unlikely that the Str-s allele transduced from CR 1 - Ml and M2 is a revertant of the original RE 26-70-7 Str-dl because of the high frequency with which sectors appear.

Attempts to show that the Mal allele was still present in CR 1, 2 and 3 failed because of the very high forward mutation rate at the MalA locus when selected with \( \text{M}^V \). Since CR 1, 2 and 3 were clearly Mal+ on Tetrazolium Maltose agar the obvious test of showing that Mal+ could be transduced from these putative diploids to a suitable Mal- strain did not occur to me. I now suspect that transduction frequencies of alleles on the exogenousomic element of a partial zygote constructed in this way can be reduced by a factor as great as 100 relative to endogenous markers. My evidence for this is confined to work on a Protex mirabilis/E. coli hybrid with \( \text{T} \) transducing phage and could of course be explained in many other ways.

The Nature of the Mal- segregating colonies obtained in the absence of streptomycin

So far, I have discussed the properties of the three Mal+ colonies which segregate Mal- which were obtained when the interrupted mating Hfr G6 thy- x RE 26-70-7 Str-dl was plated on streptomycin and Mal+ Thy+ recombinants were selected.
When the same cross was plated without streptomycin a total of 379 colonies appeared. 41 colonies segregated Mal⁻ on Tetrazolium Maltose agar and the remaining 338 failed to do so.

Most of the colonies appeared within 3 days and the remaining colonies, those subsequently shown to segregate Mal⁻ are invariably slow growing and appear on the fourth and fifth days although in some cases they are visible but extremely small on the third day. Most of the 41 segregators appeared after the third day.

Between 6 - 8 days, most of the slow growers began to produce fast growing sectors which swamp the original colony.

All 41 colonies were tested for their ability to grow on Tetrazolium Maltose agar containing 200 ug/ml streptomycin; 34 gave numerous small colonies in the well with only a few growing beyond the well. These colonies were almost entirely Mal⁻ only a rare Mal⁺ colony being observed. The remaining 7 did not show any growth in the well despite several tests, although they continued to segregate Mal⁻ on Tetrazolium Maltose agar.

A sample of 30 out of the 338 colonies which did not segregate Mal⁻ was also tested on Tetrazolium Maltose agar + streptomycin. None gave any indication of growth even in the well.

24 Mal⁻ segregants which appeared on Tetrazolium Maltose agar without streptomycin were tested for their ability to grow on streptomycin. None grew nor did they give any colonies in the well.
Nature of the Fast Growing Sectors

Because of the rapidity with which the original slow growing colonies were swamped by fast growing sectors and papillae which made maintenance of the original colony very difficult I decided to examine the fast growing sectors by transduction.

Two independent fast growing sectors JSM 1 and JSM 2 were streaked for purification on Tetrazolium Maltose agar and used to grow a F1 lysate. If the original Str-d1 allele was still present in JSM 1 and JSM 2 then it ought to be possible to transduce a suitable streptomycin sensitive strain to dependence. RE 26 was used as the recipient and a F1 lysate prepared on RE 26-70-7 Str-d1 was included as a control. No streptomycin dependent transductants were obtained from either JSM 1 or JSM 2 although 2 resistant mutants, most likely spontaneous, were found with donor JSM 1. The results are shown in Table 5.

**TABLE 5**

<table>
<thead>
<tr>
<th>Donor</th>
<th>No. of Str-d transductants per 10⁷ survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>RE 26-70-7 Str-d1</td>
<td>2840</td>
</tr>
<tr>
<td>JSM 1</td>
<td>2*</td>
</tr>
<tr>
<td>JSM 2</td>
<td>NIL</td>
</tr>
</tbody>
</table>

* Both found to be resistant.

Table 5 Transduction of the Str-d1 allele from RE 26-70-7 Str-d1 into RE 26 Str-s and failure to show transduction of Str-d from the fast growing sectors.
It will be noticed that the Str-dl allele is on the host RE 26-70-7 Str-dl chromosome and that the difficulties of transducing from the exogenotic fragment do not apply. It would appear therefore that the fast growing sectors arise by haploidization of the Str alleles, presumably by recombination which although of low frequency confers such an advantage on the haploid that it rapidly outgrows the partial diploid.

**Demonstration of the Partial Diploid Nature of the Slow Growing Colony Type**

I stated above that of the 41 Mal\(^+\) colonies which clearly segregated Mal\(^-\) on Tetrazolium Maltose agar 34 gave numerous small colonies in the well of the plate when streaked out on Tetrazolium Maltose agar containing streptomycin. It was decided to quantify this observation. Four representative slow growing colonies JSL 1, 2, 3 and 7 were streaked out on Tetrazolium Maltose agar plates and incubated at 37°C for 6 days. On each plate a few colonies were found which had not yet produced sectors. These colonies were lifted with a loop into M9 buffer and the remainder of each colony restreaked on Tetrazolium Maltose agar. Dilutions from the cell suspensions in M9 buffer were made and plated for a viable count again on Tetrazolium Maltose agar and 0.1ml aliquots were plated directly on Tetrazolium Maltose agar + 200 μg/ml streptomycin. The results are presented in Table 6.

/Table 6......
Table 6 Segregation of the Mal and Str markers in 4 of the slow growing putative partial diploids.

The seven Str-d Mal+ colonies obtained were genetically stable, i.e. Mal- colonies were not observed. 15 Str-d Mal- colonies from each of JSL 1, 2, 3 and 7 also remained genetically stable in that they failed to show growth, other than residual, when tested on Tetrazolium Maltose agar without streptomycin. Clearly then the slow growing colonies obtained by plating the CR series on Tetrazolium Maltose agar lacking streptomycin differ from the JSL series obtained by plating the cross directly on medium lacking streptomycin. In the first case, after growth in the absence of the antibiotic, the slow growing Mal+ CR colonies replate, although with low efficiency, on streptomycin medium showing the instability pattern which suggests that the cells are still heterozygous. The JSL colonies although they do plate on streptomycin medium give genetically stable recombinnants which fail to replate on the original medium.

Thus the behaviour of the partial diploids presents a puzzling paradox. On one hand the heterozygote Str-d/Str-s will grow on streptomycin and behave like a dependent while on the other hand the same Str-d/Str-s heterozygote, if selected in the absence of the antibiotic, will behave as a sensitive. As mentioned earlier, I have made an observation which helps to remove the anomaly but creates in itself deeper problems.
In 1956 Cado and Horvath discovered that a streptomycin dependent strain of E. coli could be grown on simple medium lacking streptomycin provided the medium was supplemented with acetone, methanol or ethanol at sub-lethal concentrations. The authors ascribed the effect to the action of the organic solvents on the cell membrane; although it should be pointed out that they failed to get the same effect with other organic solvents of a similar nature at sub-lethal concentrations. Later Corini et al (1967) described the phenomenon of phenotypic masking in which streptomycin dependent strains could be converted to paramomycin dependent by first growing the culture in 3% ethanol medium before exposure to paramomycin and vice versa paramomycin dependents could be converted to streptomycin dependents. This phenomenon was interpreted by Corini et al (1967) in terms of ethanol induced ambiguities of the genetic code.

I have recently shown that CR type colonies taken from streptomycin medium plate with high efficiency in the absence of streptomycin if first grown in ethanol medium before plating. JSL type colonies plate, although with a lower efficiency, on streptomycin medium if grown in a similar way.

CR-1 was taken from Nutrient Agar Streptomycin slopes and streaked on Tetrazolium Maltose Streptomycin Agar for single colonies, a Mal+ colony was restreaked on the same medium and from the second plate a culture was grown overnight in Nutrient broth + 100 µg/ml streptomycin. The culture was washed on membrane filters and a 1/10 dilution made into Nutrient broth + 3% ethanol and incubated at 37°C for about 18 hours. Dilutions were made through M9 buffer and 0.1 ml aliquots plated on Tetrazolium Maltose Agar with and without streptomycin. The plates were incubated for 3 days at 37°C and counted. A direct count was also made in a counting chamber at the time of dilution.
JSL 1 was stored on M9 maltose agar with 50 μg/ml each of proline, tryptophan and histidine. A loop from the master slope was streaked on Tetrazolium Maltose Agar which was incubated at 37°C for 3 days. Slow growing Mal⁺ colonies were transferred to a second plate and again incubated for three days to check the original phenotype i.e. slow growing colonies which produce fast growing Mal⁺ and Mal⁻ sectors and papillae. Small unsectored Mal⁺ colonies were removed from the plate with a loop into 0.5 ml of M9 buffer. 0.1 ml aliquots were then added to 1 ml volumes of Nutrient Broth + 3% ethanol and grown at 37°C for 12 hours before dilution and plating on Tetrazolium Maltose agar with and without streptomycin. Direct counts were made in a counting chamber. It was found to be essential to dispense with an initial growth stage before the ethanol stage in order to avoid massive overgrowth of the culture with segregants. The plates were incubated for three days at 37°C. Controls were performed by incubating a 1/10 dilution of CR-1 for 18 hours in Nutrient Broth without Ethanol and plating as above. The JSL 1 control was performed by transferring 0.1 ml of the M9 buffer suspension to 1 ml of Nutrient Broth, incubating for 12 hours and plating as above. The results are shown in Table 7.
TABLE 7

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total count per ml.</th>
<th>Colony forming ability on TMA* + SM</th>
<th>Colony forming ability on TMA* - SM</th>
<th>Relative plating efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR-1</td>
<td>$8.3 \times 10^8$</td>
<td>$7.7 \times 10^8$</td>
<td>$5.8 \times 10^8$</td>
<td>75%</td>
</tr>
<tr>
<td>JSL 1</td>
<td>$1.6 \times 10^7$</td>
<td>$3.1 \times 10^6$</td>
<td>$2.2 \times 10^7$</td>
<td>14%</td>
</tr>
</tbody>
</table>

TMA* = Tetrazolium Maltose Agar.

Table 7. Relative plating efficiency of ethanol grown cultures of CR-1 and JSL 1 as described in the text.

