15340

NATIONAL LIBRARY
OTTAWA

CANADA

BIBLIOTHÈQUE NATIONALE
OTTAWA

NAME OF AUTHOR.....SHIRLEY DIANNA SEMAKA.....

TITLE OF THESIS.....MOLECULAR NATURE OF R FACTORS IN.....

.....PSEUDOMONAS AERUGINOSA.....

.....

UNIVERSITY....OF ALBERTA.....

DEGREE FOR WHICH THESIS WAS PRESENTED.....M.Sc.....

YEAR THIS DEGREE GRANTED.....1973.....

Permission is hereby granted to THE NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(Signed).Shirley D Semeka.

PERMANENT ADDRESS:

1118 Casson Green

Varsity Courts

Calgary, Alberta T3B 2V6

DATED...April 27th.....1973

NL-91 (10-68)

THE UNIVERSITY OF ALBERTA

MOLECULAR NATURE OF R FACTORS IN *PSEUDOMONAS AERUGINOSA*

by

© SHIRLEY DIANNA SEMAKA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF MEDICAL BACTERIOLOGY

EDMONTON, ALBERTA

SPRING, 1973

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Molecular Nature of R Factors From *Pseudomonas aeruginosa*, submitted by Shirley Dianna Semaka in partial fulfilment of the requirements for the degree of Master of Science.

L. E. Bryan
Supervisor

Ronald L. Whitehouse

J. N. Campbell

Date Apr. 27, 1973.

TO FRANK

ABSTRACT

Several R factors from Pseudomonas aeruginosa were studied. Of these R931 and R3108 conjugally transferred to a P. aeruginosa recipient at high frequencies but failed to transfer to Escherichia coli. Other R factors (R1162, R679, R5265) were characterized by much lower transfer frequencies with P. aeruginosa recipients than R931 and R3108. However these R factors still transferred at higher frequency to P. aeruginosa than E. coli recipients. All P. aeruginosa R factors could be divided into three groups by serological reactions.

Transfer of R factor 931 to strain 280 was associated with the aquisition of satellite band in CsCl density gradient profiles of DNA isolated from strain 280. A similar satellite band could not be demonstrated for R factor R679 upon transfer to strain 280 but could be shown after transfer to E. coli. Satellite bands were present in strains 5265 and 3108. The buoyant densities of R factors varied from 1.716 to 1.719 g/cm^3. Covalently closed circular R factor DNA was isolated by ethidium bromide-CsCl density gradient centrifugation and examined by electron microscopy. DNA of two major sizes was detected ie. 25 x 10^6 Mdal and 1 x 10^6 Mdal. The relationship of the smaller molecule to the R factor DNA was unclear.

The percentage of R931 DNA relative to chromosomal DNA declined in stationary phase in drug free medium. After 126 serial subcultures in tetracycline or streptomycin the amount of R factor DNA increased over that in drug free medium and no difference existed in logarithmic or stationary phases of growth. No apparent change in buoyant density resulted.

ACKNOWLEDGEMENTS

My most sincere appreciation is extended to Dr. L. E. Bryan for his able supervision, guidance, and encouragement throughout the course of this research.

I would also like to express my earnest thanks to Dr. J. Kinnear for his invaluable assistance, encouragement and suggestions.

To Dr. R. Whitehouse, I extend my thanks for his help in electron microscopy and to Dr. K. Roy I extend my thanks for use of his fraction collector. Gratitude is also expressed to all members of the Department of Medical Bacteriology for their assistance. Finally, thanks are extended to Mrs. M. Petly-Jones for the preparation of the photographs and to Mrs. Beth Schultz for her expert typing of this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

EDTA - Disodium ethylenediaminetetraacetate

CsCl - Cesium chloride

DNA - Deoxyribonucleic acid

DNASE - deoxyribonuclease

GC - guanine plus cytosine content

RNASE - Ribonuclease

TS - Trypticase soy

TRIS - TRIS (hydroxymethyl) aminomethane

TTP - thymidine triphosphosphate

INTRODUCTION

Since the introduction of antibiotics as a means of controlling infectious disease, bacterial strains have emerged that are resistant to a wide variety of chemotherapeutic agents. An examination of the mechanisms of resistance reveals a variety of ways in which these organisms survive the presence of antibiotics. According to the laws of genetics, mutant strains should acquire resistance to only one antibiotic at a time and organisms resistant to several drugs should arise in nature only by the accumulation of successive mutations. Hence it is amazing that there has been a dramatic increase in the frequency of Enterobacteriaceae with multiple drug resistance in essentially all countries in which the problem was examined. In general the multiple drug resistance of these microorganisms appears to have been acquired simultaneously. Genetic analysis has revealed that multiple drug resistance is specified by an extrachromosomal element which is referred to as a drug resistance or R factor.

Interest in drug resistance began in Japan where it centered around the problem of shigellosis. Shortly after World War II a large amount of sulfonamide was used for the treatment of bacillary dysentery. However, the effectiveness of the drug lasted for about five years and sulfonamide (SA)-resistant Shigella strains then appeared rapidly. Fortunately the production of antibiotics such as streptomycin (SM), tetracycline (TC), and chloramphenicol (CM) started in Japan around 1951, and these were at first fairly effective against Shigella bacillary dysentery. Few isolates resistant to SM, TC and CM were detected between 1951 and 1955 with just 3,7,11 and 4 isolates resistant

to either SM or TC reported in Japan in 1952, 1953, 1954 and 1955 respectively (61,64). However with the increased use of these antibiotics, the rate of isolation of antibiotic resistant Shigella strains increased enormously. By 1966, 79% of Shigella isolated were antibiotic resistant (64).

A multiple-resistant Shigella strain was demonstrated first in 1952 by Suzuki et al (61). This multiple resistance involved streptomycin, tetracycline, and sulfanilamide. In 1955, a strain of Shigella flexeri 4a was isolated and was shown to be resistant to 4 drugs: SM, CM, TC and SA (61). A third isolation of multiple-resistant Shigella strains from an epidemic occurred in 1956 (61). However other cases of bacillary dysentery caused by multiple-resistant strains were reported in 1956 from about one hundred thousand reported cases of bacillary dysentery (64). The spread of multiple-resistant Shigella strains in Japan at that time was not accounted for by epidemic spread of a strain resistant to 4 drugs because of the difference in serotypes of such strains.

In 1957, Mitsuhashi isolated Echerichia coli strains resistant to 4 drugs (TC, CM, SM and SA) during an epidemic caused by S. flexneri 3a resistant to the same 4 drugs (61,64). Subsequently in 1958 Mitsuhashi found in one patient E. coli, E. freundii and S. flexneri 2a resistant to TC, CM, SM and SA (61). In another patient from whom S. flexneri 2a was isolated which was resistant to CM, SM and SA Mitsuhashi isolated E. coli resistant to the same agents (64).

A survey in 1960 showed that 1.3% of healthy human subjects

carried multiple-resistant E. coli (61). In contrast 61.0% of inpatients treated with CM and 20.5% of inpatients with tuberculosis and treated with SM carried multiple-resistant E. coli (64).

These appearances of multiple-resistant Shigella and E. coli strains and the rapid increase in number of such strains attracted the attention of microbiologists especially from the standpoint of epidemiology.

In 1959, it was reported independently by Ochiai et al (61) and Akiba et al (1) that multiple-resistance was transferable by mixed cultivation of Shigella and E. coli strains. This transfer of multiple resistance was not mediated by bacteriophages, deoxyribonucleic acids or other filterable agents but required direct cell-to-cell contact (59,64). Following mixed cultivation drug resistance was transmitted from drug-resistant E. coli K 12 F^- or Hfr to sensitive Shigella regardless of the polarity of the F agent (59). These facts indicated that the transferable drug resistance is transmitted independently of the chromosomal transmission of donor strain and this agent is different from the F factor. Also, it was observed that this transferable drug resistance was spontaneously and irreversibly lost from cells during storage (61). Similarly, this transferable drug resistant property was lost artificially from resistant strains of Shigella or E. coli by treating them with acriflavin (60, 104). Also, transmission of drug resistance was interrupted by blender treatment of mixed cultures (64). These findings indicated that transfer of drug resistance was mediated by cell-to-cell contact, namely by conjugation and that the transferable

drug resistant agent exists independently of host chromosome. At the conference of infective heredity held in 1962 at the National Institute of Genetics, Mishima, Japan, Japanese geneticists agreed to use the term "R" factor for the property of transmissible drug resistance. It has been found that R factors are transferable among all species of the family of Enterobacteriacea (40), the Vibrio group (7), and Pasteurella pestis (37).

Since the discovery of R factors in Japan extensive epidemiological studies carried out in Japan (64), England (3,24), the Netherlands (29), Switzerland (29) and the United States (86) have shown that in numerous clinical situations, R factors are the agents responsible for transferable resistance to antibiotics in Enterobacteriaceae. The occurrence of transferable drug resistance has been documented in domestic animals (4,89,100) and fish (108), particularly when antibiotics are used as routine diet additives.

In Table 1 are the different resistance markers found on different R factors.

Transduction experiments carried out in Salmonella and E. coli with phages P22 and P1 led Watanabe and Fukasawa (103) to propose that R factors consist of a transfer unit (RTF segment) linearly linked to drug resistance determinants (r determinants). The transfer unit mediates transmission of the plasmid during bacterial conjugation while the r determinants carry genetic information specifying resistance to antimicrobial agents. Recent studies by Anderson and his collaborators (2,5) on Salmonella and by Mitsuhashi and coworkers (46, 62) have shown that the transfer unit of at least certain classes of R

TABLE 1. Resistance markers found on different R factors (29)

Resistance to:	References
Ampicillin	27
Chloramphenicol	84,93
Kanamycin	10,71
Neomycin	10,71
Streptomycin	12,71
Spectinomycin	12,90
Gentamicin	11,109
Sulfonamides	105
Tetracycline	42
Colicinogenic factors	83
Mercury	88
Nickel and cobalt	88
Viruses	95,96
Ultraviolet light	57

factors can be transmitted alone as well as in combination with resistance determinants. Furthermore, both groups of investigators have found that ordinarily non-transmissible R determinants occurring in nature, can interact with RTF units which are then able to effect their passage into recipient (R^-) bacteria. Once within a recipient cell, the linked transfer and resistance units replicate independently of each other (6).

The earliest studies on the molecular nature of R factors showed that heterogenous satellite bands of DNA are associated with the presence of R factors in several bacterial species (32,76). However, the method of nucleic acid extraction (54) that was used in these experiments yielded DNA having a maximum molecular weight of 10-15 million daltons (76) while the molecular weight of R factor DNA was estimated at more than three times this size (76). Because the R factor was not intact in these studies it could not be determined whether the several R factor satellite bands identified by cesium chloride (CsCl) gradient centrifugation reflected intramolecular heterogeneity with a single fragmented R factor species or whether they were physically distinct R factor subunits. Recently, procedures were developed to obtain high molecular weight plasmid and chromosomal DNA from bacteria (9,30) and methods have become available to separate DNA species which have only small differences in nucleotide base composition (66).

Studies on the molecular nature of R factors R1 and R6 in Proteus mirabilis by Cohen et al (20,21) have revealed that the total amount of R factor DNA and relative amount of each of the satellite

band components of this DNA varied markedly at different stages of bacterial growth. In the early logarithmic phase bacterial growth the R factor DNA represents 3% of the total DNA and only a single satellite peak banding at a density of 1.710 g/cm^3 in CsCl. Later in the logarithmic phase growth a second satellite band having a buoyant density of 1.718 g/cm^3 appeared and the fraction of the total DNA represented by the R factor increased by about three fold. After eight to twelve hours in the stationary phase, the R factor comprised almost 50% of the total DNA and consisted almost entirely of the DNA species banding at 1.718 g/cm^3. Similar findings have been observed by Rownd et al (78). Using cesium sulfate-mercury gradient centrifugation Cohen and his associates were able to resolve the 1.710 peak into two separate component units having buoyant densities of 1.709 and 1.711 g/cm^3. Electron microscopic examination of the DNA samples taken from all three fractions of R factor DNA showed molecules appearing as both closed, tightly twisted coils and relaxed circles.

A summary of the R factor species isolated from Proteus mirabilis is shown in Table II.

The approximate molecular weights were calculated from the contour lengths of R factor DNA using the relationship of 2.07 megadaltons/um (51). The values shown for contour lengths of R1 indicate one standard deviation.

Thus the buoyant density observed for the largest of the three circular DNA species agrees with the calculated buoyant density that

TABLE II. Summary of R factor species isolated from Proteus mirabilis (20).

Buoyant density in CsCl (g/cm^3)	R factor R1 Contour length	Cal. mol. wt.	% Guanine plus cytosine content
1.709	28 ± 1 um	58 x 10^6daltons	50
1.711	33 ± 0.8 um	68 x 10^6daltons	52
1.717-1.718	5 ± 0.5 um	10 x 10^6daltons	58

would result from the joining of the two smaller units of the observed buoyant densities. Also, the sum of the two contour lengths of the smaller unit is approximately equal to the length of the large species.

Since different levels of antibiotic resistance can be conferred by the same R factor in different host bacterial species (102) and prominent differences exist in the amount of R factor DNA present in Proteus mirabilis versus E. coli (76) Cohen et al examined the R factors R1 and R6 in E. coli.

The R factor DNA cannot be separated from chromosomal DNA in E. coli by CsCl equilibrium centrifugation because the buoyant densities of the E. coli chromosome and R factor DNA are too similar. However, the circularity of R factor DNA (19,20,33,67) provided a means of achieving separation of the R factor from the E. coli

chromosome. Vinograd and his collaborators (98) have shown that covalently closed circular DNA has enhanced resistance to both heat and alkali denaturation. In addition, the interaction of closed circular DNA with ethidium bromide and certain other intercalative dyes yields DNA of a different buoyant density than is observed when these dyes interact with linear DNA or with circular DNA having one or more single strand scissions. By using alkali denaturation Cohen et al (20,21) was able to show the buoyant density of chromosomal DNA increased, while a fraction comprising less than 5% of the total DNA remained at the previous buoyant density (ρ = 1.710) suggesting a covalently closed circular form. Similar results were obtained by Rownd et al (79,78). Preparative centrifugation of ^{3}H-labelled DNA in CsCl in the presence of ethidium bromide revealed in addition to the major peak a second peak banding at a buoyant density shown by Vinograd and his collaborators (98) to be characteristic of closed circular DNA. Electron microscopic examination of the R factor showed that most molecules were 31 to 38 um in length (20). Thus the R factor species in E. coli appear to express both the resistance and transfer function of R factor in this host and is indistinguishable in buoyant density and molecular weight from the largest of the three R factor species identified in Proteus. The dissociation and replication of R factors into separate independent plasmids is under relaxed control in Proteus whereas both replication and dissociation of R factor DNA are more stringently controlled in E. coli. Thus, the conflicting genetic evidence of Watanabe (102) on the one hand and of Anderson and

Mitsuhashi (5, 46, 62) on the other can now be reconciled.

The R factor density profiles in Proteus mirabilis depend on the conditions under which host cells are cultured (78,79,80). When R^+ P. mirabilis is cultured in drug free medium for a long period the R factor DNA forms a satellite band of density 1.712 g/cm^3 whose proportion is about 8% of chromosomal DNA. After prolonged growth in medium containing any of the drugs to which the R factor confers resistance (except tetracycline) a larger satellite band whose proportion is 60% of chromosomal DNA of density 1.718 g/cm^3 is observed. These two types of density profiles are interconvertible. In these transitions a broad and diffuse band of intermediate density is intermediate in this transition. This band is apparently a collection of molecules having a broad spectrum of density between 1.712 and 1.718 g/cm^3 (78,79,80).

According to the model presented by Rownd et al (78,79,80) the R factor dissociated spontaneously into two DNA components of density 1.712 and 1.719 g/cm^3 when harboured by P. mirabilis.

The 1.712 g/cm^3 component is the RTF and it also harbors the genes specifying TC resistance. This element replicates under relaxed control: i.e., there are multiple copies per cell. The 1.719 g/cm^3 contains the remaining drug resistant genes and this element replicates under a stringent control mechanism; only one round of r-determinant replication occurs during each division cycle. However, when r-determinants are attached to an RTF-TC to form an R factor, the composite structure replicates under relaxed control.

In medium containing appropriate drugs there is selection for cells that harbour the most r-determinants because these cells have the highest drug resistance and grow most rapidly. Such cells result from the incorporation of multiple copies of r-determinants into individual R factors. This situation restores the potential for cells to harbour multiple copies of r-determinants by virtue of their attachment to RTF-TC and thus their replication under the RTF replication system. The DNA of R factors that harbour only a few copies of r-determinants is manifested as a broad band of intermediate density. The density of R factors with many copies of r-determinants is essentially the same as that of r-determinants themselves since most of the R factor DNA is from r-determinants. In cells so grown the proportion of DNA in the satellite band is considerably greater than that in cells grown in drug-free medium because incorporation of additional copies of r-determinats has increased the size of R factor.

When P. mirabilis is cultured in drug-free medium, R factor dissociation should proceed to completion since there would be no selection for high levels of drug resistance. The RTF would replace R factors in the multicopy pool of episomes that replicate under relaxed control. Thus, there is a shift in density of R factor DNA to 1.712 g/cm^3. A band corresponding to r-determinants would not be observed since a single copy of r-determinants per cell would be too small in proportion to register as a detectable satellite band.

Experiments similar to those by Rownd et al (78,79,80) but using R^+ E. coli and R^+ Serratia marcescens have shown that there is no change in the density profile of R factor DNA during growth in a

medium containing drugs (78,79). Thus the available evidence suggests that the R factor exists as a composite structure in both these species and that the transfer factor and r-determinants do not dissociate and reassociate as they do in Proteus mirabilis.

