A GENETIC ANALYSIS BY TRANSFORMATION OF A GROUP OF URACIL-REQUIRING MUTANTS OF *DIPLOCOCCUS PNEUMONIAE*[1]

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RECOMBINATION between closely linked markers in pneumococcus has been studied mainly among genes related to drug resistance. Because of the individuality of the degree of resistance corresponding to each particular marker in some loci, it may be surmised that the mutants produce a partly functional gene product, and therefore only a restricted selection of all possible gene alterations are accessible. On the other hand, the fastidiousness of pneumococcus limits the availability of nutritional markers, and this route to the variety of mutations that has proved useful with other organisms has been difficult. The work of LACKS (1966) and of LACKS and HOTCHKISS (1960 a,b) with the amylomaltase locus constitutes the only substantial genetic studies based on the enzyme-deficient phenotypes that are familiar with other organisms.

The defined medium of RAPPAPORT and GUILD (1959) partially solved the problem of defined environment, but in this laboratory failed to support the growth of small numbers of cells. RAPPAPORT and GUILD's medium was modified to produce one which supported the growth of small inocula. With this medium, mutants requiring uracil, lysine and phenylalanine were readily recovered. A genetic analysis of a group of uracil-requiring mutants was undertaken.

Twelve of the 40 uracil mutants isolated were utilized in recombination experiments in which all possible crosses were made. It became evident in this work that the mutant strains showed variable transforming efficiency when compared in transformations utilizing a reference marker. Differences in efficiencies of this type were first reported by LACKS and HOTCHKISS (1960a) in the amylomaltase locus of pneumococcus. In a recombination analysis, the disagreement in the values for reciprocal crosses and the inability to arrange certain mutations with respect to others, pointed to the necessity for the allowance of recipient variation. A complex index introduced by LACKS and HOTCKISS (1960a) provided a possible solution. A statistical analysis was made of the ratios of reciprocal crosses comparing these when expressed by a simple index to those expressed by the LACKS-HOTCHKISS index. The two sets of ratios were clearly different. Further, an unambiguous linear arrangement of mutations which utilized the former index could not accommodate the most efficient recipient whereas, a satisfactory linear arrangement including all linked markers was obtained with the latter index.

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Negative interference was found in two-point crosses of eight linked uracil mutants. Three-point crosses with more distantly spaced markers, made possible by the synthesis of a double mutant, showed no interference in agreement with the results of GRAY and EPHRUSSI-TAYLOR (1967) obtained on the *amiA* locus.

MATERIALS AND METHODS

Bacterial strains: The strain FMS of rough pneumococci is a streptomycin- and sulfanilamide-resistant, mannitol-oxidizing strain. Throughout these studies this strain was used as the donor of the streptomycin resistance marker. A rough, highly transformable strain designated as A strain was used as a recipient in transformation experiments. Both FMS and A were originally obtained from DR. R. D. HOTCHKISS and were used in studies by LERMAN and TOLMACH (1957). An R-6 rough pneumococcus used principally for obtaining mutants was obtained from DR. H. P. RAPPAPORT. Irregularity in its transformability made it necessary to transfer mutations from this strain to the A strain.

Minimal medium: A modification of the medium described by RAPPAPORT and GUILD (1959) was used both for the selection of mutants and for the recombinational analysis of mutants. Colony counts of strains A and R-6 were entirely comparable on this medium to those obtained on a complex medium. Major changes were made in the V_2 solution and the addition of vitamin-free casein was essential for high plating efficiency. In order to make the details of preparation clear, the entire composition and methods of storing and combining are given in Table 1.

Supplemented medium: For certain experiments the ingredients in Table 1 were supplemented with either uracil or uracil plus thymine, L-lysine, L-phenylalanine and L-tryptophan at a concentration of 0.01 mg/ml each.

Catalase preparation: PPPD, a partially purified protein derivative from citrated human blood had a catalase activity at a dilution of one to a thousand estimated by the capacity to induce frothing of dilute hydrogen peroxide. For its preparation 2 vol of distilled water were added to each vol of washed packed human red cells. To each liter of the resulting laked cells was added 500 ml alcohol-chloroform mixture, 3:1 parts, respectively. Hemoglobin was removed by filtration through coarse paper. The filtrate was dialyzed against physiological saline containing 10^{-3} M phosphate at pH 6.8–7.0, filtered for sterilization, and stored in the refrigerator for periods up to several years.