It would appear that when a partial diploid heterozygous Str-d/Str-s is formed it may most commonly be capable of growth in the absence of streptomycin or less commonly may require streptomycin for growth and that these two phenotypes are not normally readily interconvertible. However, they are easily interchanged by growth in the presence of 3% ethanol. There would appear to be no simple explanation of this phenomenon at the present time but the importance which it has for our understanding of streptomycin action on the cell and hence for ribosome function suggests that the action of the simple organic solvents on the cell ought to be rigorously investigated.
A spontaneously occurring streptomycin resistant mutant of Hfr G6 Thy' was selected by growing G6 Thy' overnight in Nutrient Broth with vigorous aeration at 37°C centrifuging and resuspending in $\frac{1}{10}$ Vol. and plating on Nutrient agar + 200 µg/ml streptomycin. 10 plates prepared in this way, containing approximately $2-5 \times 10^9$ cells per plate, generally give a few streptomycin resistant colonies after 3 days incubation at 37°C. About half are usually found to be simply resistant, the remainder being dependent upon streptomycin.

The selection procedure was the same as in the previous section. Hfr G6 Thy' Str-r was grown overnight at 37°C in Nutrient broth, diluted into fresh prewarmed broth and incubated for 2 hours to give a culture of log phase cells which were mated with a washed overnight culture of RE 26-70-7 Str-dl. The mating was interrupted after 30 minutes and the cells washed and plated on selective media with and without streptomycin. As before, the selective plates were M9 maltose agar containing proline, tryptophan and histidine.

In this case, 194 Mal+ colonies were obtained in the presence of streptomycin and 181 with streptomycin omitted from the plates after 3 days of incubation at 37°C. No obvious differences in growth rates as judged by colony size were observed. All colonies were tested on Tetrazolium Maltose agar, with or without streptomycin, according to whether the colonies were originally obtained in the presence of streptomycin or not.
Only 7 out of the 194 Mal\(^+\) colonies obtained in the presence of streptomycin segregated Mal\(^-\) colonies. None of the 191 colonies selected in the absence of streptomycin segregated Mal\(^-\).

Plating of the seven colonies, designated DS 1-7, on streptomycin free Tetrazolium Maltose agar gave a number of colonies which were generally confined to the well. They were predominantly Mal\(^+\). In the absence of the antibiotic, there was no suggestion of growth other than residual, i.e. the paths of the loop become heavy with growth and at the extremities pin-point sized colonies are just visible but inviable on testing.

These observations were quantified by picking isolated DS colonies from Tetrazolium Maltose agar + streptomycin into M9 buffer and plating a series of dilutions on Tetrazolium Maltose agar + streptomycin for a viable count and plating on Tetrazolium Maltose agar for a count of cells capable of growing in the absence of streptomycin. The results are shown in Table 8.

### Table 8

<table>
<thead>
<tr>
<th>Strain</th>
<th>Viable Count per ml on TMA + Sm.</th>
<th>Count per ml of Str-r Mal(^+)</th>
<th>Count per ml of Str-r Mal(^-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS 1</td>
<td>(1.4 \times 10^6)</td>
<td>548</td>
<td>17</td>
</tr>
<tr>
<td>DS 2</td>
<td>(3.3 \times 10^6)</td>
<td>511</td>
<td>9</td>
</tr>
<tr>
<td>DS 3</td>
<td>(3.1 \times 10^6)</td>
<td>803</td>
<td>33</td>
</tr>
<tr>
<td>DS 4</td>
<td>(0.9 \times 10^6)</td>
<td>263</td>
<td>NIL</td>
</tr>
<tr>
<td>DS 5</td>
<td>(1.9 \times 10^6)</td>
<td>490</td>
<td>26</td>
</tr>
<tr>
<td>DS 6</td>
<td>(2.7 \times 10^6)</td>
<td>715</td>
<td>16</td>
</tr>
<tr>
<td>DS 7</td>
<td>(1.1 \times 10^6)</td>
<td>288</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 8 Segregation of Str-r Mal\(^+\) and Str-r Mal\(^-\) from the partial diploid strains DS 1-7, following the procedure detailed in the text.
It seemed most probable that the D3 colonies were heterozygous Str-d/Str-r and that it would be a simple matter to show by transduction with a suitable recipient that both the Str-d and Str-r alleles were present. Unaccountable difficulties were encountered when this was attempted using a P1 transducing lysate prepared on each of D3 1-7 and NE 26 as the recipient. Selection was for streptomycin resistance after allowing 4 hours growth for phenotypic expression. 64 colonies from each transduction were picked and scored for their streptomycin phenotype. In every case only dependent colonies were found.

The transductions were repeated using AB2847 as recipient. This strain is MalA⁻ AroB⁻ Str⁺ and Huang and Pittard (1967) have shown that AroB and Str are 6% cotransducible. It was expected that if half of the transducing particles carry the AroB-Str region, picked up from the exogenous fragment, then 3% of the AroB⁺ transductants ought to be Str-r thus eliminating any considerations of phenotypic lag and competitive effects. In the event, it was found that the numbers of AroB⁺ Str-r joint transductants were so low as to require replica plating for identification. Unfortunately, the colonies were rather large and fairly crowded and two replications were required to eliminate residual growth of the Str-d colonies in order to detect the Str-r colonies and the consequent spreading may have made the counts somewhat inaccurate. Streptomycin resistant or dependent transductants were also selected directly by allowing for the phenotypic lag. The results are presented in Table 9. Clearly then the D3 isolates are heterozygous Str-d/Str-r although the cotransduction frequency of AroB and Str-r is considerably lower than would be expected i.e. 3%. The reason for this is possibly due to the difficulty in transducing exogenous markers as mentioned previously or the Str-r transductants may be at an initial growth disadvantage. However, the data presented in Table 9 do show that both alleles are present.

/Thus......
Thus the Str-d/Str-r heterozygote is dependent upon streptomycin for growth i.e. it is streptomycin dependent and the dependent allele is dominant over the resistant allele. Unfortunately, I have not been able to examine the effect of ethanol on the heterozygote so that this conclusion remains valid only for the test conditions.

**Table 9**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Aro B⁺</th>
<th>Str-d</th>
<th>Str-r*</th>
<th>AroB⁺ Str-r* cotransductants per 10¹⁰ survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS 1</td>
<td>3010</td>
<td>2830</td>
<td>63</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>2060</td>
<td>2290</td>
<td>12</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>3310</td>
<td>3370</td>
<td>58</td>
<td>26</td>
</tr>
<tr>
<td>4</td>
<td>2940</td>
<td>3100</td>
<td>78</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>2470</td>
<td>1030</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>2520</td>
<td>2050</td>
<td>26</td>
<td>19</td>
</tr>
<tr>
<td>7</td>
<td>2730</td>
<td>2770</td>
<td>77</td>
<td>27</td>
</tr>
</tbody>
</table>

Table 9  The number of Aro B⁺ and Str-r or Str-d transductants obtained in a transduction using the putative diploids DS 1-7 as donors and AB2347 as recipient. Str-d and Str-r transductants were selected as described in the Materials and Methods Section. Aro B⁺ transductants were selected on E9 glucose agar and replicated onto Nutrient agar + 200 µg/ml streptomycin to look for AroB⁺ Str-r cotransductants. The cotransductants are recorded per 10¹⁰ survivors.

* Determined by replica plating.
Isolation and Properties of a New Class of Streptomycin Resistant Mutation

The procedures adopted for the selection of streptomycin resistant cells in a population rely upon the resistant cell being able to grow in the presence of streptomycin. It seems reasonable to ask whether a mutant may arise within the population which is insensitive to the lethal action of streptomycin but remains unable to grow in its presence.

A culture of RE 26 Str-s was treated with nitrosoguanidine and subsequently grown in broth for four hours to allow phenotypic expression. 1 ml aliquots were then transferred to 10 ml of nutrient broth containing 100 ug/ml streptomycin and incubated at 37°C for 1 hour. Penicillin G was then added to a final 2000 units/ml and the culture incubated for a further 24 hours. The culture which was really a lysate at this stage was then filtered through a 0.45 u membrane filter and washed with 100 ml of prewarmed M9 buffer. The filter was then transferred to 5 ml of broth and incubated at 37°C. In all cases following mutagenesis but only rarely otherwise the broth became cloudy within two days. The cultures were then streaked for single colonies on nutrient agar. Two independent colonies designated Str-i1 and Str-i2 were obtained in this way and examined.

Fig. 1 shows that there is no loss in viability when the cultures are exposed to streptomycin.
Fig. 1. Effect of streptomycin at a final concentration of 100μg/ml on \text{RE26} str-1 and str-12 and the parent strain \text{RE26}. The cells were grown overnight in nutrient broth, diluted into 10 ml of fresh broth to give a density of $10^8$/ml and shaken at 37°C for 30 mins. before adding the streptomycin. Dilutions were made through prewarmed M9 buffer.

\begin{itemize}
\item \text{RE26}
\item \text{RE26} : str-11.
\item \text{RE26} : str-12.
\end{itemize}
Mapping of Str-i

The original mapping experiments were performed by transduction using F1 lysates prepared on RD26 Str-i1 and Str-i2 with an EMS induced streptomycin dependent spontaneous MalA^- N^R mutant of strain 20, 20 MalA^- (A^R) Str-d. The reason for this choice of recipient was that it seemed reasonable to examine the streptomycin non-dependent transductants first. Indeed it appeared, subsequently, to be necessary to do no more than that. 15/15 streptomycin non-dependent transductants from both donors showed the Str-i character. The transductions were performed according to the procedures given in the Materials and Methods section.

The streptomycin non-dependent transductants were tested by purifying the colonies by restreaking and picking colonies into 1ml volumes of Nutrient Broth + 200 ug/ml streptomycin in bacteriological tubes. A loopful was withdrawn after 16 hours incubation at 37°C and spread over a nutrient agar plate. With Str-i mutants this results in a large number of colonies per plate whereas with RD26 Str-s this procedure gave no colonies.