Initially various workers reported that there was no transferrable drug resistance in Pseudomonas (31, 64). However, recently transfer of multiple drug resistance in Pseudomonas utilizing E. coli strains as recipients was shown (36,74,86,94). Apart from the clinical importance of intergeneric transfer of this type, the survival and effective functioning of drug resistance genes in such dissimilar organism are of interest. Since E. coli did not appear to be a good physiological choice, Bryan et al (14) explored the possibility that intrageneric matings would permit higher frequency of drug resistance transfer and patterns not detected by intergeneric matings. By mating three donor strains of P. aeruginosa they found the hybrid strain obtained generally demonstrated a slightly lower resistance to streptomycin than the donor; although the level was considerably higher than when the R factor was transferred to E. coli. These strains 931, 679, 1162 transferred double drug resistance by conjugation to a P. aeruginosa recipient 280 at frequencies of 10^{-2}, 10^{-3}, and 10^{-4} respectively. Two of the strains 1162 and 679 transferred to E. coli at much lower frequencies 10^{-7} and 10^{-8} respectively while 931 could not be demonstrated to do so. Transfer of drug resistance from P. aeruginosa to E. coli has been reported to occur with low frequencies (87) except in a few instances (36, 94). Stanish and his collaborators (91) have also described the transfer of R factors between P. aeruginosa strains.

The molecular nature of R factors originating in P. aeruginosa was first described by Grinsted et al (39). RP1 was a covalently closed circular DNA of molecular weight 40 million daltons and of buoyant density 1.719 g/cm^3 (60% GC). This R factor was freely transmissible between strains of E. coli, P. aeruginosa and P. mirabilis at a frequency between 10^{-3} and 10^{-5}. Analytical CsCl gradient centrifugation revealed a satellite band in E. coli and in Proteus mirabilis containing the R factor RP1; however no satellite band was seen in P. aeruginosa containing this R factor. This was stated to be due to insufficient resolution by their equipment. Using ethidium bromide-cesium chloride density gradient centrifugation, two molecules of closed circular RP1 DNA per chromosome equivalent was calculated for E. coli whereas in P. aeruginosa one molecule of covalently closed circular RP1 DNA per chromosome equivalent was estimated. In the profile of analytical CsCl gradient centrifugation, satellite DNA was about 12% of chromosomal DNA in P. mirabilis whereas in E. coli satellite DNA was about 5% of chromosomal DNA. Hence, there are more molecules of RP1 DNA per chromosome equivalent in P. mirabilis than in E. coli indicating that replication of RP1 DNA is more strictly controlled in P. aeruginosa than in P. mirabilis.

In P. aeruginosa, Grinsted's R factor RP1 transferred drug resistance at frequencies of 10^3 to 10^4 fold greater than the R factors 1162, 679 do to an E. coli or P. mirabilis recipient. One P. aeruginosa strain 931 did not transfer drug resistance to E. coli or P. mirabilis. However, these R factors 1162, 679 and 931 isolated by

Bryan et al (14) transferred resistance from P. aeruginosa to a P. aeruginosa recipient at frequencies of 1 to 10^{-4} whereas RP1 transferred resistance to a P. aeruginosa recipient at the same frequencies as it does to E. coli or P. mirabilis (i.e. 10^{-4}). The difference between the R factors described by Bryan and those of Grinsted appear to be that these R factors 1162, 679 and 931 are host restricted. As a result, these could be a unique group of R factors. Even within the P. aeruginosa mating system the R factors have an unusual behaviour. R factor 931 acts as a naturally occurring derepressed strain in the original host but undergoes significant decline in transfer to the recipient P. aeruginosa strain 280. It appears to act repressed in the recipient.

Besides this, there exists the medical importance of transfer of plasmids conferring multiple drug resistance especially when P. aeruginosa are widely distributed throughout nature.

MATERIALS AND METHODS

MATERIALS

Reagents: All chemicals were of reagent grade and were obtained from commercial suppliers. Ribonuclease and deoxyribonuclease (2400 units/mg) were obtained from Worthington Biochemical Corporation, Freehold, New Jersey, U. S. A. Brij-58 (polyoxyethylene (20) cetyl ether) was obtained from Atlas Chemical Industries, Wilmington, Delaware, U. S. A. Lysozyme, sodium lauryl sulfate, and ethidium bromide were obtained from Sigma Chemical Company, St. Louis, U. S. A. Cesium chloride was obtained from British Drug Houses Limited, Poole, England and from Fisher Scientific Company, New Jersey, U. S. A. ^{32}P and tritiated thymidine triphosphate were obtained from New England Nuclear, Boston, Massachusetts.

METHODS

A. Organisms and Genetic Transfer

1. Organisms

The following organisms originally obtained from clinical specimens were kindly provided by Bryan and coworkers. Recipient *P. aeruginosa* strains used in conjugations were rifampicin resistant derivatives of strain 280 (280 rif^r). Donor *P. aeruginosa* strains were 931,679, 1162, 5265, 716, 3819, 503 and 3108. *E. coli* K-12 F^- (ATCC 14948) was used as a recipient in intergeneric matings. RP4, another *P. aeruginosa* donor strain was kindly provided by G. A. Jacoby, Massachusetts General Hospital. The nomenclature used to designate R^+ recipients is the strain number followed by the number of the R factor. Thus strain 280 which has received the R factor from strain 931 is 280R931.

2. Media

Media used was Trypticase Soy (TS;BBL), and MacConkey agar (Difco).

3. Strain Characterization

The strains were characterized by Provincial Laboratory of Public Health using the methods of Cowan and Steel (23) except acetamide hydrolysis (16) and growth in triphenyl tetrazolium chloride (99).

4. Mating System

Mating mixtures were prepared with a 1:1 or 1:10 donor to recipient cell ratio. Recipient and donor strains were grown in TSB and adjusted to a cell density of 0.5 A_{600} unit (Beckman

DBG spectrophotometer) prior to mixing. Five ml of mating mixture was added to a 150 ml medicine bottle which was then positioned flat side down in a 37°C incubator. Mating was interrupted by vigorous agitation and the cell density was adjusted to 0.5 absorbance units at 600 nm with TSB.

5. Selection Methods

Selection was carried out after mating the donor x 280 rif^r and then by plating 0.1 ml of serial ten fold dilutions on TS agar containing rifampicin (100 ug/ml) and the drug to which resistance was transferred. For recipient strain 280 the concentration of streptomycin, tetracycline and gentamicin incorporated in the medium was 16, 5, 25, and 1 ug/ml respectively. Selection in intergeneric matings was carried out by mating donor x E. coli K-12 F^- and selecting with pyocines. Pyocines which were produced by the method of Farmer and Herman (34) were generously donated by H. Van Den Elzen, Department of Medical Bacteriology. Donor organisms were screened for initial sensitivity to pyocines by applying 25-uliter drops of undiluted pyocine to a lawn of test organisms on TS agar prepared exactly as for Kirby-Bauer disc antibiotic testing (8). The plates were incubated at 37°C for 18 and 42 hours and if fewer than 20 colonies grew in the pyocine drop zone that pyocine was considered satisfactory for selection provided it failed to inhibit the recipient strain.

For use in pyocine selection after mating, the pyocine was titrated with the donor and recipient strains. Volumes of 0.1 ml

containing 10^8 organisms were added to 1.9 ml volumes of serial twofold dilutions of the pyocine (in TS broth) and incubated in 50 ml Erlenmeyer flasks at 37°C for 20 minutes with vigorous shaking; 0.1 ml volumes were applied to TS agar and incubated as above. The pyocine dilution allowing growth of fewer than 20 colonies was used for selection after mating. E. coli was totally insensitive to the pyocine used.

Pyocine selection of the resuspended broth matings in TS broth (A_{600} = 0.5) was carried out by adding 0.1 ml of that material to 1.9 ml of the appropriate pyocine dilution selected as above and incubated as for the pyocine titration. Appropriately diluted 0.1 ml volumes were added to MacConkey agar containing streptomycin (16.5 ug/ml) and to MacConkey agar for plate counts. The plates were incubated anaerobically. In some cases before the final plating the E. coli mixture was concentrated 20 fold by centrifugation at 7000 x g. After selection by either method, about 10 colonies were picked and tested for antibiotic susceptibility by the disc method (8). Frequencies are based in the total number of donor cells present at the initiation of mating.

B. Growth in Medium Containing Drugs

P. aeruginosa strain 931 was serially cultured in broth containing 500 ug/ml of streptomycin and in broth containing 125 ug/ml of tetracycline.

C. Minimal Inhibitory Concentration Determinations

The test was done by convential tube dilution procedures.

The usual volume of medium was 2.5 ml (in 18 x 125 mm tubes) and the inoculum was 0.05 ml containing 1 x 10^5 organisms. The MIC was determined visually after incubation at 37°C for 15 hours.

D. Antigenic Classification of R Factors.

1. Preparation of Bacterial Antigen

Ten ml of P. aeruginosa strains 280R679, 280R931 and 280RP4 grown at 37°C to an absorbance of 0.5 at 600 nm (Beckman DBG spectrophotometer) were centrifuged for 10 minutes at 17,000 x g in the Sorvall RC2-B centrifuge. The cells were washed once in saline, and recovered as above. The pellet was resuspended in 3.0 ml of saline and subjected to ultrasonic vibration with a Bronson Biosonik III (0.95 cm probe) for 20 seconds.

2. Production and Preparation of Antibodies

A mature rabbit was injected intramuscularly with 3.0 ml of a sonicated bacterial suspension. At weekly intervals for 3 weeks the rabbit was injected with another 3.0 ml of sonicated bacterial suspension. On the fourth week after the first inoculation, three times the normal amount of sonicated bacteria was injected into the rabbit. A week later a sample of blood was removed from the rabbit and the antibody titre was measured by making doubling dilutions of the serum with a final volume of 0.5 ml. One-half ml of the respective hybrid bacterial strain having an A_{600} of 0.2 to 0.3 was added. The tubes were incubated at 50°C for 1 hour, then kept at 4°C overnight. The titre of a serum, read the following morning was the highest dilution with visible or microscopic agglutination.

If the antibody titre was less than 1/640 at this time three to four times the amount of sonicated bacteria previously used was injected and the antibody titre was determined one week later after injection. Once there was a sufficient antibody titre, the rabbit was bled, the serum collected and absorbed with the recipient strain 280. Twenty ml of P. aeruginosa strain 280 having an absorbance at 600 nm of approximately 0.25 were centrifuged at 14,000 x g in the Sorvall RC2-B for 10 minutes and the packed cells were resuspended in 1.0 ml of serum. This mixture was incubated for 2 hours in a water bath with vigorous shaking and centrifuged as just described. The serum was decanted into the sedimented bacteria from another 20 ml of broth and incubated as above. Next the mixture was kept at 4°C for 1 to 7 days and then centrifuged at 28,000 x g for 1 hour in the Sorvall RC2-B centrifuge. To determine if all the antibodies directed against the antigens of 280 were removed, an agglutination reaction was performed using 0.1 ml of a 1/10 dilution of the absorbed serum in TS broth and 0.9 ml of P. aeruginosa donor strain and recipient strain 280. The tubes were incubated at 37°C in a water bath with vigorous shaking for 30 minutes then kept at 4°C overnight. The following morning the agglutination reaction was determined. This procedure of incubation, refrigeration and centrifugation was repeated until there was no microscopic agglutination of the serum with strain 280.

3. Serological Test for Grouping of R Factors

The same method was used as described previously for detecting the presence of antibody directed against strain 280 in absorbed serum.

E. Analytical CsCl Gradient Centrifugation

1. Growth Conditions

The cultures were grown in TS broth overnight at 37°C in a water bath shaker. Stationary phase cells had an absorbance at 600 nm of at least 3.0 (Beckman DBG spectophotometer) whereas exponential phase cells had an absorbance between 0.3 and 0.5.

2. Isolation of DNA

DNA was prepared by the method of Cohen and Miller (20). At the appropriate stages of growth approximately 5×10^{10} cells were centrifuged at 17,000 x g for 8 minutes in a Sorval RC2-B centrifuge. The packed cells were washed twice in 0.05 M Tris (pH 8.0) and resuspended in 10.0 ml of 25% sucrose in 0.05 M Tris HCl, 1.0 ml of freshly prepared lysozyme (10 mg/ml) and 2.0 ml EDTA (0.5 M, pH 8.2). Following incubation for 10 minutes at 25°C, the resulting spheroplasts were treated with Brij-58 to produce a final concentration of 0.5% (w/v). Lysates were then incubated with ribonuclease (final concentration 50 ug/ml) at 37°C for 60 minutes. Following the incubation sodium dodecyl sulfate was added to a final concentration of 0.2% (w/v). DNA was extracted three times with phenol that had been redistilled and subsequently equilibrated with 2 x st. sal. cit. (standard saline citrate is 0.15 M NaCl, 0.015 M sodium citrate). The

extracted DNA was dialyzed against 2 x st. sal. cit. containing 0.01 M EDTA, pH 8.0, prior to use for centrifugation studies.

3. Centrifugation of DNA

Centrifugations were performed in a Beckman Model E analytical ultracentrifuge equipped with a standard u.v. light source. A charcoal filled Epon centerpiece, 12 mm, 4^{o} single-sector was used. In the lower position was a quartz window and in the upper position was a 1^{o} negative wedge. The cell was torqued to 120 inch pounds. The same cell was used for every run and it was filled with 0.7 ml of a solution containing CsCl in water and 5 ug of DNA per ml. Concentrations of DNA were estimated spectrophotometrically, assuming that an absorbance of 20 at 260 nm is equivalent to a concentration of 1 mg/ml. The density of the solution determined pycnometrically was 1.71 ± 0.02 g/ml. An An-D rotor was used. The speed control was set at 44,000 and in most cases speed was checked by the revolution counter over an extended period of time with an electronic timer. Centrifugation was carried out for 25-30 hours at 25^{o}C. Films were developed using routine methods under standard condition and were traced by a Beckman analytrol densitometer with film attachment.

4. Calculation of Buoyant Density and GC Content

Densities were calculated by using the position of standard DNA as a reference (82). The compositional density gradient was then used in the following equation to determine the buoyant density of the DNA at a distance r from the center of rotation.

$$\rho = \rho_o + 4.2w^2 (r^2 - r_o^2) \times 10^{-10} \text{ g/cm}^3$$

ρ_o = density of standard DNA

w = speed of rotation in radians sec^{-1}

r_o = distance of standard DNA from center of rotation

Chromosomal DNA of E. coli and Proteus morganii were used as density markers both having a buoyant density of 1.710 g/cm^3 (92).

A linear relationship exists between buoyant density and GC content (55,75,92). Based on a value of 1.710 g/cm^3 for DNA from E. coli the following relationship is obtained (82).

$$\rho = 1.660 + 0.098(GC)$$

ρ refers to buoyant density and GC to the mole fraction of guanine plus cytosine.

F. Preparative CsCl Density Gradient Centrifugation

1. Growth Conditions for Labelling DNA with Tritiated TTP

To label DNA of P. aeruginosa with tritiated TTP the cells were grown in minimal medium (70). The composition of the medium is as follows:

Base:

$NH_4H_2PO_4$	3 g/l
$FeSO_4 \cdot 7H_2O$	0.5 mg/l
K_2HPO_4	3 g/l
H_2O	850 ml

The pH of the base was adjusted to 7.4 with 5N KOH and then sterilized. For the complete medium fifty ml of sterilized

glucose and fifty ml of sterilized $MgSO_4 \cdot 7H_2O$ was added to one liter of the base. The concentrations of glucose and $MgSO_4 \cdot 7H_2O$ were 100 g/l and 10 g/l respectively.

2. Labelling DNA with Tritiated TTP

An attempt to label DNA from P. aeruginosa with tritiated TTP was made using the method of Moses and Richardson (63). Slight modifications of the method were made. Strain 931 was grown in minimal medium until the A_{600} (Beckman DBG spectrophotometer) was 0.3. Fifteen ml of these cells were centrifuged in an RC2-B centrifuge for 10 minutes at 17,000 x g. The sediment was resuspended in 3 ml of 0.05 M potassium phosphate buffer (pH 7.4) and agitated for 10 minutes at 37°C with 1% toluene. The reaction mixture (6.0 ml) contained 70 mM potassium phosphate buffer (pH 7.4) 0.13 mM $MgCl_2$, 1.3 mM ATP, 3.7 uM (^3H)-TTP (4.5mCi), 33 uM of dGTP, dTTP, dCTP, and 1.5×10^9 toluene-treated cells. After 15 minutes incubation at 37°C, 33 uM of cold TTP was added and the mixture was incubated for another 30 minutes. The cells were then washed twice in 0.05 M potassium phosphate buffer (pH 7.4) and resuspended in 0.4 ml of a spheroplast forming mixture (1mg of lysozyme and 100 mg sucrose per ml of TES buffer (0.05 M Tris HCl, 0.005 M EDTA, 0.5 M NaCl pH 8.0)). After incubation of the mixture for 10 minutes, 1.6 ml of 10% cold TCA-0.1 M PPi was added. Then the sample was divided in half. One sample served as a control for the total amount of label present, whereas the other was assayed for DNase sensitivity. The latter sample was neutralized with 0.02 N NaOH, and 1 ug/ml DNase was added.

After overnight incubation at 37°C, 2 ml of cold 10% TCA-0.1 M PPi was added. Then the DNase treated and untreated samples were filtered through a Whatman GF/A glass filter (2.4 cm) and washed three times with 3.0 ml of cold TCA-PPi, followed by three washes of three ml each with cold 0.01 M HCl. After the filters were dried, they were placed in counting vials containing 5.0 ml of toluene scintillation fluid (4.0 g PPO, 0.1 g POPOP per liter of toluene) and counted in a Beckman model LS-250 liquid scintillation counter.

3. CsCl Density Gradient Centrifugation of Tritiated TTP Labelled DNA

The same procedure was followed as described above for labelling DNA and for the formation of spheroplasts. However after ten minutes of incubation at 37°C, the mixture was cooled in an ice bath for five minutes. Subsequently 0.2 ml of 2% sarkosyl was added and the mixture was drawn in and out of a pipette, four to five times. To 0.3 ml of the sheared lysate, crystalline CsCl and water was added till the final volume was 4.0 ml and the buoyant density was 1.71 $\pm$ 0.03 g/cm^3. The buoyant density was determined pycnometrically. Lysates were then centrifuged in a SW 56 rotor in a Beckman L-2 preparative ultracentrifuge.