Medium for the growth of competent cells: WILSON's broth was devised for the maintenance of cultures and for the growth of highly competent cells. An extract of peptones[1] was diluted 1 to 2.5 in distilled water, absorbed with Norit, 100 g/l solution and cleared with egg white. After brief autoclaving and filtration this stock concentrate was refrigerated until needed. For culture medium, the stock was diluted ten-fold, made 0.04 M with Tris buffer, adjusted to pH 7.8 and autoclaved in 40 ml volumes. Prior to inoculation each volume was supplemented with 0.005 M $NaHCO_3$, 3 mg/ml glucose, 2.5 mg/ml bovine serum albumin (Armour crystallized) and papain heart digest (PHD), approximately 2 ml per 40 ml volume of broth.

Papain heart digest: PHD was prepared by removing the fat from fresh beef hearts. Each 200 g of fat free heart was homogenized for 15 to 20 sec with 300 ml 0.15 M saline in a Waring blendor. Two grams of papain (Nutritional Biochemical Corporation) in 10 ml 0.1 M mercaptoactetate was added and the tissue digested for 3–4 hrs at 45°C. The homogenate was autoclaved 5 min to inactivate the enzyme, cooled and cleared with egg white, and reautoclaved an additional 5 min, then filtered through coarse paper. The filtrate was frozen and sterilized as needed by Selas candle filtration.

Differential medium: Minimal medium was supplemented with PHD to make a differential medium for uracil independent and dependent colonies. PHD was initially added to pneumococcal cultures as a growth stimulating agent. As the concentration of PHD was decreased in minimal medium, uracil dependent colonies decreased to pinpoint size (approximately 0.1 mm diameter)

[1] Protopeptone 627 was purchased from Wilson and Co., Res. and Tech. Div., 4200 South Marsh Field Ave., Chicago 9, Illinois.

TABLE 1

Synthetic medium

Mineral solution

NH_4Cl	33.33 g
NaCl	83.4 g
KCl	6.66 g
$MgSO_4\ 7H_2O$	7.34 g
$CaCl_2$	0.433 g
$NaH_2PO_4\ H_2O$	3.38 g
$Fe(NO_3)_3\ 9H_2O$	13.22 mg
Distilled H_2O	500 ml

Dissolve each separately. Add together. Store in refrigerator.

Amino acid solution

L-Arginine—HCl	240 mg
DL-Aspartic Acid	2080 mg
L-Histidine—HCl H_2O	40 mg
DL-Isoleucine	2400 mg
DL-Leucine	1600 mg
DL-Threonine	480 mg
DL-Valine	1600 mg
Distilled H_2O	1800 ml

Dissolve in water bath at 60°C

V_2 solution

	Solution	Amount
Asparagine	20 mg/ml	18.75 ml
Catalase preparation	See below	18.75 ml
Biotin	0.75 μg/ml	7.5 ml
Niacin	0.5 mg/ml	7.5 ml
Pantothenate, Ca	2.5 mg/ml	7.5 ml
Sodium Acetate	1M	3.75 ml
$NaHCO_3$	0.5M	7.5 ml
Pyridoxine	0.5 mg/ml	7.5 ml
Riboflavin	0.1 mg/ml	37.5 ml
Thiamine	0.5 mg/ml	7.5 ml
Choline chloride	5.0 mg/ml	7.5 ml
Adenine	2.5 mg/ml	15.0 ml

V_2 solutions are filtered and stored separately in the refrigerator except for asparagine, sodium acetate, and adenine which are stored at room temperature.

Mixture of minerals and amino acids

Amino acid solution	1880 ml
Mineral solution	120 ml
Tris-(hydroxymethyl) aminomethane	19.36 grams

Adjust with HCl to pH 7.8. Store in freezer in 500 ml amounts.

Complete minimal medium

Amino acid-mineral mixture	500 ml
V_2 solution	50 ml
Glucose solution (300 mg/ml)	8.3 ml
Glutamine (25 mg/ml)	15 ml
Cysteine HCl (25 mg/ml)	5 ml

This mixture can be frozen and thawed as needed. It is filtered just after thawing. To 150 ml amounts (filtered), add 25 ml autoclaved 5% vitamin-free casein. Dilute 1:2 in distilled water for minimal broth. For plates, add 7 ml to 5 ml 2.0% washed agar solution.

Amino acids were purchased from Mann Research Laboratories and vitamin-free casein from Nutritional Biochemicals Corp., in Cleveland, Ohio.

compared to uracil independent colonies 1 to 2 mm in diameter. Pinpoint colonies were then tested on minimal plates with and without uracil to ascertain their uracil dependence.