It was later found that this test could be adapted to a replica plating test by replicating small colonies at a suitable density onto nutrient agar + streptomycin where Str-i mutants soon cease growth and after 24 hours incubation at 37°C the plate was replicated onto a nutrient agar plate which produced good colonies within a further 24 hours in the case of Str-i mutants only. Str-r mutants could be distinguished in a mixed culture by the clearly growing colonies which appear on the streptomycin plates.

The essential point which emerges in both these tests is that the Str-i (sib^-) mutants cease growth on streptomycin quite rapidly but nevertheless retain their viability.

/Alternatively......
Alternatively, colonies could be tested by spreading a small patch on Nutrient agar + streptomycin, incubating for 24 hours at 37°C and sampling the patch onto a similar sized area on a Nutrient agar plate which is incubated at 37°C. In this way 16 colonies could be accurately tested using two plates.

Apirion (personal communication) has found that genes which affect ribosomal proteins can be very closely linked and may require the examination of many more than 16 transductants before separation of two different loci can be achieved. Therefore, I returned to a re-examination of the Str-i mutants by transduction.

In this case the recipient was a streptomycin dependent AB2847, MalA^− AroB^− Str-d. The transduction was performed as described in the Materials and Methods section. Mal^+ and AroB^+ transductants were selected in the absence of streptomycin and examined by replica plating as described above to distinguish the Str alleles.

All Mal^+ Str-independent transductants were found to be streptomycin sensitive and between 4.2% and 4.8% of the AroB^+ Str-independent transductants were found to be Str-i. The results are shown in Table 10. Obviously Str-i is not an allele of the streptomycin locus but is a closely linked locus. I have, therefore, renamed the locus the Sib gene for Streptomycin induced bacteriostasis. Sib^+ is the wild type allele and Sib^- corresponds to the Str-i class described above. The mapping of the Sib locus clearly shows that it is distal to the streptomycin locus with respect to the MalA locus and that the order is likely to be MalA - AroB - Str - Sib.

/Table 10...
Table 10  AB2847 Str-d1 was treated with a transducing lysate of P1 prepared on RE 26 Str-il and RE 26 Str-i2. Aro B+ and Mal+ recombinants were selected in the absence of streptomycin. The recombinants, which must have obtained the Str-s Aro B region and the Str-s Mal region were then checked by replica plating to determine the response to streptomycin as described in the text.

**Dominance of Sib+ over Sib-**

This work was actually carried out under the impression that I was dealing with an allele at the streptomycin locus but the results are nonetheless valid.

**Isolation of a Rec A- Str-s Recipient**

Much of the tedium associated with the construction and testing of the partial diploids arises from the use of RE 26-70-7 which has a low but significant level of recombinase activity thus making it necessary to test a great many colonies in order to obtain a few partial diploids. A further difficulty arises from recombination within the diploid itself as it grows to form a colony which often makes it difficult to distinguish the properties of the partial diploid from those of recombinants arising within the colony.

/Brookes-Low.......

<table>
<thead>
<tr>
<th>Donor</th>
<th>Aro B+ Str-s</th>
<th>Aro B+ Str-i</th>
<th>Mal+Str-s</th>
<th>Mal+Str-i</th>
</tr>
</thead>
<tbody>
<tr>
<td>RE 26 Str-il</td>
<td>1697,1771</td>
<td>74,1771</td>
<td>380,380</td>
<td>0,380</td>
</tr>
<tr>
<td>RE 26 Str-i2</td>
<td>1363,1434</td>
<td>71,1434</td>
<td>322,322</td>
<td>0,322</td>
</tr>
</tbody>
</table>
Brookes-Low (1968) has shown that recA recipients give a very low frequency of recombinants when mated with an Hfr and that on further examination all such apparent recombinants are in fact partial diploids heterozygous for the selected allele. Unfortunately the recA mutation is in a streptomycin resistant cell. It ought, however, to be possible to construct Hfr's carrying the recA allele and use these strains to transfer the recA mutation to any desired recipient strain.

Hfr Bll was crossed with AB1157 recA by mixing 0.1 ml of cells of an overnight culture of each on prewarmed nutrient agar plates which were incubated at 37°C for approximately four hours before being washed off the plate with M9 buffer and plated for Gal<sup>+</sup> Pro<sup>+</sup> recombinants, on M9 galactose agar supplemented with threonine, leucine, arginine, histidine and thiamine.

The recombinants were purified by restreaking, tested for donor ability and tested for UV sensitivity using a simple spot test. UV sensitive donor strains were designated Bll RecA. The first Bll RecA isolate tested transferred thy<sup>+</sup> to AB2647 thy<sup>−</sup>, an aminopterin selected mutant. All Thy<sup>+</sup> recombinants tested were UV sensitive. All were found to be streptomycin sensitive and Aro<sup>−</sup>. With these new recA recipients it became possible to examine the dominance/recessivity of various combinations of str- alleles without the difficulty of a background level of recombination.

A spontaneous Sib<sup>−</sup> mutation was selected in AB2647 RecA renamed All as previously described for RE 26 str-s. This mutant was crossed with Hfr G6 Thy<sup>−</sup>. The mating was interrupted after 30 minutes by blending and after exhaustive washing on filters the cells were plated to select for AroB<sup>+</sup> Mal<sup>+</sup> recombinants. The donor was counter-selected by thymine starvation.
In this series 38 putative diploids were obtained. All segregated mal$^-$ and all mal$^-$ segregants were found to be AroB$^-$. All were tested for sensitivity to streptomycin by growing overnight in nutrient broth, diluting $\frac{1}{10}$ into fresh broth and adding streptomycin sulphate to give a final concentration of 100 µg/ml. Samples were removed for counting at zero time and again after three hours. 29 of the original 38 showed the Sib$^-$ character.

Before any conclusions can be drawn regarding the dominance of Sib$^-$ over Sib$^+$ it is essential to show that both Sib$^-$ and Sib$^+$ are present in the same cell. Accordingly P1 phage was prepared on all 38 isolates and used to transduce AB2847 str-d to streptomycin independence. The transductants were tested as described earlier by replica plating onto streptomycin nutrient agar plates, incubating for 24 hours at 37°C and then replicating once more onto nutrient agar plates. The streptomycin independent colonies appeared at a density of 80–120 per plate which made replica plating fairly reliable. To prevent the colonies becoming too large the plates were kept in a refrigerator when the colonies reached a suitable size. Streptomycin sensitive colonies were detected at a low frequency, between 2–4%. The results are presented in Table 11.
<table>
<thead>
<tr>
<th>DX- 1 +</th>
<th>DX-11 -*</th>
<th>DX-21 +</th>
<th>DX-31 +</th>
</tr>
</thead>
<tbody>
<tr>
<td>DX- 2 +</td>
<td>DX-12 +</td>
<td>DX-22 +</td>
<td>DX-32 +</td>
</tr>
<tr>
<td>DX- 3 +</td>
<td>DX-13 -*</td>
<td>DX-23 +</td>
<td>DX-33 -*</td>
</tr>
<tr>
<td>DX- 4 +</td>
<td>DX-14 +</td>
<td>DX-24 -</td>
<td>DX-34 -*</td>
</tr>
<tr>
<td>DX- 5 +</td>
<td>DX-15 +</td>
<td>DX-25 +</td>
<td>DX-35 +</td>
</tr>
<tr>
<td>DX- 6 +</td>
<td>DX-16 -*</td>
<td>DX-26 +</td>
<td>DX-36 -*</td>
</tr>
<tr>
<td>DX- 7 -*</td>
<td>DX-17 -*</td>
<td>DX-27 -</td>
<td>DX-37 +</td>
</tr>
<tr>
<td>DX- 8 +</td>
<td>DX-18 -</td>
<td>DX-28 +</td>
<td>DX-38 +</td>
</tr>
<tr>
<td>DX- 9 +</td>
<td>DX-19 +</td>
<td>DX-29 -</td>
<td></td>
</tr>
<tr>
<td>DX-10 -</td>
<td>DX-20 -*</td>
<td>DX-30 +</td>
<td></td>
</tr>
</tbody>
</table>

Table II  The 38 putative partial diploids for Sib+/Sib^- were used to prepare Fl phage lysates which were used to transduce AB2847 Str-dl to streptomycin independence. The transductants were then tested for the Sib character by replica plating as described in the text.

* The original 9 which did not show the Sib^- character.
+ Both Sib^+ and Sib^- detected by replica plating.
- Sib^- only detected.

The evidence presented in this section indicates that mutations occur which, although preventing further growth in the presence of streptomycin, eliminate the bacteriocidal effect of the antibiotic. This mutation defines the Sib locus i.e. Streptomycin Induced Bacteriocidal Stasis and mapping by Fl transduction has shown the locus to be on the distal side of the Str locus to the AroB locus in the order MalA - AroB - Str - Sib. Examination of heterozygotes has shown that Sib^- is dominant over Sib^+. Further studies on this locus would no doubt be of extreme value in understanding the nature of the bacteriocidal effect of streptomycin.
Isolation and Characterization of Mutations which
simultaneously inactivate the streptomycin and spectinomycin genes

Spectinomycin is a broad spectrum antibiotic classified with the aminoglycoside group to which streptomycin, kanamycin and neomycin etc. belong. Flaks et al (1966) have shown that the gene for high level resistance to spectinomycin is approximately 7% linked to the streptomycin locus. It was further shown by Flaks et al that spectinomycin inhibits protein synthesis in a similar manner to streptomycin thus implicating the 30 s sub-unit of the ribosome in spectinomycin action. These facts taken together with the evidence presented by Dubnau et al (1965) that in Bacillus Subtilis the aminoglycoside resistance markers and the locus coding for rRNA are closely linked prompted Cox et al (1965) to suggest that a similar situation may exist in E. coli. It seemed of some interest, therefore, to design an experiment which could detect mutations which would simultaneously inactivate two ribosomal proteins. Such mutations could be of the extreme polar type if all the ribosomal proteins were under control of an operator.

Since mutations which inactivate ribosomal proteins will almost certainly be lethal it is essential to construct a partial diploid system which will supply in an easily identifiable way the genetic information which the mutation eliminates.