4. Growth Conditions for Labelling DNA with ^{32}P

For labelling DNA with ^{32}P, P. aeruginosa strain 931 was grown in low phosphate medium (LPM) according to a method modified by Cheng et al (17).

NH_4Cl	0.02 M
KCl	0.02 M
NaCl	0.02 M
Tris HCl	0.12 M
Glucose	0.5%
Bactopeptone (Difco)	0.5%
$MgSO_4$	0.0016 M

The pH was adjusted to 7.5, then sterilized. Various concentrations of phosphate i.e. 1×10^{-3} M, 5×10^{-4} M, 2.5×10^{-4} M, 5×10^{-5} M were added to the low phosphate broth to determine the optimum growth conditions.

5. Uptake of ^{32}P from the Medium

The uptake of ^{32}P by P. aeruginosa was determined by adding 5 uC/ml to a diluted culture in low phosphate broth having an absorbance of 0.1 at 600 nm. At intervals of an hour 0.2 ml samples were removed and centrifuged in a Beckman 152 Microfuge for 5 minutes. Ten microliters of supernatant was added to 5.0 ml Brays scintillation fluid (13) and counted in a Beckman Model LS-250 liquid scintillation counter.

6. Labelling of cultures with ^{32}P

Cultures to be labelled with ^{32}P-phosphate were grown overnight in low phosphate medium. In the morning the culture was diluted to an absorbance of 0.05 at 600 nm (Unicam SP 1800 ultraviolet spectrophotometer) and radioactive phosphate (12.5 uC/ml) was added. The culture was incubated at 37°C in a water bath with vigorous shaking until the A_{600} of the culture reached

0.5

7. Lysis of Radioactive Culture and Ethidium Bromide-CsCl Gradient Centrifugation

Direct dye-buoyant density centrifugation of labelled lysates was carried out by the method of Bazaral and Helinski (9) Six ml of labelled cultures were washed twice in TES buffer (0.05 M NaCl-0.05 M Tris- 0.005 M EDTA pH 8.0) at 4°C. The pellet was resuspended in 0.4 ml of a spheroplast-forming mixture (1 mg of lysozyme, 500 ug of ribonuclease and 100 mg of sucrose per ml of SET buffer (0.05 M Tris,0.015 M NaCl, 0.1 M EDTA pH 8.0)) and incubated at 37°C for 10 minutes without shaking. The spheroplast preparation was cooled in an ice bath for 5 minutes. Subsequently the spheroplasts were lysed to release DNA by adding 0.2 ml of 2% sarkosyl, and gently mixed by drawing the preparation through a 1 ml pipette. An additional 0.4 ml of SET buffer was then added and the DNA was sheared by slowly drawing in and out of the pipette (20 times). A 0.8 ml amount of the sheared lysate was added to 1.6 ml ethidium bromide solution (700 ug ethidium bromide/ml of phosphate buffer pH 7.0) and 3.0 ml of water. Crystalline CsCl (5.2 g) was added and, after mixing by inversion the solution was transferred to a cellulose nitrate tube and overlaid with sufficient light mineral oil to fill the tube. Samples were centrifuged for 60 hours at 40,000 rpm at a temperature of 20°C in a Ti-65 fixed-angle centrifuge rotor using a Beckman Spinco model L2 preparative ultracentrifuge. Gradients were then fractionated by piercing the

bottom of the tube and collecting 60-70 fractions for each tube.

8. Estimation of Radioactivity of ^{32}P Labelled DNA

The collected fractions were digested with 2 M NaOH at 37°C for at least five hours, neutralized, and then 20 ug of yeast RNA was added to each sample to act as a carrier. Subsequently the DNA was precipitated with 10% trichloroacetic acid, and filtered on Whatman's GF/A glass fiber filters. The filter papers were placed in an oven at 80°C until they were dry. The filters were then washed three times by dropping them into 20 ml of 1% trichloroacetic acid (v/v) for fifteen minutes. After the papers were again dried in an oven at 80°C they were placed in counting vials containing 5 ml of toluene scintillation fluid (4 g PPO, 0.1 g POPOP per liter of toluene) and counted in a Beckman model LS-250 liquid scintillation counter.

G. Electron Microscopy of R Factor DNA

R factor DNA was isolated for electron microscopy by dye-buoyant density centrifugation of cell lysates. DNA from 3 or 4 pooled samples of the denser satellite band characteristic of covalently closed circular molecules was dialyzed against 500 ml of 0.15 M ammonium acetate (pH 7.0) for four to five hours at 4°C. After dialysis single strand scissions were introduced into the DNA molecule by storage for seven days at 4°C or by DNase I treatment. For DNase I treatment 1-2 ug/ml DNA was incubated at 37°C with 0.1 ng/ml pancreatic DNase for 20 minutes. Grids for electron microscopy were prepared by the technique of

Kleinschmidt (47). A protein and nucleic acid solution was prepared containing 2 ug of DNA per ml, 100 ug cytochrome c per ml and ammonium acetate having a final concentration of 1 M. This solution was floated down a glass slide which served as a ramp leading to the surface of a trough containing 0.25 M ammonium acetate. The extent of the protein film which formed was visualized by putting a few talcum particles on the subphase (0.25 M ammonium acetate) near the ramp. These were pushed along by the film as it spread out showing the boundaries of the film. Grids of 3 mm diameter covered with polyvinylformaldehyde were touched horizontally to the protein-nucleic acid monolayer on the surface of the trough. Using forceps the grids were removed from the monolayer and without delay was placed for approximately 30 seconds in 5% uranyl acetate dissolved in 95% methanol, followed by two washes in 95% ethanol.

Photographic negatives of the R factor were projected on a screen and the contour lengths were measured using a map measurer.

RESULTS

A. Host Ranges of P. Aeruginosa R Factors

Table III demonstrates the ability of P.aeruginosa to act as a more effective recipient than E. coli. In strains 931 and 3108, the frequency of transfer of P. aeruginosa R factors to P. aeruginosa recipient strain 280 is at least 10^7 fold higher than with an E. coli recipient. In the case of 679 and 5265, the difference in mating frequency between an E. coli or P. aeruginosa strain 280 as recipients is much less pronounced. This restriction of the transfer of R factors by the host was not observed by Datta et al. for the R factor RP4 (25). Thus P. aeruginosa R factors especially 931 and 3108 appear to represent a separate group of R factors with a different host range than that of previously described R factors. Further evidence supporting host restriction has been presented by Bryan et al (14,15). R factors 931 and 3108 have never been observed to transfer to an E. coli recipient.

B. Serologic Grouping of P. Aeruginosa R Factors

An attempt to classify R factors in P. aeruginosa was made by serological studies. Antisera which were produced against R factors 679 and 931 in strain 280 were absorbed with strain 280 as described. Little success was obtained in producing antibodies to the R factors RP4 and 5265 as agglutination titres were consistently less than 1/100. Using the two absorbed antisera, three different antigenic classes of R factors resulted as shown in Table IV. Group I R factors reacted only with absorbed 280R931 antiserum; Group II reacted

TABLE III. Frequency of transfer of *P. aeruginosa* R factors to *E. coli* and *P. aeruginosa* recipients.

Donor *P. aeruginosa* strain	Recipients	
	E. coli	*P. aeruginosa* strain 280
931	$< 5 \times 10^{-8}$	0.5
3108	$< 5 \times 10^{-8}$	0.1
679	2×10^{-7}	5×10^{-6} to 2.5×10^{-4}
5265	2×10^{-8}	2×10^{-5}
280RP4	9×10^{-5}	1×10^{-4}
280R931	$< 5 \times 10^{-8}$	1×10^{-4}

only with absorbed 280R679 antiserum and Group III reacted with neither antiserum. Compatability studies carried out by Bryan et al revealed that 3108 and 931 belong to the same compatability group. Hence these compatibility results conflict with the results obtained by serological studies. Due to this problem, further serological studies were not pursued.

C. Additional Studies on the P. Aeruginosa R Factors

In addition to the property of host restriction P. aeruginosa R factor 931 has been shown to transfer resistance at a high frequency in strain 931 (Table III) and in recipient strain 1310 (15). This is an unusual property as most other R factors transfer at a low frequency (25,26). However R931 exhibits more irregular behaviour in that in recipient strain 280 it transfers at a low frequency (Table III). Since R931 is an anomalous R factor the study of it and its relationship to other P. aeruginosa was deemed necessary. Also R931 represents a significant group of Pseudomonas R factors. Four of seven P. aeruginosa R factors examined belonged to this group (15).

D. Analytical CsCl Gradient Centrifugation

Densitometer traces of photographs taken after CsCl gradient centrifugation from strain 280 showed only a single peak (Fig. 1a). Strain 931 exhibited a satellite peak of 1.718 g/cm^3 in addition to the major peak of chromosomal DNA (Fig. 1b). Following mating of strains 931 and 280, analytical CsCl gradient analysis revealed a satellite band of density 1.718 g/cm^3 or 59% GC (Fig. 1c, Table VI). Thus, the acquisition of R factor 931 by 280 was accompanied by the appearance of

TABLE IV. Serological grouping of P. aeruginosa R factors based on agglutination reactions.

Group	R factor	Agglutination with antisera to	
		280R679	280R931
I	931	-	4+
	3819	-	4+
II	679	4+	-
	1162	4+	-
	3108	4+	-
III	503	-	-
	716	-	-
	5265	-	-

The antisera were diluted 1/100 in TS broth.

Agglutination reactions were graded from 1+ to 4+

1+ indicates slight agglutination

4+ indicates strong agglutination

- indicates no agglutination

satellite DNA. Using the chromosomal DNA of E. coli and P. mirabilis as markers the buoyant density for the genome from 931 and 280 was determined to be 1.7255 g/cm^3 or 67% GC (Table V). This value agrees with values generally obtained for P. aeruginosa strains (53).

Equilibrium centrifugation profiles of DNA isolated from three other P. aeruginosa strains are seen in Figs. II and III. Strains 3108 and 5265 exhibit a satellite band at 1.716 g/cm^3 and 1.718 g/cm^3 respectively (Fig. IIa and IIb, Table VI). However no satellite band was seen in P. aeruginosa strain 280R679 (Fig. III). After mating strain 280R679 with E. coli K-12 F$^-$ (ATCC 14948) a satellite peak could be demonstrated having a buoyant density of 1.719 g/cm^3 (Fig. IIIb, Table VI). Figure IIIa shows no such satellite peak in E. coli K-12 F$^-$ prior to conjugation. This situation is similar to that described by Grinsted et al. (39) for R factor RP4. No satellite band was detected in traces of photographs after CsCl gradient centrifugation of DNA from P. aeruginosa strains containing RP1 but was seen in E. coli strains containing the R factor. Furthermore the buoyant densities of RP1 and R679 are the same. The apparent absence of a satellite of density 1.719 g/cm^3 after analytical CsCl gradient centrifugation of DNA from strain 280R679 could be due to insufficient resolution by our equipment.

R factors 931 and 5265 have buoyant densities similar to the value obtained by Grinsted et al. (39) for R factor RP1. Finley and Punch (35) described two satellite bands for a P. aeruginosa strain 31. The R factors 931 and 5265 have the same buoyant density as one

FIGURE I.

Analytical CsCl gradient centrifugation of DNA from P. aeruginosa strains. Samples of DNA were centrifuged to equilibrium in CsCl and then ultraviolet photographs were taken and traced with a densitometer.

Trace (a) DNA from strain 280;

Trace (b) DNA from strain 931;

Trace (c) DNA from strain 280R931.

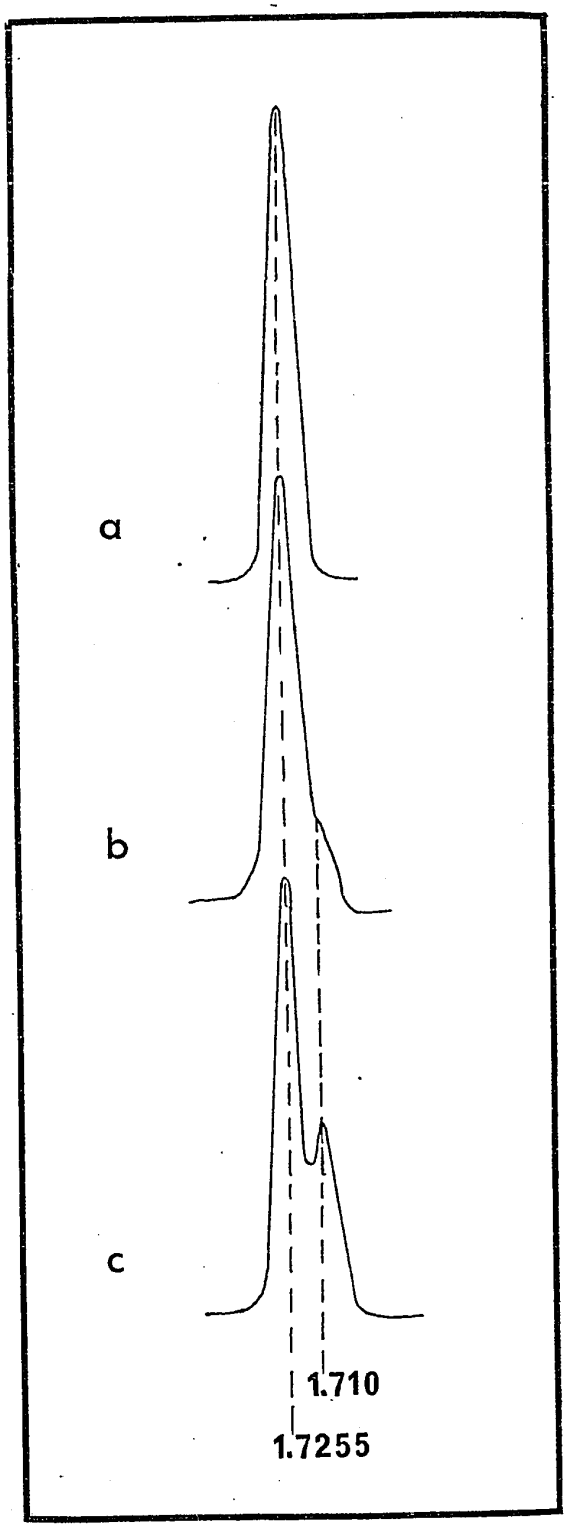

a
b
c
1.710
1.7255

TABLE V. Buoyant density of chromosomal DNA from P. aeruginosa

Strain	Buoyant Density (g/cm^3)
280	1.7255 ± 0.001
931	1.7255 ± 0.0005
3108	1.726 ± 0.001

The values shown for buoyant density indicate two standard deviations.

TABLE VI. Buoyant density of R factor DNA from P. aeruginosa

R factor	Buoyant Density (g/cm^3)
931	1.718 ± 0.0005
5265	1.718 ± 0.001
3108	1.716 ± 0.001
679	1.719 ± 0.001
931 - 126 transfers in streptomycin[a]	1.718 ± 0.0005
931 - 126 transfers in tetracycline[a]	1.718 ± 0.0005

a - see methods for procedural details

The values shown for buoyant density indicate two standard deviation.

FIGURE II.

Analytical CsCl gradient centrifugation of DNA from strains of P. aeruginosa.

Trace (a) DNA from strain 3108, and E. coli which was used as marker.

Trace (b) DNA from strain 5265.