/The...
The spontaneous mutation rate at the Str locus is approximately $1 - 5 \times 10^{-10}$ per cell per generation for Str-s $\rightarrow$ Str-r mutations and a similar figure applies to the spectinomycin locus for Spc-s $\rightarrow$ Spc-r. Clearly then the chances of two spontaneous mutations occurring to give the doubly resistant mutant will be of the order of $10^{-20}$, such a rare event that it is most unlikely to be encountered.

The rationale was simply to establish a partial diploid carrying Str-r Spc-r on the exogenomic element and look for the spontaneous occurrence of doubly resistant mutants. The initial experiments used the rec strain RE 26-70-7 which is both streptomycin and spectinomycin sensitive as the recipient and Hfr G6 Thy as the donor.

Hfr G6 Thy was grown overnight in nutrient broth with vigorous aeration at 37°C. The culture was concentrated by centrifugation and 0.1 aliquots – approximately $10^9$ cells – were plated on M9 glucose containing histidine, thymine and 200 μg/ml streptomycin to select Str-r mutants. The mutants were purified and verified as Str-r and the procedure was repeated to give a doubly resistant streptomycin and spectinomycin resistant mutant.

Hfr G6 Thy Str-r Spc-r was crossed with RE 26-70-7. The mating was interrupted after 30 minutes and the cells were thoroughly washed on membrane filters and plated on M9 maltose agar with proline, tryptophan and histidine. 363 Mal+ colonies were examined by streaking on Tetrazolium Maltose agar with and without streptomycin and Tetrazolium Maltose agar with and without spectinomycin. Only seven colonies were obtained which gave positive indication of the presence of both the Str-r and Spc-r alleles. The results obtained with one such partial diploid designated CMS-1 are presented in Table 12.
The data clearly confirm that spectinomycin resistance is recessive to spectinomycin sensitivity (Apirion, personal communication.)

**TABLE 12**

<table>
<thead>
<tr>
<th>Viable Count on TMA</th>
<th>Count on TMA</th>
<th>Count on TMA</th>
<th>Count on TMA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TMA⁺ Sm⁻ Mal⁺</td>
<td>TMA⁺ Sm⁺ Mal⁻</td>
<td>TMA⁺ Sm⁺ Sm⁺</td>
</tr>
<tr>
<td>4.4 x 10⁸</td>
<td>7.3 x 10⁴</td>
<td>few</td>
<td>1.1 x 10⁵</td>
</tr>
<tr>
<td></td>
<td>1.3 x 10³</td>
<td>Nil</td>
<td></td>
</tr>
</tbody>
</table>

TMA = Tetrazolium Maltose Agar  
Sm = 200 µg/ml Streptomycin  
Sp = 100 µg/ml Spectinomycin

Table 12 CMS-1 was grown overnight in Nutrient broth. A series of dilutions were made in order to determine the viable count by plating on Tetrazolium Maltose agar. Dilutions were also plated on Tetrazolium Maltose agar with antibiotics as indicated. The small number of doubly resistant cells relative to the singly resistant is probably due to the slow growth rate of double resistants.

It had always been realised during the course of this work that considerable effort would be required to distinguish true mutants from recombinants.
It was originally thought that if mutations similar to the 00 class in the lactose operon arose these could be identified by the fact that they would revert to give the streptomycin and spectinomycin sensitive phenotype. Thus the problem of identification of the mutants resolves itself into one of detecting the sensitive phenotype. We now know that 00 mutations are really extreme polar chain terminating mutations which are suppressible by amber and ochre suppressors. This does not, however, interfere with the detection procedure.

Davies et al (1965) have shown that spectinomycin is a bacteriostatic agent whose action is essentially reversible.

I have shown that in a mixture of approximately 1 : 10^3 spectinomycin sensitives: resistants the sensitive can be easily detected by first growing the mixture in Nutrient broth + 100 μg/ml spectinomycin for 1 hour at 37°C and then adding penicillin to 2000 units final concentration. After 12 hours of incubation the culture is filtered and washed on Millipore filters, the filter is added to fresh broth and incubated for 4 - 8 hours at 37°C before plating. This method would, of course, also detect Spc-r ———> Spc-s revertants.

The experiment was performed simply by plating about 10^8 cells of CMS-1 on Nutrient Agar + 200 μg/ml streptomycin + 100 μg/ml spectinomycin. This gave between 300 - 500 colonies per plate. 18 colonies were examined as described above. 4 gave spectinomycin sensitives which remained streptomycin resistant. Obviously the low level of recombinant forming ability in RE 26-70 is too great to allow the recovery of true mutants. When the All = AB2847 RecA strain became available I returned to this aspect of the work.
A 11 was mated with HfrG6 Thy^- Str-r Spc-r and the mating interrupted by blending after 30 minutes. The cells were washed on Millipore filters and plated on M9 Maltose Agar to select for Mal^+ AroB^+ recombinants. The Hfr was counterselected by thymine starvation. 12 colonies were picked and tested on Tetrazolium Maltose agar. All twelve segregated Mal^- colonies which proved to be Aro^- on further testing. All Mal^+ and Mal^- colonies tested were streptomycin and spectinomycin sensitive. One of the partial diploids AllXS2 was selected for further study.

AllXS2 gave rise to streptomycin and spectinomycin resistant colonies individually with a frequency of between 10^-6 and 10^-7. None of the 24 Str-r colonies tested proved to be dependent. I interpret this finding as meaning that by far the most common class of mutations occurring at the two loci are inactivating mutations which then allow expression of the recessive resistant allele on the exogenote. Only a very small fraction, 10^-2 - 10^-3, result in a viable mutation which is resistant to the antibiotic.

When AllXS2 was plated on both antibiotics doubly resistant mutants were detected at one per 10^9 - 10^10 cells plated. These are clearly single events since double mutants are unlikely to occur independently at a frequency less than 10^-12.

Complete confirmation that AllXS2 is indeed heterozygous Str-s/Str-r Spc-s/Spc-r was provided by preparing a Pl lysate on AllXS2 and using it to transduce AB2847. The selected markers were MalA^+, AroB^+, Str-r and Spc-r. The results are shown in Table 13. Obviously the Str-r and Spc-r alleles must have come from AllXS2 which is nevertheless phenotypically sensitive to both antibiotics. This taken together with the high frequency of Str-r and Spc-r mutations in AllXS2 provide compelling proof that AllXS2 is a partial diploid of the following composition:

/All....
TABLE 13

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient AB2847</th>
<th>Recombinants per $10^9$ viable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mal$^+$</td>
</tr>
<tr>
<td>AllXS2</td>
<td></td>
<td>414</td>
</tr>
</tbody>
</table>

Table 13. A Pl lysate was produced on AllXS2 and used to transduce AB2847 for mal$^+$, aro-B$^+$ str-r and spo-r as described in the Materials and Methods section. The reason for the low recovery of transductants is not clear and would probably merit further investigation.
Selection of Mutations which result in Simultaneous Inactivation of the Endogenous Str-Spc Genes

AllXS2 was grown overnight with vigorous aeration at 37°C in Nutrient broth + 0.2% glucose. The culture was centrifuged and resuspended in 1/10 volume from which 0.1 ml aliquots were plated on Nutrient agar + streptomycin + spectinomycin each at 100 µg/ml. 28 colonies were obtained in three series of experiments, 15 of which must be of independent origin.

Unfortunately there is no sound simple criterion which can be applied to decide whether the original mutations are deletions or reverting point mutations. The approach which I adopted relied upon the bacteriostatic action of spectinomycin as described previously. If a reverse mutation results in the cell becoming spectinomycin sensitive then it would be possible to isolate such a revertant by growing the culture in spectinomycin + penicillin. The antibiotics are subsequently washed out by membrane filtration and the survivors plated. Controls show virtually no loss of viability and reconstruction experiments show that 1 sensitive cell per 10⁸ resistsants can be recovered.

In all 28 cases I have failed to find a single survivor which is spectinomycin sensitive and streptomycin sensitive. This suggests but in no way proves that the mutants are deletions.

The 15 independent mutants were then grown overnight in Acridine Orange Broth. Morphologically all presented the picture seen on plates 1a and 1b. Grossly elongated cells with very few normal sized cells seemed to be typical of the acridine orange treatment.

/Viability.....
FIGURE 2

Photomicrographs of A11x22 after 24 hours growth in Antibiotic No. 3 broth supplemented with Acridine orange.
Viability was estimated by counting the cells in a counting chamber and by plating suitable dilutions for viable counts. Viabilities appeared to range from as high as 7% to as low as 0.02% but little real value can be placed on the accuracy of the counting chamber because of the extreme length of the cells, which made counting very difficult.

It is essential to test whether the mutants AllXS2 - Δ1 to Δ15 are true deletions. Phage ϕ1 was grown on AllXS2 and the "deletion" mutants derived from it. Attempts were then made to transduce AB2847 str-d to streptomycin independence and to examine the nature of the transduced allele. The results are presented in Table 14.

The very high cotransduction figure for Str-r and Spc-r of between 40% and 77% causes some concern. Flaks et al (1966) give a value of 7% so it is possible that the original exogenomic fragment carries both the streptomycin and spectinomycin resistance alleles but some interspersed non-essential genes are deleted resulting in an increased cotransduction frequency. Thus it would appear unlikely that the ribosomal protein cistrons are under control of an operator.

/Table 14....
### TABLE 14

<table>
<thead>
<tr>
<th>Donor</th>
<th>Selected Marker</th>
<th>Percent Str-ind*</th>
<th>Percent Str-r</th>
<th>Percent Str-s</th>
<th>Percent Str-r Spc-r</th>
</tr>
</thead>
<tbody>
<tr>
<td>A11XS2</td>
<td>217</td>
<td>8.3%</td>
<td>91.7%</td>
<td>39% (7/18)</td>
<td></td>
</tr>
<tr>
<td>A11XS2 - Δ 1</td>
<td>262</td>
<td>100%</td>
<td>NIL</td>
<td>66%</td>
<td></td>
</tr>
<tr>
<td>&quot; - Δ 2</td>
<td>166</td>
<td>100%</td>
<td>NIL</td>
<td>61%</td>
<td></td>
</tr>
<tr>
<td>&quot; - Δ 3</td>
<td>318</td>
<td>97.2%</td>
<td>2.8%</td>
<td>77%</td>
<td></td>
</tr>
<tr>
<td>&quot; - Δ 4</td>
<td>382</td>
<td>100%</td>
<td>NIL</td>
<td>72%</td>
<td></td>
</tr>
<tr>
<td>&quot; - Δ 5</td>
<td>119</td>
<td>100%</td>
<td>NIL</td>
<td>66%</td>
<td></td>
</tr>
<tr>
<td>&quot; - Δ 6</td>
<td>251</td>
<td>100%</td>
<td>NIL</td>
<td>69%</td>
<td></td>
</tr>
<tr>
<td>&quot; - Δ 7</td>
<td>382</td>
<td>95.8%</td>
<td>4.2%</td>
<td>76%</td>
<td></td>
</tr>
<tr>
<td>&quot; - Δ 8</td>
<td>62</td>
<td>100%</td>
<td>NIL</td>
<td>62%</td>
<td></td>
</tr>
<tr>
<td>&quot; - Δ 9</td>
<td>274</td>
<td>99.4%</td>
<td>0.6%</td>
<td>56%</td>
<td></td>
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<tr>
<td>&quot; - Δ 10</td>
<td>202</td>
<td>100%</td>
<td>NIL</td>
<td>67%</td>
<td></td>
</tr>
<tr>
<td>&quot; - Δ 11</td>
<td>191</td>
<td>100%</td>
<td>NIL</td>
<td>70%</td>
<td></td>
</tr>
<tr>
<td>&quot; - Δ 12</td>
<td>411</td>
<td>97.6%</td>
<td>2.4%</td>
<td>58%</td>
<td></td>
</tr>
<tr>
<td>&quot; - Δ 13</td>
<td>90</td>
<td>100%</td>
<td>NIL</td>
<td>77%</td>
<td></td>
</tr>
<tr>
<td>&quot; - Δ 14</td>
<td>235</td>
<td>100%</td>
<td>NIL</td>
<td>70%</td>
<td></td>
</tr>
<tr>
<td>&quot; - Δ 15</td>
<td>129</td>
<td>100%</td>
<td>NIL</td>
<td>66%</td>
<td></td>
</tr>
</tbody>
</table>

Table 14  A Fl phage lysate was prepared on A11XS2 and the 16 deletion mutants. The lysates were used to transduce AB2847 Str-dl to streptomycin independence. The nature of the transduced streptomycin allele and the response to spectinomycin was determined by replica plating. Since the double resistant was very slow growing it was necessary to incubate the plates for 3 - 4 days before replica plating.

*Streptomycin not required for growth; includes both Str-r and Str-s classes.
These results are rather difficult to interpret. The majority, $11/15$, of the mutations would appear to be deletions on the basis of this rather crude test as indicated by the failure to detect streptomycin sensitives among the transductants. However, $\text{AllXS2} = \underline{3}, \underline{7}, \underline{9}$ and $\underline{12}$ clearly require some comment. The recovery of streptomycin sensitive transductants could be the consequence of recombination occurring between the $\underline{3}, \underline{7}, \underline{9}$ and $\underline{12}$ mutant sites and the Str-dl site in AB2847 which would imply that the mutants could be deletions extending through the Spc locus into the Str locus but stopping short of the Str-dl site. Alternatively they could be point mutations which simultaneously inactivate both genes although the failure to obtain Spc-s Str-s revertants in each case makes this seem rather unlikely.

Obviously this finding opens the way for fine structure mapping of both the Str and Spc locus particularly with the use of the closely linked shikimic acid requiring mutations AroB and AroE described by Pittard and Wallace (1966.)
Attempt to Map the Ribosomal DNA Cistrons

Yankovsky and Speigelman (1962) have shown the ribosomal RNA is coded for by about 0.3% of the _E. coli_ genome and they suggest that their data implies that the region(s) coding for rRNA are grouped together rather than spread throughout the genome. The evidence presented by Dubnau et al (1965) in _Bacillus subtilis_ strongly favours the contiguity of at least some rDNA genes and the aminoglycoside resistance markers. It would therefore appear to be reasonable to examine the MalA-Spc region in an attempt to locate the rDNA cistrons. Apirion and Gorelic (personal communication) have done just that by constructing a partial diploid carrying an _F. mal^-Spc_. Their evidence is that the rDNA is indeed coded in that region.

The technique which was devised by Nygaard and Hall (1963) and modified by Gillespie and Speigelman (1965) relies upon DNA - RNA hybridization. DNA is prepared from both the parent strain and the partial diploid by a gentle extraction to minimize shearing and the DNA is denatured i.e. converted to the single strand form by either boiling or alkali treatment. Long single-stranded DNA has the property of sticking to nitrocellulose filters of the Schleicher and Schuell B6 type. When such DNA bearing filters are incubated with a radio-active labelled RNA species which is derived from or phylogenetically closely related to the DNA binding of the labelled RNA to the DNA occurs.

/Unrelated.......


Unrelated RNA passes through the filter as does double-stranded DNA. The amount of RNA bound to the DNA on the filter is determined by placing the filter in a suitable scintillation counting fluid and finding the counts per minute bound to the filter.

My initial attempts to locate the rDNA cistrons depended upon the observation made by Falkow et al that F's could be transferred from E. coli to Proteus mirabilis and that such hybrids possessed a heavy satellite DNA band when hybrid DNA was centrifuged in a cesium chloride gradient.

I subsequently showed that Hfr transfer also occurs from E. coli to P. mirabilis provided suitable markers are present in P. mirabilis to identify and stabilise the transferred material. By using the heavy satellite DNA band associated with the transferred material I was able to show that the satellite DNA for the Spc- Mal region did in fact hybridise with E. coli rRNA to a very significant extent. This work will be reported in detail in the following section.

I now know that I erroneously assumed that I was using the MalA locus at 66 minutes on the Taylor map whereas I was in fact using the MalB locus at 79 minutes. I have since shown that some of the rDNA cistrons are close to minute 74 using F's for that region together with an ilv mutant of P. mirabilis thus confirming the mapping of Vermeulen and Atwood (1970).
However, I used AllXS2 to seek confirmation of both my early mapping and that of Apirion and Gorelic. I argued that AllXS2 DNA should possess twice the rRNA hybridizing capacity. AllXS2-A1 should show the same capacity as the parent strain and a culture of AllXS2-A1 treated with acridine orange should have a substantially lowered capacity.

The two strains AllXS2 and the A1 derivative were grown overnight to give 10 ml volumes in nutrient broth. Each 10 ml was then inoculated into 1 litre volumes of nutrient broth containing 50 μg/ml acridine orange. After overnight incubation the respective cultures were pooled, washed and the viability determined. 10 litres of AllXS2-A1 gave between 3 and 4 gms wet weight of cells. The viability of AllXS2 was around 80% and the viability of the deletion mutant A1 dropped to between 2% and 0.6% in the various flasks. All the cultures of A1 presented the grossly elongated form seen in plates la and lb. The total count of A1 rose from about 4 x 10^6/ml to 7 x 10^7 - 10^8/ml. All and AllXS2 were grown up similarly with the omission of acridine orange. AllXS2 was plated at suitable dilutions on Tetrazolium Maltose agar + streptomycin to check that there had been no massive loss of the exogenomic element.

The cells were harvested by centrifugation and washed twice with standard saline-citrate (0.15M NaCl, 0.015M Na2citrate, pH7.)
Fig. 3A. Titration of $^3$H labelled denatured DNA from AllFX22 and the parent All with $^{32}$P r RNA. The results are the mean of two separate experiments done in duplicate.

- $\circ\circ\circ\circ$ All DNA. The result of two experiments in which 34 µg and 42 µg respectively of DNA were adsorbed onto the filters.

- $\times\times\times\times$ AllFX22 DNA. The result of two experiments in which 31 µg and 47 µg respectively of DNA were adsorbed onto the filters.

\[ p^{32} rRNA = 11600 \text{ cpm/µg} \]
The cells were lysed by several freeze-thaws in 1% SLS at pH 8. Pronase was added to a final 500 µg/ml and the mixture incubated at 37°C for four hours before the phenol extraction as described in the Materials and Methods section. The DNA preparation was dialyzed overnight against 0.1 x SSC. The dialyzed preparation was then treated with RNase previously heat treated to eliminate DNase activity. The treatment was continued for 60 minutes at 37°C. The mixture was again deproteinized three times with SSC saturated phenol. DNA-RNA hybridization was performed as described in the Materials and Methods section using P32 labelled rRNA prepared as described in the Methods section.

It was not possible in this case to label the DNA satisfactorily so that it was necessary to estimate the quantity of DNA retained on the filters spectrophotometrically by measuring the OD 260 before and after filtration assuming that for denatured DNA E 1%, 1 cm = 200.

It would appear from the hybridization data that if AllX32 Δ1 is a true deletion extending into the Str and Spc genes which are only 7% cotransducible there is no evidence to support the contention that the rDNA cistrons are included in the deleted material. This does not, of course, preclude the possibility that the rDNA cistrons are in the Str-Spc region but at one or other end of the deleted segment.
If all of the rDNA cistrons i.e. 0.3% of the total DNA were in this region then AllX32 ought to give a hybridization value of 0.6% which it clearly does not. Hence it appears that the rDNA cistrons do not lie within the MalA <sup>-</sup> Spc region.

Alternatively we can assume that the rDNA cistrons consist of two regions split 20:80 with the smaller region deleted. This leads to the conclusion that AllX32 ought to give a value of $0.24 + 2 \times 0.06 = 0.36\%$ whereas following acridine orange treatment, even assuming 10% of the population retain the exogenote, the value would drop to $0.24 + 0.006 = 0.246\%$.

Differences of this magnitude have not been observed so again the conclusion is that no rDNA material is in that region.
Falkow et al. (1964) made a most useful and exciting discovery when they found that F primes could be transferred to *Proteus mirabilis* from *E. coli*. Of particular interest is their observation that such hybrids give a heavier satellite DNA component when a DNA preparation is banded in a cesium chloride gradient and that this satellite is uniquely associated with the presence of the F prime.