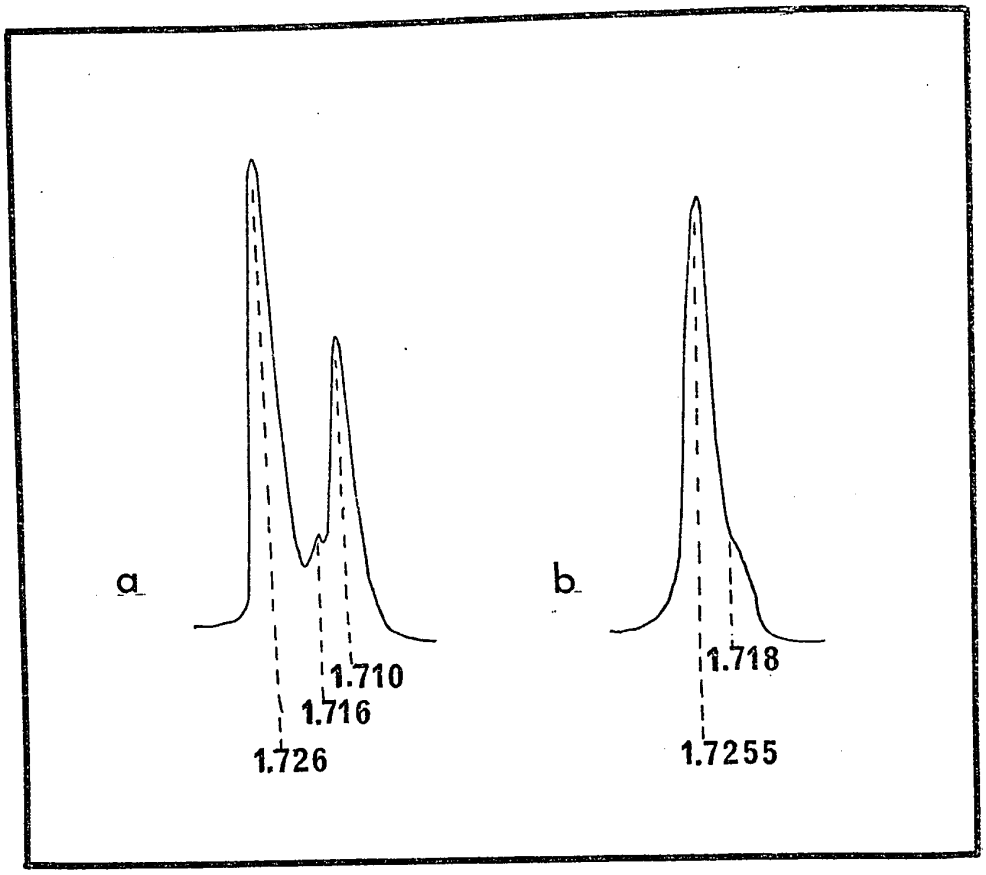

a
1.726
1.716
1.710
b
1.7255
1.718

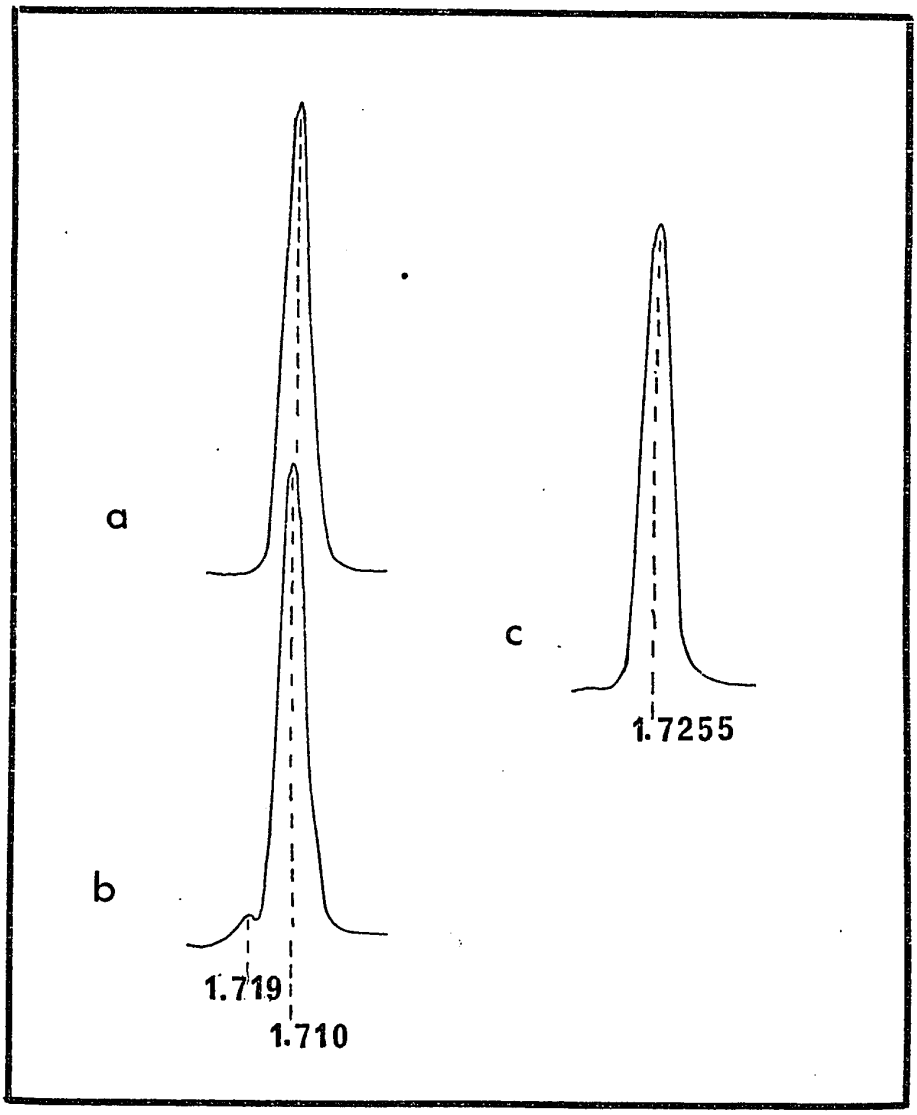
FIGURE III.

Analytical CsCl gradient centrifugation of DNA from *E. coli* and *P. aeruginosa*.

Trace (a) DNA from *E. coli* K-12 F^-;

Trace (b) DNA from *E. coli* R679;

Trace (c) DNA from *P. aeruginosa* strain 280R679.


a
b
c
1.719
1.710
1.7255

of the satellite bands of strain 31, i.e. 1.718 g/cm^3. A second satellite band was not detected with either strain 931 or 5265.

A buoyant density of 1.716 g/cm^3 for 3108 is surprising because compatability studies suggest that R931 and R3108 are members of the same compatability group (15).

E. Labelling DNA with ^{3}H-TTP.

Moses and Richardson (63) reported DNA synthesis in E. coli treated with toluene. Such cells maintain many of their physiological functions but have become permeable to deoxyribonucleoside trisphosphates. Although these cells are no longer viable, it has been possible to obtain extended semi-conservative replication, and to distinguish this process from a repair type synthesis. DNA in P. aeruginosa cannot be selectively labelled with either thymine or thymidine.[1] Therefore, an attempt was made to label R factor DNA with ^{3}H-TTP. When P. aeruginosa strain 931 was treated in a similar manner to that described above for E. coli to obtain labelled DNA, there was 10,097 cpm in the cell lysate that was DNase sensitive (Table VII). However preparative CsCl gradient centrifugation of these lysates revealed no labelled peaks. Thus, there was no detectable DNA labelled by this method. This could be due to differences in the cell wall between P. aeruginosa and E. coli. Under toluene treatment, the P. aeruginosa cell wall may not become permeable to nucleotides.

---

1 Unpublished experiments, Bryan L.E.

TABLE VII. DNase sensitive counts in strain 931 after treatment with toluene and exposure to ^{3}H-TTP.

	cpm/3.0 ml
Before DNase treatment	21,615
After DNase treatment	11,518
Net	10,097

Toluene treated strain 931 cells were incubated in reaction mixture containing phosphate buffer, Mg^{++}, ATP, dATP, dGTP, TTP, dCTP, and ^{3}H-TTP. After incubation the cells were washed and lysed. Then an aliquot of the cell lysate was treated with DNase. TCA was added to the treated and untreated cell lysate. The samples were filtered and after three washes, the filters were dried and the radioactivity measured.

F. Growth Conditions for Labelling DNA with ^{32}P.

Figure IV. shows the growth curve of strain 931 obtained by the addition of various concentrations of Na_2HPO_4 to low phosphate growth medium. Of the various added phosphate concentrations the addition of 1×10^{-3} sodium phosphate buffer (pH 7.4) to the medium produced maximal growth of strain 931. This concentration of sodium phosphate was chosen for labelling DNA with ^{32}P because it allowed growth of strain 931 to 2.5 A_{600} unit before a phosphate deficiency occurred which limited growth.

G. Uptake of ^{32}P from the Medium.

The uptake of ^{32}P by strain 931 grown in low phosphate medium is seen in Table VIII. Labelling studies of DNA were carried out in log phase cells which have an $O.D._{600}$ of 0.5. In order to increase the specific activity of the labelled DNA, the amount of ^{32}P was increased 2.5 fold over the conditions shown in Table VIII to 12.5 uC per ml.

H. Ethidium Bromide-CsCl Gradient Centrifugation.

Covalently closed circular DNA can be separated from other conformations of DNA on ethidium bromide-CsCl gradient centrifugation. This observation has been used to isolate R factor DNA . Lysates of P. aeruginosa strain 931 labelled with ^{32}P were analyzed by ethidium bromide-CsCl gradient centrifugation. Figure V illustrates a major band and a minor peak on the more dense side of the main band indicating the DNA was in the covalently closed circular form. In several repeated trials, the ratio of radioactivity in the satellite

FIGURE IV.

Growth curve of strain 931 in low phosphate medium containing various phosphate concentrations.

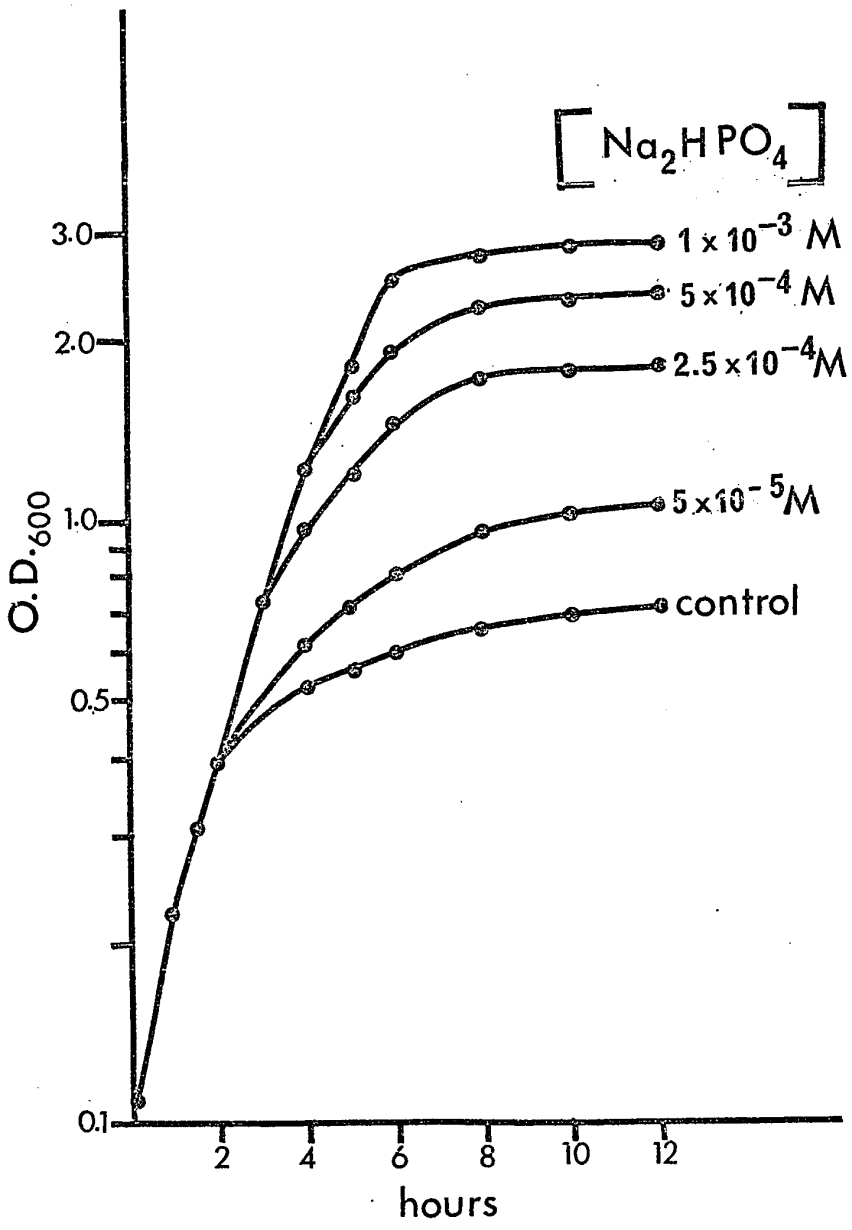

[Na2HPO4]
1 x 10-3 M
5 x 10-4 M
2.5 x 10-4M
5 x 10-5M
control
O.D.600
3.0
2.0
1.0
0.5
0.1
2
4
6
8
10
12
hours

TABLE VIII. Amount of ^{32}P remaining in the supernatant at various times of growth of P. aeruginosa strain 931.

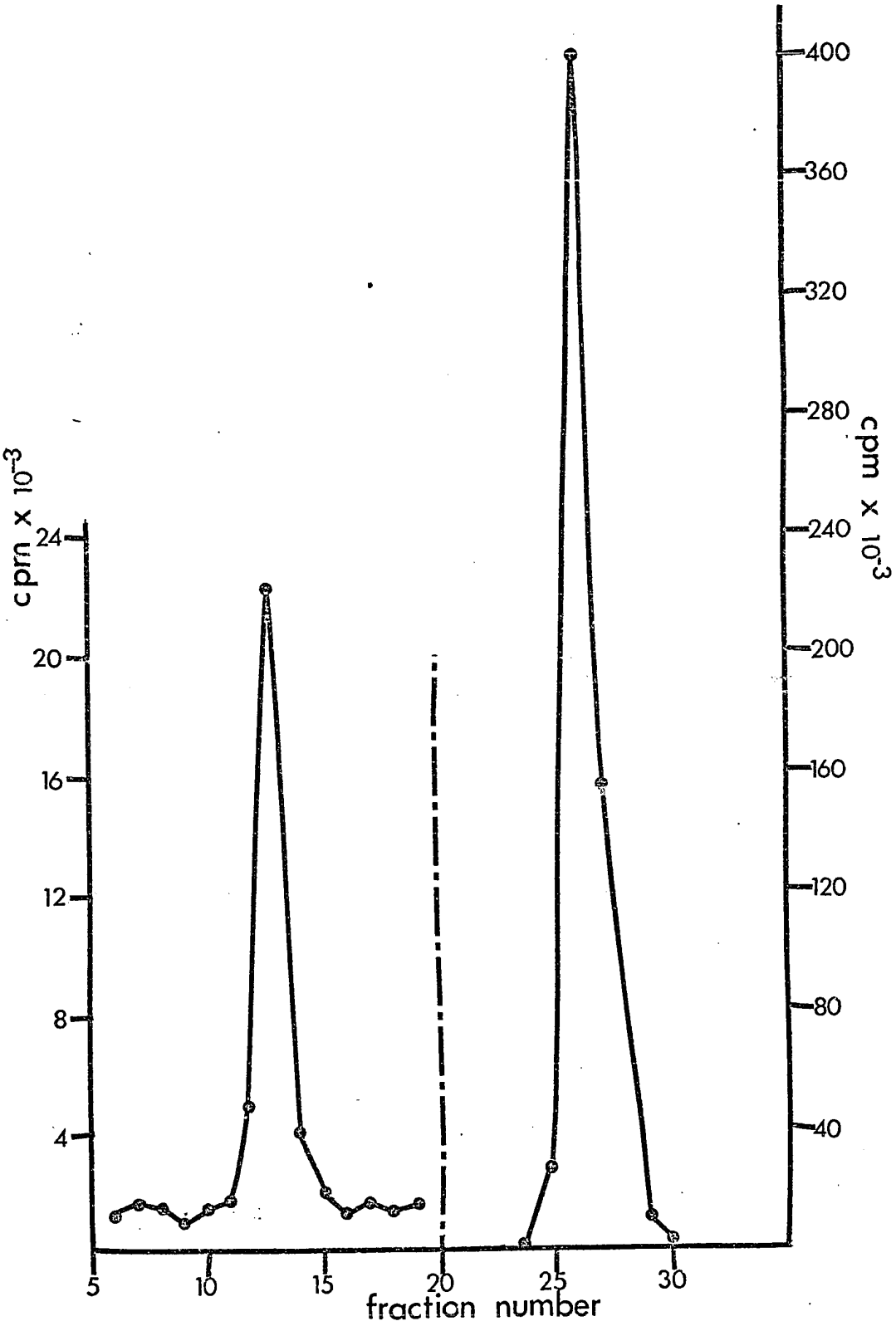
Time (hr)	$O.D._{600}$	Cpm	% of cpm remaining in supernatant
0	0.09	133,264	100
2	0.22	117,098	87.9
4.5	0.65	89,226	67.0
6.5	1.80	15,053	11.3
8.5	2.72	3,775	2.8

Strain 931 was grown in LPM containing ^{32}P. At regular intervals, 0.2 ml samples were removed and centrifuged. Ten ul of supernatant was added to Bray's scintillation fluid and counted.

FIGURE V.

Ethidium bromide-CsCl gradient centrifugation of DNA from strain 931.

Preparative centrifugation of strain 931 DNA labelled with ^{32}P, isolated by detergent lysis, and centrifuged in CsCl in the presence of ethidium bromide. Note the change of scale at fraction 20.


cpm x 10^{-3}
24
20
16
12
8
4
400
360
320
280
240
200
160
120
80
40
cpm x 10^{-3}
5
10
15
20
25
30
fraction number

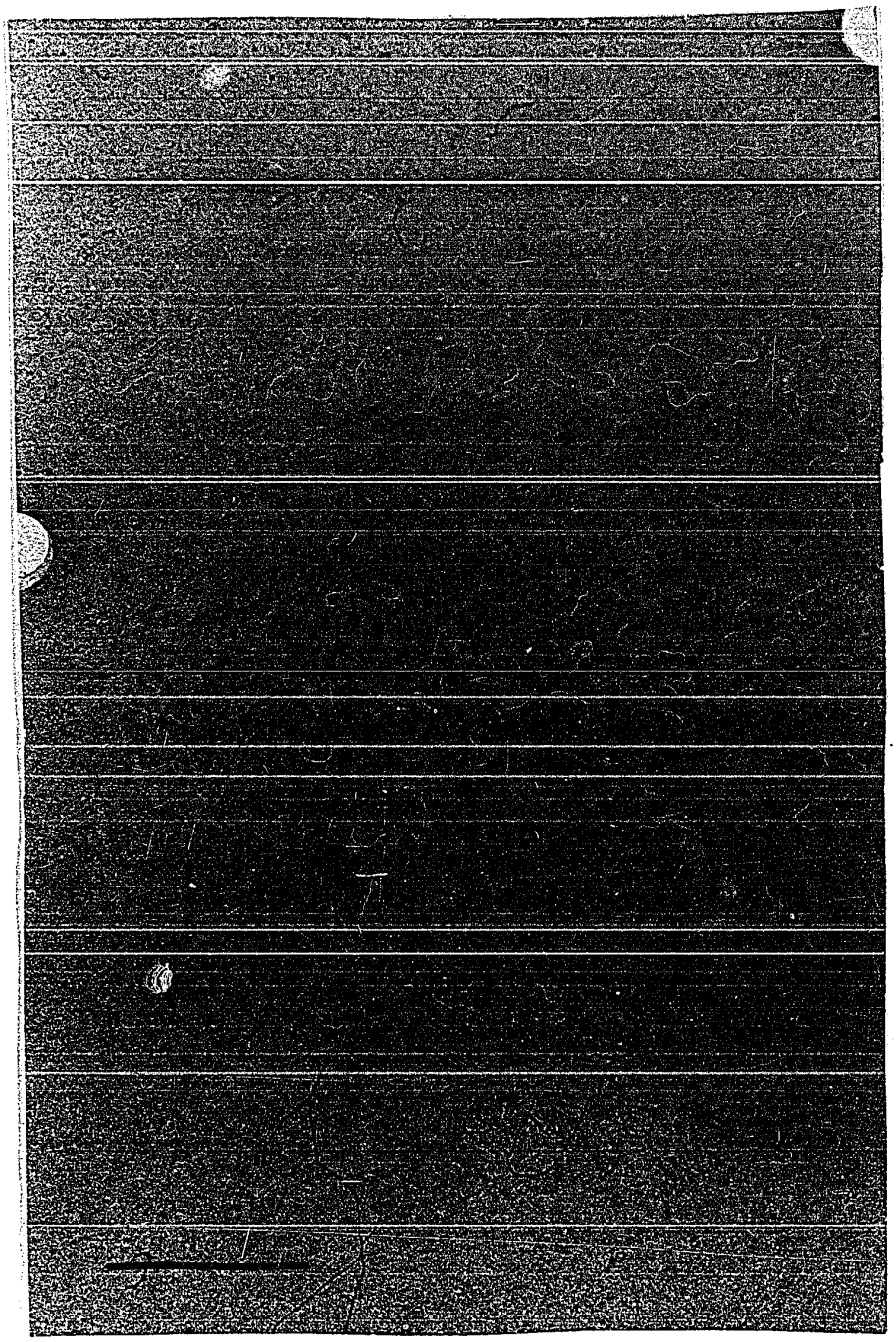
band to that in the main band was 5.5%.

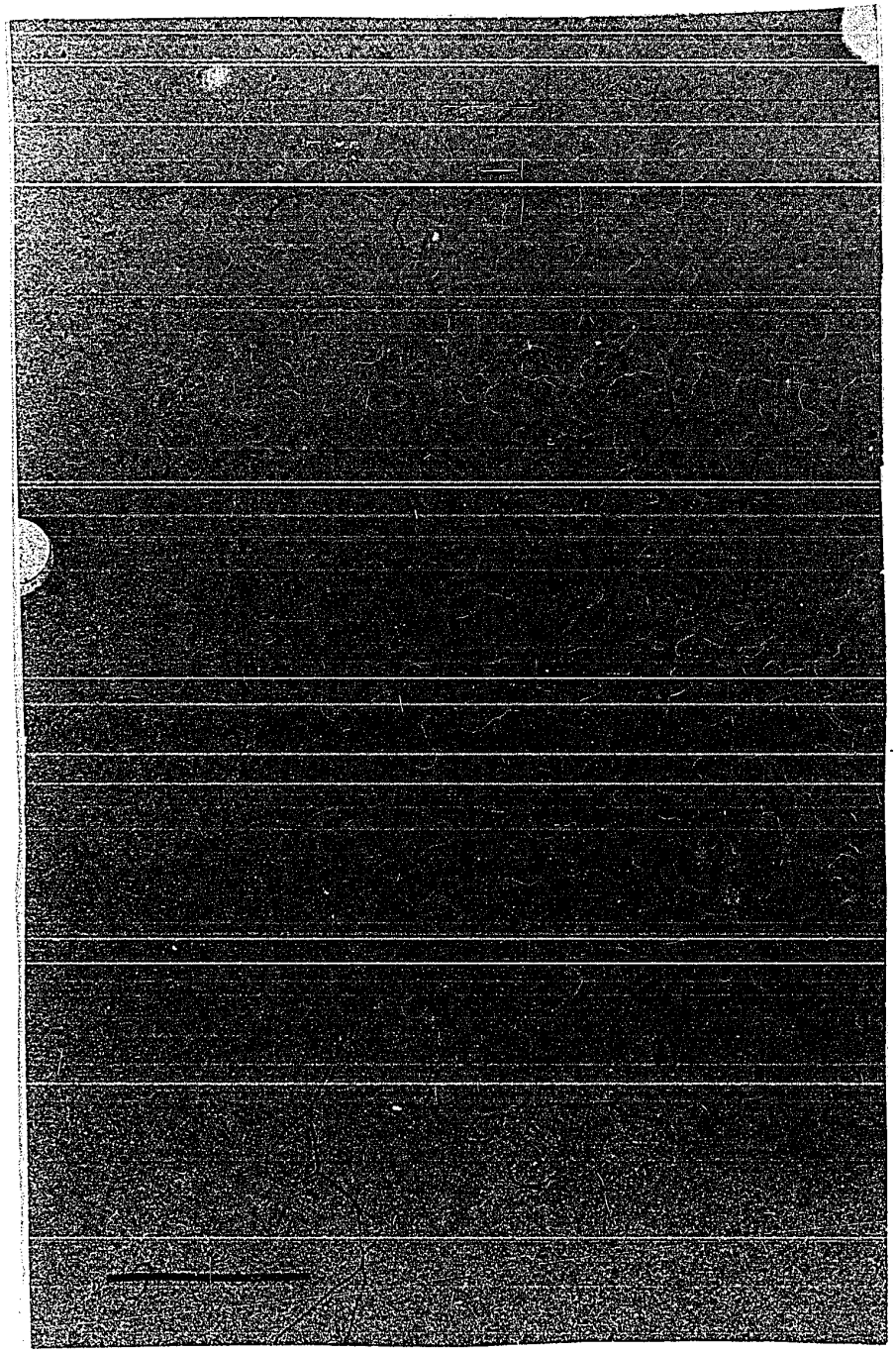
I. Electron Microscopy

Electron microscopic examination of R factor DNA revealed two distinct sizes of DNA molecules (Plates I to III). Before DNase I treatment or storage, the majority of the molecules were super-twisted forms of DNA (Plate I and Plate IIIa) whereas after treatment with DNase I or storage for seven days at $4^{o}C$ most of the molecules were converted to the open circular form (Table IX, Plate II and Plate IIIb). The small molecule of DNA is distinguished from fragments of chromosomal DNA by the method used in isolation, the uniformity of length, the presence of forms intermediate between covalently closed circular and open circular, and the effect of DNase I (Table IX).

In Table X, the lengths of the two major sizes of DNA as determined by measuring the contour length of projected photographic negatives. Based on a value of 2.07×10^6 daltons/um (51), the larger molecule has a molecular weight of 25×10^6 daltons and the smaller molecule 1×10^6 daltons. The relative proportion of the sizes is about 3.6 to 4.6 small DNA molecules to 1 large DNA molecule (Table X). Estimations of the relative proportions of the DNA molecules is subject to error because the larger molecule is probably more susceptible to nicking and thus fewer molecules would be isolated by ethidium bromide-CsCl gradient centrifugation. Occasional circular molecules about 35 um, 1.0 um, and 1.5 um in size have been detected.

The relationship between the two major molecules is at present unclear. The small size of the 0.5 um molecule and the error





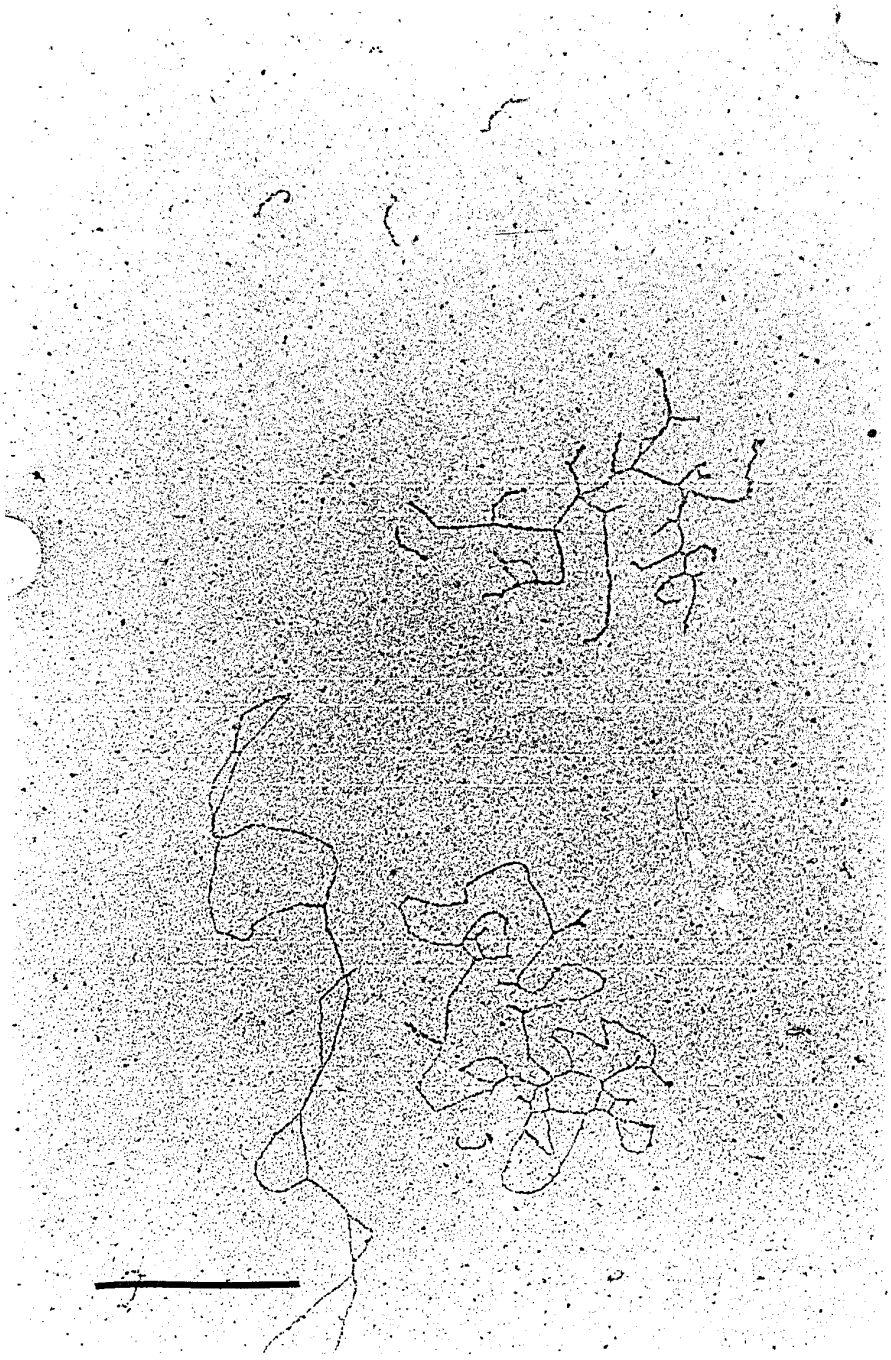
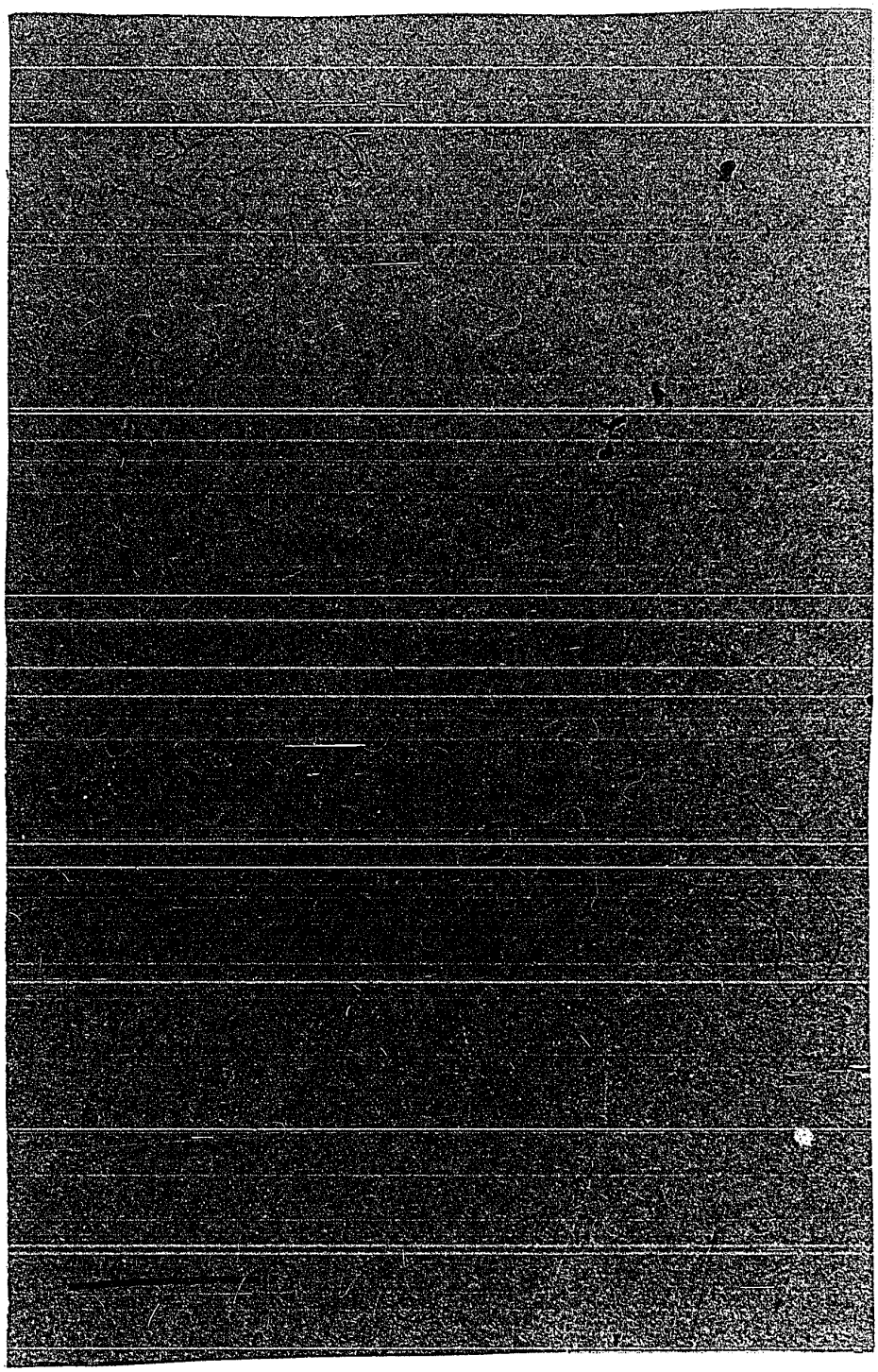


PLATE I.

Electron micrographs of twisted circular molecules isolated from strain 931. Bar represents 1 um.