An initial difficulty lay in the lack of a suitable F prime for the maltose-streptomycin region despite repeated attempts to obtain such an F prime by the classical method of selecting for early transfer of a suitable terminal marker by an appropriate Hfr strain.

However, during many discussions with Dr. J. Davison it became apparent that there may be no need to restrict one's considerations to F primes. Accordingly I was able to show that *E. coli* Hfr donors will transfer markers to *P. mirabilis*.

The investigation was carried out using two Hfr strains, Hfr G6 thy− which transfers the streptomycin-maltose A region early and Jla-19, a Bll type of Hfr which carries the cmlA gene conferring resistance to chloramphenicol which is linked to gal, both early markers for Hfr Jla-19, Reeve (1966) and Reeve and Suttie (1967).
Table 15 shows the results obtained from the crosses Hfr G6 thy⁻ x Proteus mirabilis - 1 with selection for mal⁺ and Jla - 19 x IM - 1 with selection for either gal⁺ or chloramphenicol resistance. In the first cross the mal⁺ cells were scored for their response to streptomycin and in the second cross gal⁺ were scored for chloramphenicol resistance and vice versa.

Table 15 shows that approximately 15% of the cells receiving the maltose marker also became streptomycin sensitive. The streptomycin sensitives readily segregated large numbers of resistent in the well of the plate with few colonies appearing beyond the well.

<table>
<thead>
<tr>
<th>Cross</th>
<th>G6 thy⁻ x IM-1</th>
<th>Jla - 19 x IM - 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mal⁺</td>
<td>gal⁺</td>
</tr>
<tr>
<td>Str-r</td>
<td>Str-s</td>
<td>Cml²</td>
</tr>
<tr>
<td>183</td>
<td>37</td>
<td>99</td>
</tr>
</tbody>
</table>

Table 22 The two Hfr strains G6 thy⁻ and Jla - 19 were crossed with the P. mirabilis strain IM-1 by plate mating as described in the Materials and Methods section. In the first cross mal⁺ hybrids were selected by plating on M9 maltose agar supplemented with vitamin B₁₂ and nicotinic acid. The donor was counter-selected by thymine starvation. In the second cross gal⁺ hybrids and cmn⁻ hybrids were selected on either M9 gal or M9 glu+ chloramphenicol at 15 μg/ml with supplemented as above. The donor was counterselected both nutritionally and by streptomycin killing.

/In.....
In the case of selection for the Gal\(^+\) marker and scoring the selected colonies for chloramphenicol sensitivity or resistance it is found that approximately 50% received the linked but distal dominant CmlA marker. Selection for chloramphenicol resistance and subsequent scoring for the proximal Gal marker results in a lower recovery of Gal\(^+\).

The reason for this is probably related to the ease and rapidity with which chloramphenicol resistant mutants arise in PM-1; these would, of course, be scored as Gal\(^-\).

In order to get additional data regarding the segregation of the exogenote, a Mal\(^+\) Str-s colony and a Gal\(^+\) Cml\(^-\) colony were grown overnight in Nutrient broth and plated at a suitable dilution on Tetrazolium Maltose agar and Tetrazolium galactose agar respectively. In the first case, a sample of 40 Mal\(^+\) and 40 Mal\(^-\) colonies were tested for their response to streptomycin and in the second case, a similar number of Gal\(^+\) and Gal\(^-\) colonies were tested on chloramphenicol. The results are shown in Table 16.

<table>
<thead>
<tr>
<th>Mal(^+) colonies</th>
<th>Mal(^-) segregants</th>
<th>Gal(^-) segregants</th>
<th>Gal(^+) colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Str-r</td>
<td>Str-s</td>
<td>Cml(^r)</td>
<td>Cal(^r)</td>
</tr>
<tr>
<td>14</td>
<td>26</td>
<td>17</td>
<td>33</td>
</tr>
<tr>
<td>Str-r</td>
<td>Str-s</td>
<td>Cal(^s)</td>
<td>Cal(^s)</td>
</tr>
<tr>
<td>22</td>
<td>18</td>
<td>23</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 16 Showing the segregation of the sugar linked antibiotic markers in the Proteus mirabilis - E. coli hybrids as described above.
At this stage it was thought that the difficulties of establishing and maintaining the Mal⁻ Str region from E. coli in Proteus mirabilis were really too great to make any hybridization results very meaningful.

Even after overnight growth only 65% of the Mal⁺ cells were likely to be carrying the E. coli Str locus. Obviously some method had to be found of exerting a selection pressure in favour of the required hybrid.

An acceptable solution to this problem emerged from the chance observation that the hybrid MM-1 Str-r/E. coli Str-s, unlike the situation in E. coli, is not killed by streptomycin but merely inhibited. This was first noticed in experiments designed to examine the streptomycin resistant segregants from the Mal⁺ Str-s hybrids obtained in the MM-1 x Hfr G6 Thy” cross. Many anomalous results obtained initially were due to the carry-over of inhibited but viable Str-r/Str-s cells from the background of the Str-r segregant. The revised procedure was simply to produce a number of starter cultures as before but to treat the cultures with streptomycin followed by penicillin to lyse all streptomycin resistant cells so that the pooled starters were free of segregants.

Another major improvement was to replace MM-1 by P6, a prototrophic streptomycin sensitive strain of Proteus mirabilis obtained from Dr. H. Böhme.

/MM-1....
HM-1 had the great disadvantage of growing very slowly on M9 glucose medium supplemented with vitamin B\textsubscript{12} and nicotinic acid whereas P6 grew extremely well.

Double streptomycin and spectinomycin resistant mutants were selected in P6 by two successive steps. The double mutant P6 Str-r 5 Spc-1 was mated with Hfr G6 Thy\textsuperscript{-} by the plate mating method, and Thy\textsuperscript{+} Mal\textsuperscript{+} recombinants selected on M9 maltose agar with vitamin B\textsubscript{12} and nicotinic acid. The donor was counterselected by thymine deprivation. 64 Mal\textsuperscript{+} colonies were examined by streaking on Tetrazolium Maltose agar + 200 \textmu g/ml streptomycin and Tetrazolium Maltose agar + 100 \textmu g/ml spectinomycin. All 64 colonies readily segregated Mal\textsuperscript{-} colonies and all Str-s colonies retained their viability when streaked on Streptomycin Nutrient agar and then transferred to Nutrient agar after 12 hours of incubation. The analysis of the 64 colonies is presented in Table 17.

\begin{table}
\centering
\begin{tabular}{l}
\textbf{P6} & \textbf{Mal\textsuperscript{-}} & \textbf{Str-r} & \textbf{Spc-r} \\
\hline
\textbf{chromosome} & \ldots & \ldots & \ldots \\
\textbf{Hfr G6 Thy\textsuperscript{-}} & \textbf{I} & \textbf{II} & \textbf{III} \\
\textbf{chromosome} & \textbf{Mal\textsuperscript{+}} & \textbf{Str-s} & \textbf{Spc-s}
\end{tabular}
\caption{}
\end{table}
TABLE 17

No. of Mal\textsuperscript{+} colonies examined

<table>
<thead>
<tr>
<th></th>
<th>Class 1</th>
<th>Class 2</th>
<th>Class 3</th>
<th>Class 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mal\textsuperscript{+}</td>
<td>Mal\textsuperscript{+}</td>
<td>Mal\textsuperscript{+}</td>
<td>Mal\textsuperscript{+}</td>
<td></td>
</tr>
<tr>
<td>Str-r Spc-s</td>
<td>Str-s Spc-S</td>
<td>Str-s Spc-r</td>
<td>Str-r Spc-r</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>17</td>
<td>1</td>
<td>39</td>
<td></td>
</tr>
</tbody>
</table>

Table 17. Hfr G6 thy\textsuperscript{-} was crossed with F6 Str-r5 Spc-1 by the plate mating technique as described in the Materials and Methods section. The cells were washed from the plates and washed several times by centrifugation before being plated on M9 maltose medium supplemented with vitamin B\textsubscript{12} and nicotinic acid. Colonies were tested for segregation of Mal\textsuperscript{-} and resistance to streptomycin and spectinomycin, as explained in the text.

Class 1 would arise, referring to the diagram above Table 17, by breakage in region II either pre-transfer or breakage and elimination after transfer. Class 2 results from breakage in region III and class 4 from breakage in region I. Class 3 is more difficult to explain unless one accepts a treble breakage in regions I, II and III followed by elimination of the E. coli Str-s locus.
It will be recalled that the preceding table shows that the closely linked markers in *E. coli* Mal and Str and Gal and Cml appear to segregate independently suggesting that physical linkage could be easily broken in the hybrid.

Two hybrids from class 2 were selected and designated H1 and H2.

Since the intention was to isolate the satellite DNA band and use it in a DNA-*E. coli* r-RNA hybridization test an important requirement was a control hybrid which does not possess the *E. coli* maltose-spectinomycin region.

In the course of earlier unrelated work I isolated an F prime carrying the markers ser/gly and thy in *E. coli* strain E34 thy−. E34 is a his− auxotroph in which Dr. J. Bishop isolated a ser/gly mutant which I subsequently identified as being the ser A marker at minute 56 on the Taylor and Trotter (1967) map.

F' ser thy was transferred to RM-1 thy− by overnight plate mating followed by selection on M9 glucose + casamino acids + nicotinic acid + streptomycin to counterselect the donor.

Since it does not appear to be possible to transfer F primes from *P. mirabilis* to *E. coli* it is difficult to assess the extent of the genetic material present in the hybrid. However, overnight growth of a hybrid in Nutrient broth + thymine with 50 ug/ml acridine orange resulted in a population, 40/40 tested which was thymine requiring.

/ Figures.....
Figures 4a, 4b and 5 clearly show that a substantial satellite band is present in the hybrids.