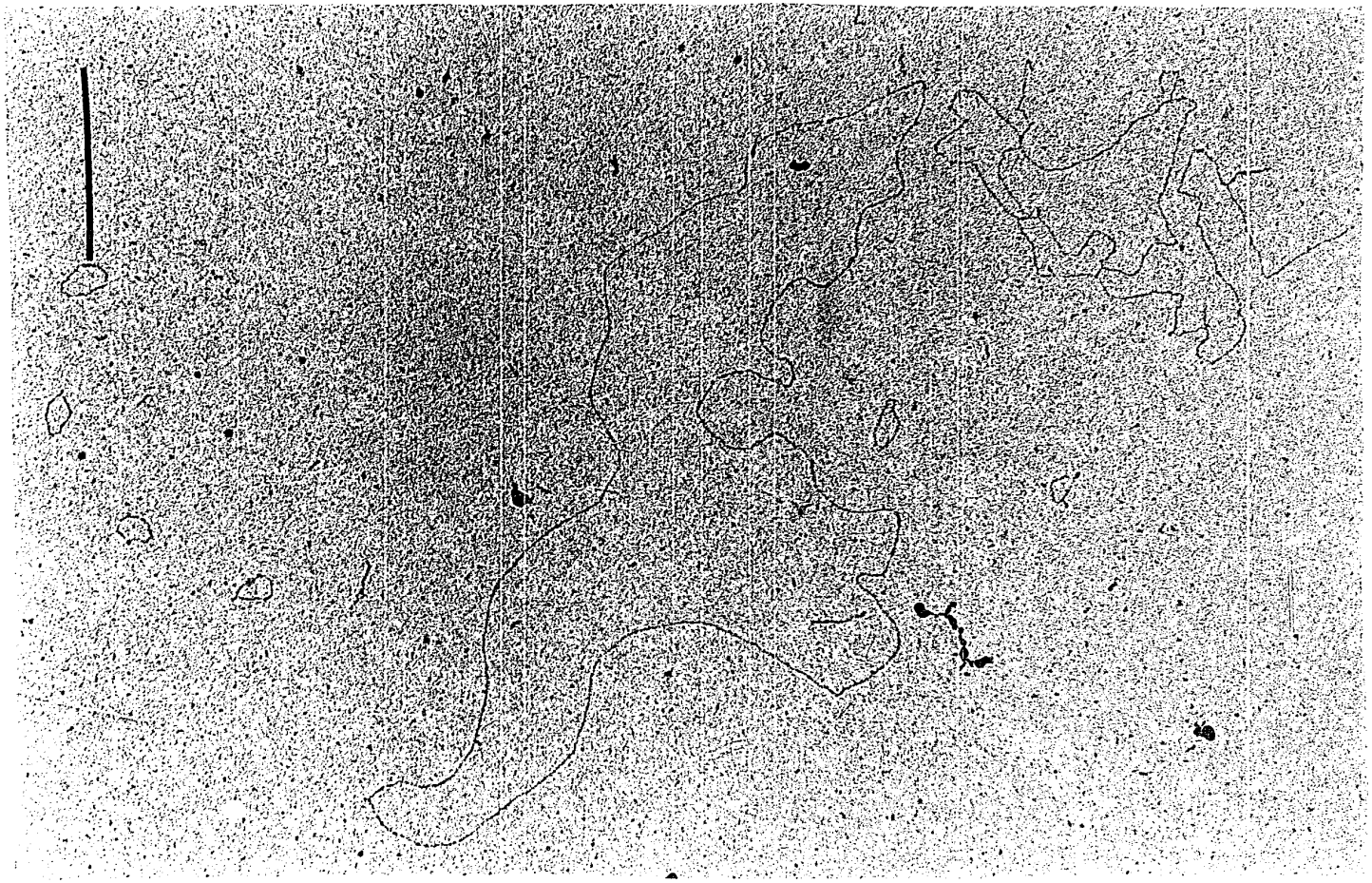
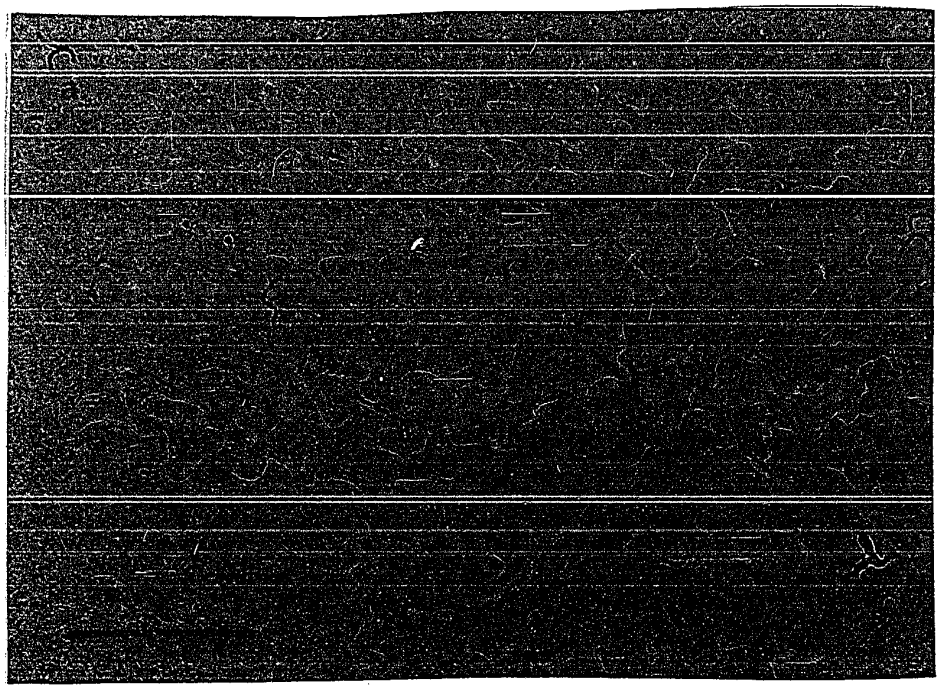
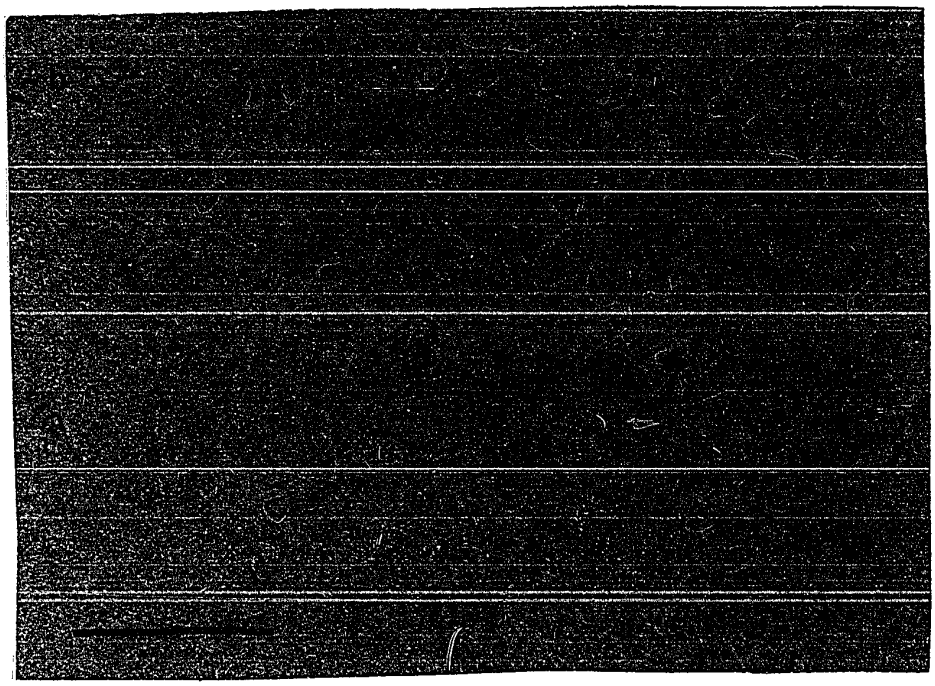
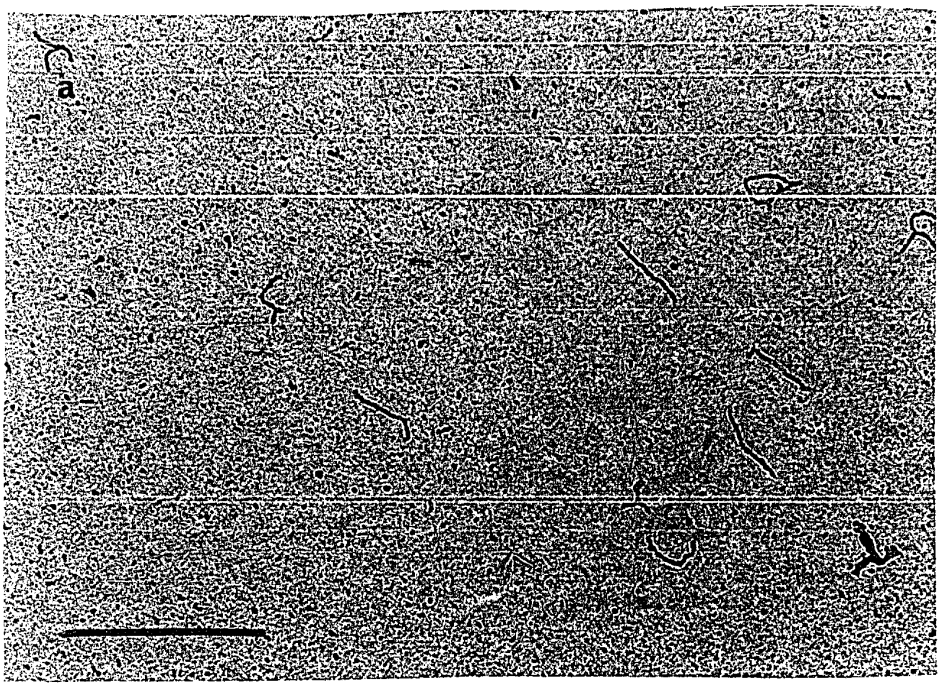


PLATE II.

Electron micrographs of R factor DNA taken from satellite band after dye buoyant density centrifugation of strain 931. Super-twisted forms and open circular forms of the large and small molecules are shown. Large open circular molecule has a contour length of 12.0 um. Bar represents 1 um.






a

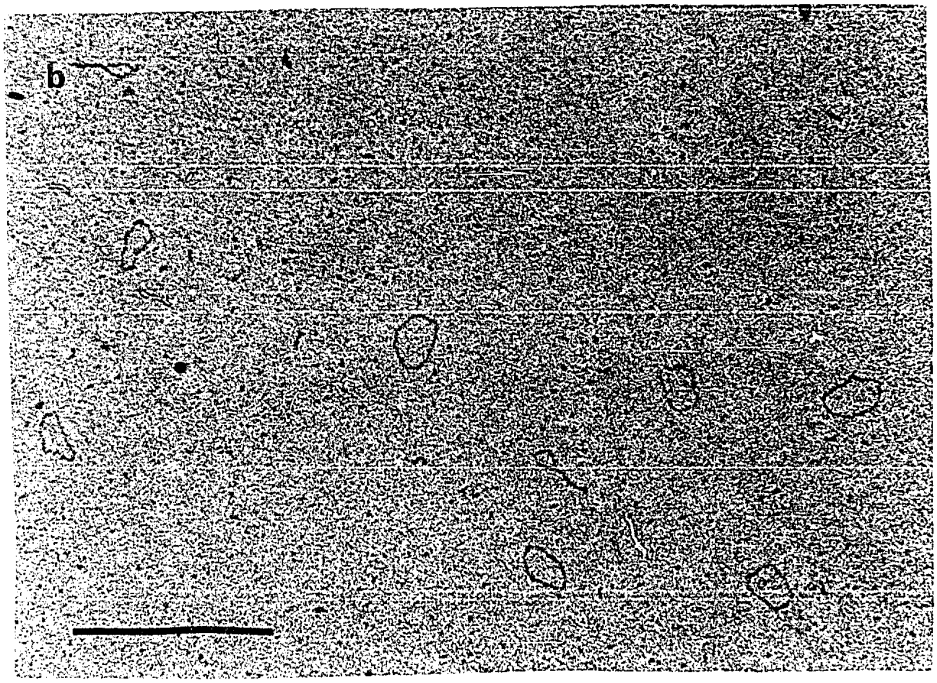

b

PLATE III.

Small circular DNA from strain 931 before (a) and after (b) treatment with deoxyribonuclease I. Bar represents 1 um.

TABLE IX. Effect of DNase I on molecules isolated by ethidium bromide-cesium chloride gradient centrifugation.

Molecule size or ratio of molecules	Conformation	Number of Molecules[a] Pre-DNase	Post-DNase
Large	ccc[b]	80	12
	oc[c]	159	104
Small	ccc	764	238
	oc	90	299
Σsmall/ Σlarge		854/239 (3.6/1)	537/116 (4.6/1)
Σsmall oc/ Σlarge		90/239 (.38)	299/116 (2.57)

a - a unit of area of the grid was counted for both oc and ccc forms of DNA.

b - covalently closed circular

c - open circular

of measurement of the larger molecule (Table X) prevents any conclusion of the former molecule being integrated with the latter molecule. However, the large excess of small molecules relative to the 12 um molecule tends to rule out a 1:1 relationship between the small and large molecules. The possibility remains that the 1 x 10^6 dalton molecule could have a completely separate function.

J. DNA Content in Satellite Bands Under Various Growth Conditions

The percentage of the total DNA represented by R factor DNA estimated by planimetry of densitometer tracings is shown in Table XI. These estimations were based on the assumption that the peaks were symmetrical in shape. Considering the inherent error present in these measurements (Table XI), it appears that 5265, 931, and 3108 have similar percentages of R factor DNA in stationary phase. The content of R factor DNA in 679 is different from the above three R factors since it cannot be detected in 280 (Figure IIIc). However, R679 in E. coli K-12 F^- represents 4% of the total DNA. This value is similar to other values reported for the percentage of R factor DNA in E. coli (20,78).

The percentage of R931 in 280 in stationary phase value is surprising since this value is approximately four fold greater than that obtained for R931 in strain 931. Probably R931 is subject to a different control mechanism in these two hosts. Another difference between the two strains is that R931 acts derepressed for conjugation in strain 931, whereas in strain 280, it acts repressed (Table III). Thus R factor 931 was studied in the naturally occurring state host

TABLE X. Contour length measurements, molecular weights and relative frequency of episomal DNA from P. aeruginosa strain 931.

Number of molecules measured[c]	Contour length ± SSD[a] (um)	Molecular weight (Mdal)	Relative frequency[b]
39	12.3 ± 0.7	25	1
28	0.05 ± 0.03	1.0	3.6 - 4.6

a - SSD, sample standard deviation

b - based on 653 molecules

c - rare molecules of other sizes occurred but represented <0.01 relative frequency.

TABLE XI. Content of R factor DNA relative to chromosomal DNA

R factor	Host strain	Growth Phase	R factor DNA[a] % ± SSD[6]
R931	931	logarithmic	18 ± 3
"	931	stationary	11 ± 3
"	280	stationary	40 ± 5
R679	280	stationary	not detected
"	*E. coli*	stationary	4 ± 1
R3108	3108	stationary	8 ± 2
R5265	5265	stationary	12 ± 3

a - R factor DNA is expressed relative to total DNA. Values are based on 5 or more planimetry measurements of one or more separate CsCl centrifugations.

b - sample standard deviation.

i.e. strain 931 so that further studies could be carried out to compare the effects of other host strains on R931.

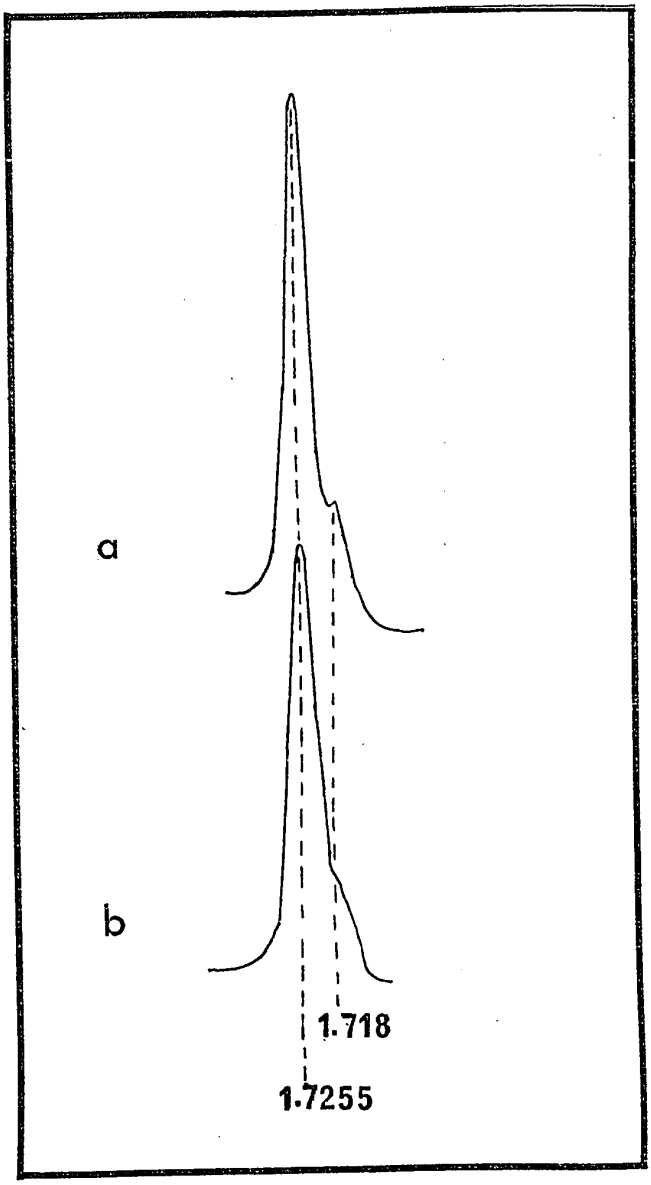
R factor DNA in 931 declines from 18% in logarithmic phase to about 11% in stationary phase (Table XI, Figure VI). Due to the presence of a shoulder in the CsCl centrifugation profile of DNA in stationary phase, there is a large error in the measurement of peak area. However, the value obtained for the percentage of R factor DNA in stationary phase cells was reproducible in all three separate determinations. Thus this apparent decline of R factor DNA in stationary phase cells is surprising because it does not resemble any of the other R factor systems.

In Proteus mirabilis, there is a definite increase in the percentage of R factor DNA in stationary phase (20,78), whereas in E. coli there is either no change (20) or a very small increase (78) in the percentage of R factor DNA.

Table XII shows the percentage of R factor DNA when 931 is grown in the presence of antimicrobial agents. After 60 or 126 subcultures of strain 931 grown in the presence of streptomycin the percentage of R factor DNA increases to 23% in both log and stationary phases (Table XII). When 931 is subcultured 60 times in the presence of tetracycline, the percentage satellite DNA increases to 17%, whereas after 126 subcultures in tetracycline the percentage of satellite DNA increases to 22% (Table XII). Thus the rate of increase in the percentage of satellite DNA is slower when 931 is subcultured in the presence of tetracycline than in streptomycin. After exposure to either drug there is no evidence for change in the buoyant density of

FIGURE VI.

Analytical CsCl gradient centrifugation of DNA from exponential (a), and stationary (b) phase cultures of strain 931.


a
b
1.718
1.7255

the R factor DNA (Table VII).

The increase in the amount of R factor DNA after exposure to tetracycline or streptomycin differ from the results obtained by Finley and Punch (35). After several subcultures in a medium containing chloramphenicol there was no change in the amount of satellite DNA or buoyant density in a P. aeruginosa strain containing satellite DNA. Unfortunately Finley and Punch were unsuccessful in their attempt to transfer the satellite DNA to E. coli. This is not unlikely in view of the inability to transfer R931 to E. coli. Thus, this does not exclude the possibility of R factors. However, in a survey of P. aeruginosa R factors no evidence of transferable chloramphenicol resistance has been detected.[1] Thus it seems possible that Finley and Punch have not examined the effect of a drug containing medium on R factor DNA containing resistance for that drug and their results at present are not comparable.

The effect of drugs on strain 931 are at variance with the effects of drugs on R factors in E. coli and P. mirabilis. When E. coli is subcultured in the presence of a drug to which the R factor confers resistance there is no change in the relative DNA content or in buoyant density. On the other hand, in P. mirabilis there is an increase in the amount of R factor DNA and a change in buoyant density of the satellite DNA (78,79,80). This phenomenon occurs after multiple subcultures in the presence of chloramphenicol, but not in the presence of tetracycline. Resistance to both drugs is mediated by the R factor.

---

1 Unpublished results, Bryan, L.E.

FIGURE VII.

Analytical CsCl gradient centrifugation of DNA from P. aeruginosa strain 931 after being serially subcultured in the presence of drugs.