**Isolation of the Satellite DNA**

Cells of H1 and H2 were grown in M9 maltose medium supplemented with nicotinic acid, casamino acids and a trace of vitamin B12. A 24 hour 100 ml culture of each hybrid was transferred to 1 litre of fresh medium containing streptomycin, 100 µg/ml and penicillin, 2000 units/ml. The culture was incubated at 37°C with aeration for 4 hours. The culture was then washed several times, split into two 2 litre flasks each containing 500 ml of fresh medium without antibiotics and incubated with aeration at 37°C for 10 - 12 hours. Samples were removed to determine the degree of segregation of the exogenous markers. Each culture was washed, resuspended in SSC and given three phenol deproteinizations with recovery steps. The aqueous layer was stored in a refrigerator at 4°C until the degree of segregation was determined for each culture. Cultures were discarded if they showed segregation for the Mal - Str - Spc markers greater than 10%.

The aqueous layers obtained from the selected cultures were pooled, made up to 1.71 M with cesium chloride by adding solid salt and centrifuged for 24 hours at 42,000 r.p.m. in the 10 x 10 fixed angle rotor of the M.S.E. 3365. The satellite bands were collected as described in the Materials and Methods section and pooled. The pooled material was returned to 1.71 M CsCl and again centrifuged to remove the lighter component.

/These
UV scan trace showing satellite DNA band obtained from H1.

The gradient contained approximately 60 µg DNA in 0.1 x ssc to which was added solid CsCl to give a final density of 1.71 gms/cc.
UV scan trace showing satellite DNA band obtained from H2.
The gradient contained approximately 60 µg DNA in 0.1 x ssc to which was added solid CsCl to give a final density of 1.71 gms/cc.
OPTICAL DENSITY

FRACTION NUMBER

Fig. 4B
UV scan trace showing satellite DNA band obtained from PM-1Thy/F' ser/gly Thy. The gradient contained approximately 60 µg of DNA in 0.1 x ssc to which was added solid CsCl to give a final density of 1.71 gms/cc.
The heavier satellite material was collected, dialyzed for 24 hours against 1/10 SSC and prepared for hybridization as described earlier. The results obtained with P^{32} r RNA are shown in Figure 6, together with the results obtained for the satellite band obtained from the control EM-1 thy"/mer/gly-thy, treated in a similar way.

The percentage DNA hybridized was 1.41 in the case of H2 and 0.92 with H1. A repeat experiment performed approximately eight months later in exactly the same way but using only two P^{32} r RNA inputs gave lower values of 1.02 and 0.76 respectively. The controls in both experiments were about the same value of 0.23. The high value for the controls is probably due to the fact that despite careful extraction of DNA from Proteus mirabilis considerable breakage must have occurred thus allowing the heavier Proteus mirabilis ribosomal DNA cistrons to move into the heavier DNA fraction where their phylogenetic similarity would allow them to compete effectively for E. coli r RNA (Moore and McCarthy 1967).

Even so there can be little doubt that at least some of the cistrons which code for E. coli r RNA are present in the P. mirabilis/E. coli hybrid.

When the experiment was repeated more recently with H1 and H2, values of 0.74 and 0.61 respectively were obtained which implies that despite the selection applied to retain the Mal^+ Str-s and Spc-s markers in the hybrid there is a steady loss of the r DNA cistrons with each subculturing. Although it must be pointed out that in the last experiment it was not possible to include a control.
Fig. 6 Titration of denatured E. coli DNA obtained from various E coli - Proteus mirabilis hybrids, with P$^{32}$ r RNA.

- Filters contained approximately 26µg of denatured F' ser/gly-thy DNA. P$^{32}$ RNA = 10400 cpm/µg.
  1 experiment only in duplicate.
- Filters contained approximately 18µg of denatured E coli mal-str-spc region obtained from hybrid HII P$^{32}$ RNA = 24200 cpm/µg.
  2 experiments in duplicate.
- Filters contained approximately 18µg of denatured E coli mal-str-spc region obtained from hybrid HI P$^{32}$ r RNA = 27700 cpm/µg.
  1 experiment in duplicate.
The technique of isolating specific regions of E. coli DNA by constructing an intergeneric hybrid between E. coli and Proteus mirabilis is obviously a potentially powerful tool but suffers as used in the previous section from several disadvantages. It is likely that temperature sensitive mutants of Proteus mirabilis would prove more useful provided E. coli is capable of masking the genetic lesion.

Initially many Ts mutants were obtained by the simple procedure of mutating the culture, growing in broth at 30°C for two hours before transfer to 38°C. After 30 minutes at 38°C penicillin was added to 2000 units per ml and incubation continued for 10 - 12 hours. The cultures were then membrane filtered and resuspended in fresh broth for growth at 30°C overnight. The culture was then diluted and plated to give approximately 200 colonies per plate. Ts mutants were detected by replica plating or by incubating the plate overnight at 38°C, marking the colonies which appear and then transferring the plate to 30°C and picking colonies which appear at the lower temperature. After the penicillin step most plates showed several Ts mutants.
No mutants were obtained which were cotransducible with either the Str or Spo locus. It was interesting to note, however, that all of the Ts mutants tested i.e. those with a low reversion frequency gave intergeneric hybrids which were capable of growing at 38°C.

In view of my earlier work with the sib locus in E. coli I decided to attempt to find temperature sensitive sib\(^-\) mutants in P. mirabilis.

The selection procedure was modified to include a 30 minute exposure to streptomycin at the higher temperature before the addition of penicillin. In the first experiment four mutants - possibly allelic - were obtained which did not grow at 38°C, were not killed by streptomycin at the higher temperature and were cotransducible with Str locus as shown in Table 13.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>No of Ts(^+) transductants per 10(^9) survivors</th>
<th>% Str-r</th>
<th>% Spo-r</th>
</tr>
</thead>
<tbody>
<tr>
<td>P6 Ts1</td>
<td>4640</td>
<td>80.5</td>
<td>92.2</td>
</tr>
<tr>
<td>P6 Ts2</td>
<td>5560</td>
<td>72.3</td>
<td>88.6</td>
</tr>
<tr>
<td>P6 Ts3</td>
<td>3790</td>
<td>77.8</td>
<td>96.3</td>
</tr>
<tr>
<td>P6 Ts4</td>
<td>6200</td>
<td>81.4</td>
<td>91.4</td>
</tr>
</tbody>
</table>

Table 13  T\(\lambda\) phage was grown on P6 Str-r Spo-r and used to transduce the four Ts mutants to ability to grow at 38°C. The colonies which emerged at 38°C were replicated onto nutrient agar containing streptomycin at 200 \(\mu\)g/ml and nutrient agar with 100 \(\mu\)g/ml spectinomycin.
The two *E. coli* Hfr strains G6 Thy<sup>−</sup> and AB312 were crossed with each of the F6 Ts mutants and *Proteus mirabilis* colonies capable of growing on M9 glucose + nicotinic acid + B<sub>12</sub> at 38°C were selected, purified by restreaking at 38°C and verified as urease + ve. The donor strains were counter-selected nutritionally and in the case of the mating with AB312 which is Str<sup>r</sup>-r, several restreakings were required before streptomycin resistant urease -ve colonies failed to appear, presumably because of cross-feeding on the selective plates.

All four Ts mutants reverted to Ts<sup>+</sup> with a frequency of 1 per 10<sup>5</sup> - 10<sup>6</sup> cells plated and all formed intergeneric hybrids at about 1 per 10<sup>5</sup> with approximately equal donor and recipient input. Streptomycin was bacteriocidal for the Ts mutants at 30°C and for 16 hybrids tested from each F6 x Hfr G6 Thy<sup>−</sup> cross at both 30°C and 38°C.

Two representative hybrids were chosen from Ts1 and Ts2 and designated HTs<sup>−</sup>1 and HTs<sup>−</sup>2.

HTs<sup>−</sup>1 and HTs<sup>−</sup>2 were grown in Nutrient broth at 38°C from single colonies grown on Nutrient agar plates at 38°C. Under these conditions it proved impossible to detect directly any Ts mutants as a result of the exogenicomic element segregating out. However, Ts mutants could be shown to be present in the culture by transferring 0.1 ml of overnight culture to 10 ml of fresh broth containing streptomycin, incubating at 38°C for 30 minutes and adding penicillin to a final concentration of 2000 units per ml.

/After....
After 8 hours the culture was washed on Millipore filters and resuspended in 1 ml of M9 buffer. 0.1 ml aliquots plated on Nutrient agar and incubated at 30°C always gave between 80 and 600 colonies per plate. All colonies tested were unable to grow at 38°C. This implies that 0.1 ml of the original culture must have contained between 800 and 6000 Ts segregants per $4 \times 10^8$ cells i.e. approximately 1 per $10^5$ hybrids. Obviously then the use of temperature sensitive mutants overcomes the difficulty of massive segregation of the exogenomic element experienced in previous work.

DNA for hybridization was obtained by growing cells over-night in Nutrient broth at 38°C, diluting into fresh Nutrient broth prewarmed to 36°C and incubating until the cells reached an OD$_{660}$ 0.3 - 0.6. The cells were harvested, washed twice with SSC and the cells lysed with SLS as described in the Materials and Methods section.

DNA was prepared and banded in CsCl as described previously and the hybridizations were carried out according to the method given by Kennell and Kotoulas (1968). The basic differences are that the hybridization is performed in 0.5 ml volumes of 6 x SSC at 66°C for 20 hours. The hybridization curves for the satellite DNA bands from the hybrids HTs-1 and HTs-2 against three r-RNA input levels are shown in figure 7, together with the control. It will be readily seen that there is no support for the hypothesis that the exogenomic element carries the rDNA cistrons.
The somewhat lower hybridization levels reached in this experiment are probably due to reannealing at 66°C under which conditions less closely related hybrids would not be stable.

Obviously, the fact that Ts mutants are easily obtained in _P. mirabilis_ and the ease with which they will form intergeneric hybrids with _E. coli_ opens up possibilities for making a wide range of hybrids and using them to obtain selected regions of _E. coli_ DNA.
Fig. 7 Titration of denatured E.coli DNA from P.mirabilis-E.coli hybrids with $^{32}\text{P} r\text{RNA}$.