Trace (a) 60 serial subcultures in medium containing streptomycin

Trace (b) 126 serial subcultures in medium containing streptomycin

Trace (c) 60 serial subcultures in medium containing tetracycline

Trace (d) 126 serial subcultures in medium containing tetracycline

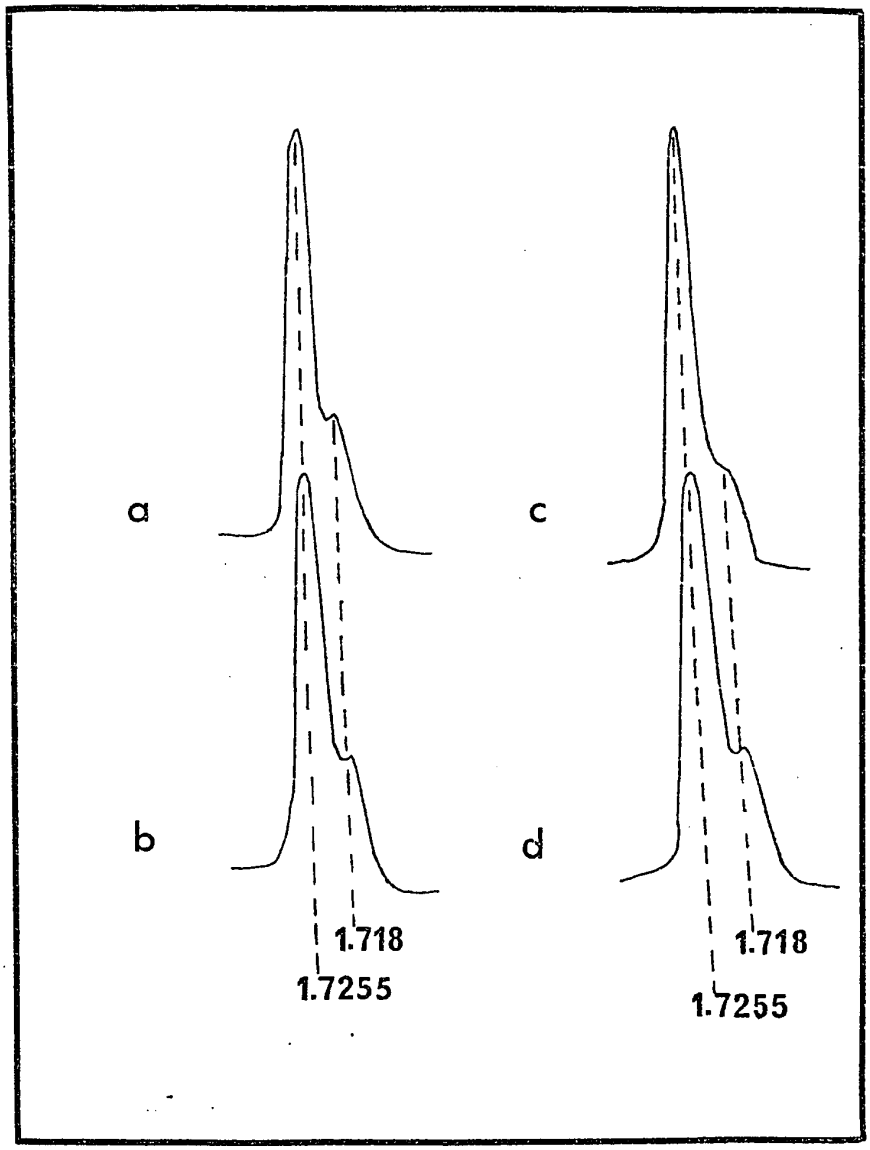

a
b
c
d
1.718
1.7255
1.718
1.7255

TABLE XII. Relative R factor DNA content in P. aeruginosa strain 931 grown in the presence of antimicrobial agents.

Number of serial subcultures	Drug	Growth Phase	Satellite DNA[a] % ± SSD[b]
60	Streptomycin	Stationary	23 ± 3
126	"	"	23 ± 3
126	"	Logarithmic	23 ± 3
60	Tetracycline	Stationary	17 ± 3
126	"	"	22 ± 3

a - R factor DNA expressed relative to total DNA. Values are based on three or more planimetry measurements of one or more separate centrifugation.

b - sample standard deviation.

K. Minimal Inhibitory Concentration

Table XIII illustrates the minimal inhibitory values (MIC) obtained for streptomycin and tetracycline with strain 931 before and after serial subcultures in the presence of these drugs. The MIC of strain 931 was examined after 80 serial subcultures in the presence of tetracycline. Organisms that were subcultured more than 80 times in the presence of tetracycline refused to grow after being stored at -20°C for five months. This could be due to a defective protein being synthesized after prolonged growth in the presence of tetracycline.

The increased MIC after 126 serial subcultures in the presence of stretomycin parallels the increase in the percentage of R factor DNA (Table XII). This phenomenon agrees well with other data where the R factor resistances involve antibiotic inactivation enzymes (50,78). Strain 931 inactivates streptomycin by producing an enzyme streptomycin phosphotransferase (14). There is a slight increase in the MIC of strain 931 after 80 subcultures in the presence of tetracycline. It is believed by Tseng and Bryan[1] that strain 931 expresses resistance to tetracycline by producing an inhibitor to tetracycline transport. In order for a strain to express tetracycline resistance, transport of tetracycline would have to be below a certain limit. Thus growth in the presence of a constant tetracycline concentration would not be expected to increase the MIC as a further decline in transport

---

1 Manuscript in preparation.

TABLE XIII. MIC[a] for strain 931 before and after serial subcultures in a drug containing medium.

Number of serial subcultures in drugs	MIC (ug/ml) Streptomycin	Tetracyline
-	1,200	100
80	-	150
126	15,000	-

a - innoculum was 10^5 organisms; grown for 18 hr.

would probably not significantly increase tetracycline resistance. Similarly a major increase in R factor DNA would not be expected. However, there is an increased percentage of R factor DNA and a small increase in MIC of tetracycline. The increase of R factor DNA appears to occur more slowly than that associated with streptomycin. The reason for the increase in R factor DNA with serial subcultures in tetracycline media is unknown. It is possible that more inhibitor or a new inhibitor for another transport system is produced. Also it is possible the slight increase in MIC could be accounted for by the error inherent in determining MIC values.

L. Plasmid Copies per Genome Equivalent.

Assuming that the molecular weight of the chromosome of E. coli which is 2.5×10^9 daltons (22) is similar to the molecular weight of the chromosome of P. aeruginosa, the number of plasmid copies per genome equivalent in strain 931 can be estimated. From previous data the satellite DNA represents 18% in log phase cells and about 11% in stationary phase cells (Table XI). The relative frequency of small to large molecules is 4.1:1 (Table X). The molecular weight is 25×10^6 daltons and of the small molecule is 1×10^6 daltons (Table X). Based on these values the number of copies of plasmid DNA per chromosome is 15 to 16 for the large molecules and 65 for the small molecules in log phase. The copy number declines to about 10 and 40 respectively in stationary phase. If the molecular weight of the chromosome of P. aeruginosa is $6\text{-}7 \times 10^6$ daltons as reported by Leth Bak et al (52), the number of copies per genome in each growth phase increases 2 to 3

fold. This indicates that both molecules are under relaxed replication. Plasmids preserving a near unity relationship in copy number between the factor and the chromosome have been termed stringent (49) in the regulation of their DNA replication. When there are ten fold or so more copies of the R factor than the chromosome, the replication of the DNA has been termed relaxed (77).

DISCUSSION

The R factor 931 appears to be representative of a unique group of R factors. It does not transfer to E. coli unlike all other R factors described so far (26). Another rare property is that R931 transfers drug resistance at a high frequency 10^{-1} to 1 in at least two hosts, i.e. 931 (Table III) and 1310 (15). This is unusual in comparison to R factors which in general transfer drug resistance at frequencies between 10^{-3} and 10^{-5}. In strain 280, the R factor 931 acts in a manner similar to the Col 1 system (69) and thus by inference seems subject to repression of fertility. CsCl analytical centrifugation shows no evidence of another plasmid present in 280 (Fig. 1a) which might produce the apparent repression.

Another unusual feature of 931 is that the percentage of R factor DNA declines in stationary phase (Table XI). In contrast, other R factors have an increased amount of R factor DNA in stationary phase (20,78,79,80). Since certain behaviour of R931, such as the frequency of drug transfer, seems dependent to some extent on the host it would be interesting to look at the relative amount of R factor DNA in 280R931 in log phase. So far the evidence shows approximately four fold more R factor DNA in strain 280 than in strain 931 in stationary phase (Table XI).

When strain 931 is subcultured in the presence of streptomycin or tetracycline, the percentage of R factor DNA in stationary phase increases to 23% from about 11% in drug free medium (Table XII). In P. mirabilis, the relative amount of R factor DNA increases in the presence of chloramphenicol but not in the presence of tetracycline (78,79,80). Resistance to both drugs in P. mirabilis is mediated by the R factor.

This phenomenon may represent one of two possibilities. The R factor 931 may dissociate in the host strain 931 and the tetracycline gene may reside on the r-determinants rather than on the RTF as postulated for P. mirabilis. On the other hand if R931 does not dissociate, then there may be an increased size of the molecule by repeating sequences or more likely there may be an increased number of plasmid copies per genome.

When 931 is exposed to streptomycin, the R factor DNA increases to about 23% in both stationary and log phases (Table XII). In P. mirabilis there is also an increase of R factor DNA in both phases of growth in the presence of streptomycin (78,79,80). However there is a greater increase in R factor DNA in stationary phase than in log phase in the presence of drugs (78,79,80). Studies on the behaviour of 280R931 after being serially subcultured in the presence of drugs would be of interest to compare the effects of the two hosts 280 and 931 on R931 after being exposed to drugs. Paralleling the increased amount of R factor DNA in strain 931 after being subcultured in the presence of streptomycin is an increased MIC level (Table XIII). This is similar to other R factor systems involving antibiotic inactivation enzymes (50,78).

This uncommon behaviour of R factor 931 in the two hosts 280 and 931 may be explained by a tentative hypothesis in which there is an initiator for R factor DNA synthesis produced by the large extrachromosomal species and by various cellular repressors for R factor DNA synthesis. The initiator would be normally controlled by the host's cellular repressor. In 280R931, the repressor would be less effective,

allowing more R factor DNA to be synthesized in this host (Table XI). The decline in the percentage of R factor DNA 931 in stationary phase could be due to an effective repressor attaining sufficient levels in the late exponential phase, thus preventing further R factor replication. The decline in mating frequency with time in 280R931 could be due to another repressor synthesized by the host which inhibits translation of DNA. However this repressor would be absent in strain 931. Also this repressor may account for the much lower MIC values obtained for 280R931 as compared to strain 931, even though 280R931 has an approximately four fold greater amount of R factor DNA than 931 (Table XI). In the presence of streptomycin and tetracycline the percentage of R factor DNA in 931 increases indicating that the cellular repressor controlling R factor replication could be sensitive to these drugs. The same relative amount of R factor DNA in log and stationary phase (Table XII) may be due to R factor replication paralleling chromosomal DNA replication in absence of the repressor.

Recently it has been reported that minicircular DNA in E. coli show homology with Col EI factors (38). Thus it is possible that the 1 x 10^6 dalton molecular weight circular DNA in P. aeruginosa strain 931 represents r determinants. DNA of similar molecular weight has been described in Shigella dysenteriae and paradysenteriae (43,81) but have no known function. Studies of homology between the small and large molecular weight DNA in strain 931 would be of much interest to determine if these molecules are related to one another. The minicircular DNA could be separated from the larger DNA by sucrose gradients or by acrilamide gels. Demonstration of genetic homology is possible

through DNA:DNA hybridization studies using either radioactive labels or electron microscopy. In the latter the region of homology between the two strands of DNA can be seen. Homologous regions or heterologous duplexes can be seen as double-stranded regions of DNA whereas the non-homologous segments remain as single strands which can be distinguished by electron microscopy both by thickness and conformation. If homology exists between the large and small molecule it would be interesting to see if there is homology between the small molecule of DNA and the r determinants of P. mirabilis.

Nine centrifugations of DNA from strain 931 on CsCl gradients were performed. None of these density profiles revealed more than one satellite band. Hence the failure to differentiate the large and small molecules of extrachromosomal DNA into two bands on CsCl gradients implies one of two possibilities. Both molecules may have very similar or identical buoyant densities or that the smaller molecular species is concealed by the chromosomal DNA. The satellite band obtained with DNA from 280R931 and log phase 931 strains is sharp. This represents either a single species of DNA or two species of extremely close buoyant densities due to the increased diffusion by the smaller molecular species. Thus the value of 1.718 g/cm^3 seems a satisfactory estimation of the buoyant density of the larger extrachromosomal DNA species (25×10^6 daltons) in strain 931.

It seems unlikely that the Pseudomonas aeruginosa R factors described here belong to the same group. Two R factors 931 and 3108 transfer at high frequency to P. aeruginosa strain 280 while R679 and R5265 do not (Table III). In addition R5265 and R679 transfer resistance

to E. coli while R931 and R3108 do not (14,15). An attempt to classify the R factors serologically was only partially successful. Problems with the immunogenicity occurred with 280R5265 and 280RP4. Three groups were defined but some of the results conflicted with the results of compatability studies done in the laboratory (15). The compatability studies showed R3108 and R931 belonged to the same compatability group. A compatability group is defined by the effect of the introduction of a second R factor into a strain containing a resident R factor. The resident R factor will significantly reduce the transfer frequency of the introduced R factor if the plasmids are closely related or permit stable coexistence if the plasmids are unrelated. Serological reaction in the past have been useful to produce subtypes of major R factor compatability groups (26).

The relationship of Pseudomonas R factors to those of the Enterobacteriaceae is vague. Investigation by compatability studies would be difficult due to the limited host range of Pseudomonas R factors. Similarly E. coli R factors transfer very poorly to P. aeruginosa. Homology studies of the R factors appear to be the most useful approach to this problem.

A physical difference between E. coli and P. aeruginosa R factors is their buoyant density or GC content. P. aeruginosa R factors have similar buoyant densities ranging from 1.716 (57% GC) to 1.719 g/cm^3 (59% GC) (Table VI). However, E. coli R factors have lower buoyant densities. E. coli R factors have a value of 1.708 (49% GC) to 1.711 g/cm^3 (52% GC) (20,21,50,67,68) except for R6K which has a value of 1.704 g/cm^3 (45% GC) (50). The buoyant density for P.

aeruginosa R factors of this study is closer to the value obtained for RP1 which is 1.719 g/cm^3.

Although the GC content of R931 is similar to that of RP1, the size of 931 is much less than that for RP1. R factor 931 has a molecular weight of 25 x 10^6 daltons (Table X). No evidence has been presented of a small molecular weight DNA species (1 x 10^6 daltons) associated with RP1.

The size of R931 is extremely different from E. coli R factors, except for R6K which synthesizes penicillinase. Generally E. coli R factors have a molecular weight of 38 to 70 million daltons. The exception R6K is the smallest known R factor that is capable of self transfer and that has fertility characteristics. This E. coli R factor has a molecular weight of 26 x 10^6 which is similar to 931 (25 x 10^6 daltons). R6K has been shown to produce catenated DNA which represents 5-10% of the total R factor DNA. Only the occasional dimer has been detected in 931. The buoyant density of R6K is very much less than that of R931.

Possibly the difference in R factors from P. aeruginosa and those from E. coli lie in the transfer moiety. R factors from E. coli 222, R1 and R6 are similar. The segregation pattern in Proteus, and particularly their molecular size and their segregant molecular size are similar. However their origins and patterns of resistance are different. Factor 222 (101) isolated in Japan (65) carries resistance to sulphonamide, streptomycin, chloramphenicol, and tetracycline. R1 originated in the United Kingdom (4) and determines re-

sistance to sulphonamide, streptomycin, chloramphenicol, kanamycin, and ampicillin whereas R6 isolated in Switzerland (18) carries sulphonamide, streptomycin, chloramphenicol, tetracycline, kanamycin and neomycin resistance. Hence these geographically and phenotypically distinct R factors may have at least the same transfer factor moiety. A preliminary report by Sharp el al (18) shows homology of Col V2, R1 and R6 with the same region of F. R1 and R6 show extensive homologies over their total length. Thus it is possible that P. aeruginosa has a different transfer factor moiety from the R factors in E. coli. This may account for the higher GC content of the R factor, the smaller size of the R factor and the lower frequency of drug-resistance transfer to E. coli recipients than to P. aeruginosa recipients. Homology studies between the P. aeruginosa R factor and the transfer unit of one of the R factors from E. coli would be of interest.

BIBLIOGRAPHY

1. Akiba, T., K. Koyama, Y. Isshibi, G. Kimura and T. Fukushima. 1960. One the mechanism of the development of multiple-drug resistant clones of Shigella. Japan J. Microbiol. 4:219-229.

2. Anderson, E.S. 1966. A rapid screening test for transfer factors in drug sensitive Enterobacteriaceae. Nature 208: 1016-1017.

3. Anderson, E.S. 1968. The ecology of transferable drug resistance in the Enterobacteria. Ann. Rev. Microbiol. 22:131-181.

4. Anderson, E.S., and N. Datta. 1965. Resistance to penicillins and its transfer in Enterobacteriaceae. Lancet I:407-409.

5. Anderson, E.S., and M.J. Lewis. 1965. Characterization of a transfer factor associated with drug resistance in Salmonella typhimurium. Nature 208:843-849.

6. Anderson, E.S., M.V. Kelemen, C.M. Jones, and J.S. Pitton. 1968. Study of the association of resistance to two drugs in a transferable determinant in Salmonella typhimurium. Genet. Res. 11: 119-124.

7. Baron, L.S., and S. Falkow. 1961. Genetic transfer of episome from Salmonella typhosa to Vibrio cholerae. Records Genet. Soc. Am. 39:59-62.

8. Bauer, A.W., W.M.M. Kirby, J.C. Sherris and M. Turck. 1966. Antibiotic susceptibility testing by a standard single disc method. Amer. J. Clin. Pathol. 45:493-496.

9. Bazaral, M., and D.R. Helinski. 1968. Circular DNA forms of colicinogenic factors E1, E2, and E3 from Escherichia coli. J. Mol. Biol. 36:185-194.

10. Benveniste, R., and J. Davies. 1971. Enzymatic acetylation of aminoglycoside antibiotics by Escherichia coli carrying an R factor. Biochem. 10:1787-1796.

11. Benveniste, R., and R. Davies. 1971. R factor mediated gentamicin resistance: a new enzyme which modifies aminoglycoside antibodics. Fed. Eur. Biochem. Sci. Lett. 14:293-296.

12. Benveniste, R., T. Yameda, and J. Davies. 1970. Enzymatic adenylation of streptomycin and spectinomycin by R factor resistant Escherichia coli. Infect. Immun. 1:109-119.

13. Bray, G.A. 1960. A simple and efficient liquid scintillator for counting aqueous solutions in liquid scintillation counter. Anal. Biochem. 1:279-285.

14. Bryan, L.E., H.M. Van Den Elzen, and Jui Teng Tseng. 1972. Transferrable drug resistance in Pseudomonas aeruginosa. Antimicrob. Ag. Chemother. 1:22-29.

15. Bryan, L.E.,S. Semaka, H.M. Van Den Elzen, J. Kinnear, and R. Whitehouse. 1973. Characteristics of R931 and other Pseudomonas R factors. Antimicrob Ag Chemother. In press 1973.

16. Bühlman, X., W.A. Vischer, and H. Bruhin. 1961. Identification of apocyanogenic strains of Pseudomonas aeruginosa. J. Bacteriol. 82:787-788.

17. Cheng, K.J., J.M. Ingram, and J.W. Costerton. 1970. Release of alkaline phosphatase from cells of Pseudomonas aeruginosa by manipulation of cation concentration and pH. J. Bacteriol. 104: 748-753.

18. Clowes, R.C. 1972. Molecular structure of bacterial plasmids. Bacteriol. Rev. 36:361-405.

19. Cohen, S.N. 1969. Isolation and characterization of R factor DNA. Bacteriol. Proc. 69:49-51.

20. Cohen, S.N., and C.A. Miller. 1970. Non-chromosomal antibiotic resistance in bacteria. II Molecular nature of R factors isolated from Proteus mirabilis and Escherichia coli. J. Mol. Biol. 50: 671-687.

21. Cohen, S.N., R.P. Silver, P.A. Sharp, and A.E. McCoubrey. 1971 Studies on the molecular nature of R factors. Annal. New York Acad. Sci. 182:172-187.

22. Cooper, S., and C.E. Helmstetter. 1968. Chromosomal replication and the division cycle of Escherichia coli B/r. J. Mol. Biol. 31:519-540.

23. Cowan, S.T. and K. J. Steele. 1965. Manual for the identification of medical bacteria. Cambridge University Press, London.

24. Datta, N. 1965. Infectious drug resistance. British Med. Bull. 21:254-259.

25. Datta, N., R.W. Hedges, E.J. Shaw, R.B. Sykes, and M.H. Richmond, 1971. Properties of an R factor from Pseudomonas aeruginosa. J. Bacteriol. 108:1244-1249.

26. Datta, N., and R.W. Hedges. 1972. Host ranges of R factors. J. Gen. Microbiol. 70:453-460.

27. Datta, N. and P. Kontomichalou. 1965. Penicillinase synthesis controlled by infectious R factors in Enterobacteriacea. Nature 208:239-241.

28. Datta, N., and M.H. Richmond, 1966. The purification and properties of a penicillinase whose synthesis is mediated by an R factor in Escherichia coli. Biochem. J. 98:204-209.

29. Davies, J.E., and R. Rownd. 1972. Transmissible multiple drug resistance in Enterobacteriaceae. Sci. 176:758-768.

30. Davern, C.I. 1966. Isolation of the DNA of the E. coli chromosome in one piece. Proc. Nat. Acad. Sci. Wash. 55:792-797.

31. Egawa, R., Y. Hara, S. Mitsuhashi, and S.N. Cohen. 1969. Demonstration of R factors in enteric bacteria isolated in a Boston City hospital. Japan J. Microbiol. 13:241-245.

32. Falkow, S., R.V. Citarella, J.A. Wohlheiter, and T. Watanabe. 1966. The molecular nature of R factors. J. Mol. Biol. 17: 102-116.

33. Falkow, S., D.K. Haapala, and R.P. Silver. 1969. Bacterial episomes and plasmids: relationships between extrachromosomal elements. Ciba Foundation Sympos. Little, Brown and Co. Boston Mass.

34. Farmer, J.J. III, and L.G. Herman. 1969. Epidemiological fingerprinting of Pseudomonas aeruginosa by the production of and sensitivity to pyocin and bacteriophage. Appl. Microbiol. 18:760-765.

35. Finley, R.B., and J.D. Punch. 1972. Satellite deoxyribonucleic acid in Pseudomonas aeruginosa. Can. J. Microbiol. 18:1003-1006.

36. Fullbrook, P.D., S.W. Elson, and B. Slocombe. 1970. R factor mediated β-lactamase in Pseudomonas aeruginosa. Nature 226: 1054-1056.

37. Ginoza, H.S., and T.S. Matney. 1963. Transmission of a resistance transfer factor from Escherichia coli to two species of Pasteurella. J. Bacteriol. 85:1177-1178.

38. Goebel, W., and H. Schrempf. 1972. Isolation of minicircular deoxyribonucleic acids from wild strains of Escherichia coli and their relationship to other bacterial plasmids. J. Bacteriol. 111:696-704.

39. Grinsted, J., J.R. Saunders, L.C. Ingram, R.B. Sykes, and M.H. Richmond. 1972. Properties of an R factor which originated in Pseudomonas aeruginosa 1822. J. Bacteriol. 110:529-537.

40. Harada, K., M. Suzuki, M. Kameda, and S. Mitsuhashi. 1960. On the drug resistance of enteric bacteria. 2. Transmission of the drug-resistance among Enterobacteriaceae. Japan J. Exptl. Med. 30:289-299.

41. Hill, L.P. 1966. An index to deoxyribonucleic acid base compositions of bacterial species. J. Gen. Microbiol. 44:419-437.

42. Izaki, K., K. Kiuchi, and K. Arima. 1966. Specificity and mechanism of tetracycline resistance in a multiple drug-resistant strain of Escherichia coli. J. Bacteriol. 91:628-633.

43. Jansz, H.S., J. Zandberg, J.H. Van de Pol, E.F.J. Van Bruggen. 1069, Circular DNA from Shigella paradysenteriae. Eur. J. Biochem. 9:156-159.

44. Jacob, F., P. Schaeffer, and E.L. Wollman. 1960. Episomic elements in bacteria. Symp. Soc. Gen. Microbiol. 10:67-91.

45. Kabins, S.A., C.R. Nathan, and S. Cohen. 1971. R factor-mediated resistance to gentamicin in a clinical isolate of Escherichia coli. J. Infect. Dis. 124:S65-67.

46. Kameda, M., K. Harada, M. Suzuki, and S. Mitsuhashi. 1969. Formation of transferable drug resistance factor by recombination between resistance determinants and transfer factors. Japan J. Microbiol. 13:255-262.

47. Kleinschmidt, A.K. 1968. Monolayer techniques in electron microscopy of nucleic acid molecules. Methods in Enzymology 12B:361-377.

48. Kondo, S.,M.K.Okaniski, R. Utahara, R. Maeda, H. Umezawa. 1968. Isolation of Kanamycin and paromamine inactivated by E. coli carrying R factor. J. Antibiot. 21:22-29.

49. Kopecko, D.J., and J.D. Punch. 1971. Regulation of R factor replication in Proteus mirabilis. Ann. New York Acad. Sci. 182: 207-216.

50. Kontomichalou, P., M. Mitani, and R. C. Clowes. 1970. Circular R factor molecules controlling penicillinase synthesis, replicating in Escherichia coli under either relaxed or stringent control. J. Bacteriol. 104:34-44.

51. Lang, D. 1970. Molecular weight of coliphages and coliphage DNA. III Contour length and molecular weight of DNA from bacteriophage T_4, T_5 and T_7 and bovine papilloma virus. J. Mol. Biol. 54:557-565.

52. Leth Bak, A., C. Christiansen and A. Stenderup. 1970. Bacterial genome sizes determined by DNA renaturation studies. J. Gen. Microbiol. 64:377-380.

53. Mandel, M. 1966. Deoyribonucleic acid base composition in the genus Pseudomonas. J. Gen. Microbiol. 43:273-292.

54. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acids from microorganisms. J. Mol. Biol. 3:208-218.

55. Marmur, J., and P. Doty. 1959. Heterogeneity in deoxyribonucleic acids. 1. Dependence on the composition, on the conformational stability of deoxyribonucleic acids. Nature 183:1427-1429.

56. Martin, C.M., N.S. Ikari, and J.A. Zimmerman. 1971. A virulent nosocomial Klebsiella with a transferable R factor for gentamicin: emergence and suppression. J. Infect. Dis. 124:S24-S29.

57. Marsh Jr., E.B., and D.H. Smith. 1969. R factors improving survival of Escherichia coli K-12 after ultraviolet irradiation. J. Bacteriol. 100:128-139.

58. Mitsuhashi, S., K. Harada, M. Kameda, M. Suzuki, and R. Egawa. 1960. On the drug resistance of enteric bacteria. 3. Transmission of the drug resistance from Shigella to F^- or Hfr strains of E. coli K-12. Japanese J. Expt. Med. 30:301-306.

59. Mitsuhashi, S., K. Harada, and H. Hashimoto. 1960. Multiple resistance of enteric bacteria and transmission drug resistance to other strains by mixed cultivation. Japanese J. Exptl. Med. 30:179-184.

60. Mitsuhashi, S., K. Harada, and M. Kameda. 1961. Elimination of transmissible drug resistance by treatment with acriflavin. Nature 189:947-947.

61. Mitsuhashi, S. 1968. Review: The R factors. J. Infect. Dis. 119 89-100.

62. Mitsuhashi, S., M. Kameda, K. Harada, and M. Suzuki. 1969. Formation of recombinants between nontransmissible drug resistance determinants and transfer factors. J. Bacteriol. 97:1520-1526.

63. Moses, R.E., and C.C. Richardson. 1970. Replication and repair of DNA in cells of Escherichia coli treated with toluene. Proc. Nat. Acad. Sci. 67:674-681.

64. Mitsuhashi, S. 1971. Transferable drug resistance factor R. University of Tokyo Press, Tokyo, Japan.

65. Nakaya, R., A. Nakamura, and Y. Murata. 1960. Resistance transfer agents in Shigella. Biochem. Biophys. Res. Commun. 3:654-659.

66. Nandi, U.S., J.C. Wang, and N. Davidson. 1966. Separation of deoxyribonucleic acids by Hg(11) binding and Cs_2SO_4 density gradient centrifugation. Biochem. 4:1687-1696.

67. Nisioka, T., M. Mitani, and R. Clowes. 1969. Composite circular forms of R factor deoxyribonucleic acid molecules. J. Bacteriol. 97:376-385.

68. Nisioka, T., M. Mitani, and R.C. Clowes. 1970. Molecular recombination between R factor deoxyrionucleic acid molecules in Escherichia coli host cells. J. Bacteriol. 103:166-177.

69. Nomura, N. 1967. Colicins and related bacteriocins. Ann. Rev. Microbiol. 21:257-284.

70. Norris, F.C., and J.R. Campbell. 1949. The intermediate metabolism of Pseudomonas aeruginosa. III The application of paper chromatography to identification of gluconic and 2-ketogluconic acids, intermediate in glucose metabolism. Can. J. Res. 27:253-268.

71. Ozanne, B., R. Benveniste, D. Tipper, and J. Davies. 1969. Aminoglycoside antibiotics: Inactivation by phosphorylation in Escherichia coli carrying R factors. J. Bacteriol. 100:1144-1146.

72. Punch, J.D., and D.J. Kopecko. 1972. Positive and negative control of R factor replication in Proteus mirabilis. J. Bacteriol. 109:336-349.

73. Radloff, R., W. Bauer, and J. Vinograd. 1967. A dye-buoyant density method for the detection and isolation of closed circular duplex DNA: The closed circular DNA in hela cells. Proc. Nat. Acad. Sci. Wash. 57:1514-1521.

74. Roe, E., R.J. Jones and E.J.L. Lowbury. 1971. Transfer of antibiotic resistance between Pseudomonas aeruginosa, Escherichia coli, and other gram negative bacilli in burns. Lancet 1:149-152.

75. Rolfe, R., and M. Meselson. 1959. The relative homogeneity of microbial DNA. Proc. Nat. Acad. Sci. Wash. 45:1039-1043.

76. Rownd, R., R. Nakaya, and A. Nakamura. 1966. Molecular nature of the drug resistance factors of the Enterobacteriaceae. J. Mol. Biol. 17:376-393.

77. Rownd, R., 1969. Replication of a bacterial spisome under relaxed control. J. Mol. Biol. 44:387-402.

78. Rownd, R., H. Kasamatsu, and S. Mickel. 1971. The molecular nature and replication of drug resistance factors of the Enterobacteriacea. Ann. New York Acad. Sci. 182:188-205.

79. Rownd. R., H. Watanabe, S. Mickel, R. Nakaya, and B. Gargan. 1971. The molecular nature and the control of replication of R factors. In Progress in antimicrobial and anticancer chemotherapy. Int. Congr. Chemotherapy. Proc. VI. Univ. of Tokyo Press, Tokyo.

80. Rownd, R., and S. Mickel. 1971. Dissociation and reassociation of RTF and r-determinants of the R factor NRI in Proteus mirabilis. Nature 234:40-43.

81. Rush, M.G., C.N. Gordon, and R.C. Warner. 1969. Circular deosyribonucleic acid from Shigella dysenteriae. J. Bacteriol. 100: 803-808.

82. Schildkraudt, C.L., J. Marmur and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acids from its buoyant density in CsCl. J. Mol. Biol. 4:430-443.

83. Siccardi, A.G. 1966. Colicin resistance associated with resistance factors in Escherichia coli. Genetic. Res. 8:219-228.

84. Shaw, W.V. 1967. The enzymatic acetylation of chlorampenicol by extracts of R factor resistant Escherichia coli. J. Mol. Biol. 247:687-693.

85. Siccard, A.G. 1969. Effect of R factors and other plasmids on ultraviolet susceptibility and host cell reactivation property of Escherichia coli. J. Bacteriol. 100:337-346.

86. Smith, D.H., and S.E. Armour. 1966. Transferable R factors in enteric bacteria causing infection of the genic-urinary tract. Lancet 2:15-18.

87. Smith, D.H. 1967. Drug resistance of enteric bacteria mediated by R factors. Antimicrobial Ag. Chem. 1966:274-280.

88. Smith, D.H. 1967. R. factors mediate resistance to mercury, nickel, and cobalt. Sci. 156:1114-1116.

89. Smith, H.W. 1969. Vetinary implication of transfer activity. Ciba Foundation Symposium on Bacterial Episomes and Plasmids. Little, Brown, Boston.

90. Smith, D.H., J. A. Janjigian, N. Prescott, and P.W. Anderson. 1970. Resistance factor mediated spectinomycin resistance. Infect. Immun. 1:120:127.

91. Stanish, A., and B.W. Holloway. 1971. Chromosome transfer in Pseudomonas aeruginosa mediated by R factors. Genet. Res. 17: 167-172.

92. Sucoka N. 1959. A statistical analysis of deoxyribonucleic acid distribution in density gradient centrifugation. Proc. Nat. Acad. Sci. Wash. 45:1480-1490.

93. Suzuki, Y., and S. Okamoto. 1967. The enzymatic acetylation of chloramphenicol by the multiple drug-resistant Escherichia coli carrying R factor. J. Biol. Chem. 242:4722-4730.

94. Sykes, R.B., and M.H. Richmond. 1970. Intergeneric transfer of a β-lactamase gene between P. aeruginosa and E. coli. Nature 266:952-954.

95. Takano, T., and T. Watanabe. 1966. Specific inactivation of infectious DNA by sonicates of restrictive bacteria with R factors. Biochem. Biophys. Res. Commun. 25:192-198.

96. Takano, T., T. Watanabe, and T. Fukasawa. 1968. Mechanism of host controlled restriction of bacteriophage by R factors in Escherichia coli K-12. Virology 34:290-302.

97. Umezawa, H., M. Okanishi, S. Kondo, K. Hamana, R. Utahara, K. Maeda, and S. Mitsuhashi. 1967. Phosphorylative inactivation of aminoglycoside antibiotics by Escherichia coli carrying R factor. Sci. 157:1559-1561.

98. Vinograd, J., J. Lebowitz, R. Radloff, R. Watson and P. Laipis. 1965. The twisted circular form of polyoma vial DNA. Proc. Nat. Acad. Sci. 53:1104-1111.

99. Wahba, A.H., and J.H. Darrell. 1965. The identification of atypical strains of Pseudomonas aeruginosa. J. Gen. Microbiol. 38:329-342.

100. Walton, J.R. 1966. Infectious drug resistance in Escherichia coli isolated from healthy farm animals. Lancet 2:1300-1302.

101. Watanabe, T., and T. Fukasawa. 1960. "Resistance transfer factor" an episome in Enterobacteriaceae. Biochem. Biophys. Res. Commun. 3:660-665.

102. Watanabe, T., and T. Fukasawa. 1961. Episome mediated transfer of drug resistance in Enterobacteriaceae. I. Transfer of resistance factors by conjugation. J. Bacteriol. 81:669-675.

103. Watanabe, T., and T. Fukasawa. 1961. Episome-mediated transfer of drug resistance in Enterobacteriaceae. III. Transduction of resistance factors. J. Bacteriol. 82:202-209.

104. Watanabe, T., and T. Fukasawa. 1961. Episome-mediated transfer of drug resistance in Enterobacteriaceae. II. Elimination of resistance factors with acridine dyes. J. Bacteriol. 81:679-683.

105. Watanabe, T. 1963. Infective heredity of multiple drug resistance in bacteria. Bacteriol. Rev. 27:87-115.

106. Watanabe, T., and T. Fukasawa. 1962. Episome-mediated transfer of drug resistance in Enterobacteriaceae. IV. Interactions between resistance transfer factor and F factor in Escherichia coli K-12. J. Bacteriol. 83:727-735.

107. Watanabe, T., C. Ogata, and S. Sato. 1964. Episome-mediated transfer of drug resistance in Enterobacteriaceae. VIII. Six-drug resistance R factors. J. Bacteriol. 88:922-928.

108. Watanabe, T., T. Aoki, Y Ogatta, S. Egusa. 1971. R factors related to fish culturing. Ann New York Acad. Sci. 182:383-410.

109. Witchitz, J.L., and Y.A. Chabbert. 1970. High level transferable resistance to gentamicin. J. Antibiot. 24:137-139.