- $\times-\times-\times$ Filters contained approximately 37μg denatured $F^1$ ser/gly-thy DNA.

- $\circ-\circ-\circ$ Filters contained approximately 44μg HTs-1 satellite DNA.

- $\circ-\circ-\circ$ Filters contained approximately 27μg HTs-2 satellite DNA.

$P^{32}\text{RNA} = 37100 \text{ cpm/μg.}$
DISCUSSION
The tendency at the present time appears to be to accept completely as central to the action of streptomycin the role of the ribosomal and indeed the evidence is overwhelmingly in favour of the ribosome interpretation. However, there are many observations which are not immediately compatible with the ribosome theory. For example, Rosenkrantz (1963) has shown that Str-d strains of *E. coli* contain high levels of alkaline phosphatase even in the presence of inorganic phosphate. Some interesting studies from Polglase's group Bragg and Polglase (1962) have shown that a streptomycin dependent strain of *E. coli* growing in a minimal glucose-salts medium excreted 10% of the substrate carbon as L-valine. This was later, Coukell and Polglase (1965) related to an increased specific activity of acetohydroxy acid synthetase although the end-product inhibition and repression control mechanisms appeared unchanged and in 1969 Conkell and Polglase (1969) showed that AHA synthetase which is normally subject to catabolite repression does not exhibit glucose repression in streptomycin dependent *E. coli*. Related to this observation is the discovery of Goodman and Spotts (1967) that both inducible and constitutive synthesis of B-galactosidase in streptomycin-dependent *E. Coli* were inhibited by streptomycin deprivation.
Coukell and Polglase (1969) conclude "There are two distinct and contrasting observations on streptomycin-dependent *E. coli* to be understood: first, the impaired carbohydrate metabolism which decreases catabolite repression; secondly, the effect of streptomycin deprivation which increases catabolite repression."

Another observation which does not appear easily compatible with the ribosome theory was made by Lederberg and St. Clair (1952) who found that L- forms of streptomycin resistant *E. coli* became sensitive to the antibiotic.

The action of the organic solvents acetone, methanol and ethanol on streptomycin dependent organisms is also extremely puzzling. My own studies (unpublished) have failed to show that the solvent is being metabolized which leaves the misreading hypothesis or the original membrane effect as proposed by Gado and Horvath (1964).

The miscoding hypothesis argues that because misreading results from the addition of the above chemicals to the in vitro system of Nirenberg and Matthai a similar effect occurs in vivo. This miscoding in fact repairs the original mutation which can also be repaired by streptomycin itself. I have attempted to isolate mutants which show a requirement for acetone, methanol or ethanol with complete lack of success. This suggests but does not prove that if the misreading hypothesis is correct mutants belonging to that class are extremely rare or are confined only to the streptomycin locus.

/Thu....
The membrane hypothesis seems the most attractive and would merit further investigation. It is possible that some relationship between ribosome and membrane is involved in this effect.

Finally and most difficult to consolidate with the ribosome hypothesis is that streptomycin does not inhibit anaerobically growing cells nor are streptomycin dependent cells capable of growth under anaerobic conditions.

It is true to say that none of the work described in this thesis has in any way clarified the situation. I have extended the work on heterozygotes at the streptomycin locus to include Str-d/Str-s and Str-r/Str-d heterozygotes. Several interesting facts emerge; the most important being that when the heterozygous Str-d/Str-s is selected in the presence or absence of streptomycin, colonies are obtained which will continue to plate normally on the selective medium but fail to plate or plate with low efficiency on medium containing the antibiotic when the original isolation was performed in its absence and vice versa.

It was subsequently discovered that a period of growth in liquid media containing ethanol increased the plating efficiencies by a considerable factor.

It was also possible to show that the Str-d/Str-r heterozygote is dependent upon streptomycin for growth and therefore that the dependent allele is dominant over the resistant allele. It was, unfortunately, impossible to extend the observation on the effect of ethanol on this class of heterozygote.
It should be noted that not all streptomycin dependent mutants are capable of growth in the presence of ethanol only. In the present work approximately $2/3$ of all the dependents isolated are capable of doing so, the remaining group apparently having an absolute requirement for streptomycin.

My own recent attempts to investigate the action of ethanol on Str-s E. coli and its Str-d derivatives have suggested that there are differences in the concentrations and ratios of NAD, NADP and NADH, NADPH, although at this time I have no theory which would help to explain these differences. One can readily appreciate, however, that such changes even though quite small could have major consequences for cellular metabolism giving rise to many of the more unusual phenomena associated with streptomycin dependence perhaps even in the manner suggested by Kacser (1957) and Hinshelwood and Dean (1963).

In this context I have had an opportunity to measure the total pyridine nucleotide concentration in nine micro organisms with a widely differing range of G + C values. My results show quite clearly that there is a high correlation of 0.68 between total pyridine nucleotide and percentage DNA adenine. Whether this indicates that DNA adenine has a role in regulation of metabolism or whether it merely reflects the stoichiometry of cellular concentrations remains to be seen.

It is hoped to publish this work in the near future.
Attention has been drawn to the complexities of streptomycin action on the bacterial cell by the discovery of a new mutant at the Sib locus. It is shown that this locus is likely to be closely linked to the classical Str locus and it is identified by the occurrence of Sib\(^{-}\) mutants which are inhibited but not killed by streptomycin. In terms of the ribosome hypothesis of streptomycin resistance it may be that the Sib locus defines a ribosomal protein which is closely associated with the streptomycin binding ribosomal protein in such a way as to prevent the sequence of events which lead to cell death.

The Sib locus could be of considerable use both for biochemical studies and as a closely linked marker which is easily selected to assist in fine structure mapping of the classical Str locus. At the moment, however, other than showing that such mutants exist I am unable to speculate further on the nature of the locus and its function in the cell.

The work of Flacks et al (1966) which showed that the resistance gene for the aminoglycoside antibiotic spectinomycin was linked to the classical Str locus and that spectinomycin also appeared to act on protein synthesis at the 30s ribosomal sub-unit level was enough to suggest that the two loci might be under control of a single operator thus ensuring stoichiometric synthesis of the ribosomal components. One might be forgiven for hoping further that other 30s sub-unit proteins were also mapped in the Str-Spc region.

//Accordingly....
Accordingly an experiment was designed to search for single mutations which inactivated both the Str and Spc loci simultaneously. The evidence presented in the results indicates that inactivating mutations do occur with a frequency many times greater than would be expected on the basis of two simultaneous mutations occurring, one at each locus. However, there is also a strong indication that many mutations can occur at either of the two loci which seem to inactivate the gene and allow expression of the recessive allele on the merozygote. My interpretation of the data is that only between \( \frac{1}{100} \) to \( \frac{1}{1000} \) of the spontaneous mutations result in a mutation which simultaneously confers resistance and retains viability.

If extreme polarity mutants do occur which result in simultaneous inactivation of both antibiotic sensitive alleles then the crude test adopted to find these mutants was unsuccessful. On the other hand the evidence is strongly in favour of at least some of the doubly resistant mutants arising as a consequence of a deletion of the endogenous alleles. One interesting aspect which emerges from this work is the possibility observed in four cases out of fifteen that recombination occurs between the streptomycin dependent allele in a recipient strain and the transduced fragment from the deletion mutants to give streptomycin sensitive recombinants.
This could occur if the endogenomic inactivation mutation was a multi-site one or a deletion which ran into but not through, nor as far as, the streptomycin dependent site. Under suitable circumstances then recombination could occur to give the streptomycin sensitive recombinants observed.

This would certainly merit further investigation as a means of constructing a fine structure map of Str-d mutational sites by a deletion analysis technique.

The final part of this thesis is concerned with my attempts to locate the ribosomal DNA cistrons. It is in this section that I have been most unhappy with the work. DNA - RNA hybridization is a difficult technique which requires a great deal of experience. In my early work I used a strain of Proteus mirabilis which carried the _E. coli_ region for maltose - spectinomycin. There were serious disadvantages associated with this work, particularly the fact that the interspecific partial diploid was constructed by Hfr transfer to _P. mirabilis_ rather than by F transfer. Thus the actual extent of the _E. coli_ material present could not be determined.

However, notwithstanding, these objections and despite the difficulties of DNA - RNA hybridization it is fairly clear that there is a greatly increased capacity for _E. coli_ ribosomal RNA hybridization when the trapped DNA was obtained from a hybrid with the spo - mal region from _E. coli_. Unfortunately, it was much later before I discovered that the maltose marker which was being selected in _P. mirabilis_ appears to be Mal B.
This, although initially a very disturbing observation made complete sense with the publication of Vermeulen and Atwood (1970) which indicates that ribosomal RNA cistrons are located around minute 74 on the Taylor map. Putting a strong selective pressure on the Maltose marker probably selected much more efficiently for material in the 79 minute region than the 65 minute region where Str is mapped. Negative evidence to support this view is afforded by the work on *Proteus mirabilis* temperature sensitive mutants where much stronger selection pressure was applied to retain the streptomycin region around minute 65. The failure to detect an increase in r RNA binding capacity possibly indicates that the initial success sprung from the use of the Maltose marker and not as imagined from the Streptomycin marker.

I have subsequently obtained conclusive evidence that this was so by selecting an ilv mutant in *Proteus mirabilis* F6 and transferring an F' ilv from *E. coli* to the mutant. This work is reported in a short paper which I am hoping to have published in the near future.
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Since revising and resubmitting this thesis the publication by Birnbaum and Kaplan (1971) has come to my attention. They use a very similar method to show that approximately half of the rRNA genes are coded in the region 74-77 minutes. It is interesting to note that Yu, Vermeulen and Atwood failed to find any indication of rRNA genes at any other locus. My experience with P. mirabilis - E. coli hybrids is that they can be extremely unstable and that regions between two conserved markers may be readily lost. Birnbaum and Kaplan give no figures to support the implication that no loss of exogenote has occurred during the several stages of growth required to produce a culture large enough to give manageable amounts of DNA. However their results do confirm my conclusion that the ilv-mal region contains at least some of the rRNA cistrons.