Assay of transformants: Transformations to streptomycin resistance and to uracil independence were carried out in WILSON's broth according to the techniques of LERMAN and TOLMACH (1957). Cultures were then placed at 37°C for 45 to 60 min to allow time for expression in this complete medium before transfer to minimal plates. Dilutions were made in WILSON's broth and plating for both streptomycin resistance and prototrophy was done simultaneously. The total number of colony forming units was scored on minimal medium with addition of uracil and on blood agar plates after 40 to 48 hrs at 37°C.

DNA: The procedure for the preparation of DNA was that of LERMAN and TOLMACH (1957). This preparation was modified for strain A because of its refractiveness to lysis. Harvested cells suspended as described in sodium deoxycholate were frozen in a dry ice-cellosolve bath. Upon thawing they were immediately placed in an ice bath for 2 to 3 hrs with frequent agitation until lysis. The lysate was made 3 molar with solid NaCl and alcohol precipitation carried out as given. DNA in all preparations was measured according to BURTON's modification of the diphenylamine reaction (BURTON, 1956).

Isolation of auxotrophic mutants: Populations derived from single clones of strains R-6 or A were exposed to nitrous acid according to KAUDEWITZ (1959). Mutants were selected by the penicillin enrichment technique. Plates were prepared using minimal medium and 0.1 units penicillin/ml. Cultures were diluted so that 200 to 1000 colony forming units survived the 5 hrs exposure to penicillin at 37°C. To each plate was added after 5 hrs, 0.5 ml supplemental amino acids and pyrimidines, 0.3 ml human blood, 3.0 ml soft agar (double agar concentration) and 2 ml penicillinase (Mann, 10 mg/ml). Plates were refrigerated for 1 hr in order to allow penicillin destruction before growth of cells resumed. Colonies were picked after 48 hrs at 37°C for testing on minimal and supplemented media.

Synthesis of a double mutant: A strain combining the uracil alterations of two mutants, 84 and 47, was isolated by transforming mutant 84 with DNA from strain 47. After transformation and growth for 1 to 2 hrs, the culture was plated on blood agar. Of 100 colonies streaked on sectors of differential medium, a majority showed areas of opaque growth where reversion had occurred (a property of strain 84). Colonies which appeared not to revert were checked in a plate transformation using 47 DNA (RAVIN 1954). Finally 16 colonies remained for tube transformations. One of these showed a clear-cut double mutant genotype. It was transformed by wild-type DNA but failed to be transformed with 84 or 47 DNA. DNA recovered from this strain did not transform 47 or 84 to uracil independence.

RESULTS AND DISCUSSION

Uracil-dependent mutants: Auxotrophic mutants requiring uracil, phenylalanine, and lysine were obtained from the R-6 and A strains, at a higher frequency however from the former strain. Phenylalanine and lysine mutants grew to a slight extent on minimal medium because of the presence of casein, whereas uracil mutants showed no growth and consequently were the organisms selected for further investigation. From 40 uracil-requiring mutants, 12 were chosen for recombinational analysis. Mutants obtained from R-6 were not routinely competent. Therefore their uracil requirements were transferred to the highly transformable A strain.

Transformability of mutant strains: The efficiency of transfer of the wild-type marker to any mutant is given by Index I:

$$\frac{\text{Transformants to Uracil Independence (UI)}}{\text{Transformants to Streptomycin Resistance (SR)}},$$

where SR is the reference marker. Some values for this index are shown in Table 6. The standard deviations on three or four determinations for each mutant ranged from 0.013 to 0.097. The mean Index I value for the double mutant 84–47 was 0.542. The high transformability of the latter should be noted.

The span of efficiency for various markers, from about 0.12 to 1.0 relative to streptomycin resistance, agrees with the higher single-site mutations reported by LACKS (1966), but his lowest values are below 0.03. It should be noted that measurable rates of reversion to wild type are presented below for both the highest efficiency marker and two others of quite low efficiency. Attempts to explain the different marker efficiencies have been offered by LACKS (1966), by GRAY and EPHRUSSI-TAYLOR (1966), and others. Relevant molecular data have been provided by GURNEY and FOX (1968).

Index I was tested for its variability to such conditions as changes in the competence of the recipient culture and variation in the DNA concentration. The ratio of transformants to UI and to SR remained constant during a 200-fold change in the competence of the cells generated during the growth of a culture. Similarly, a 100-fold change in DNA concentration had no effect on this index.

Expression of uracil independence: In transformation to streptomycin resistance at least 90 min is required before the newly introduced genetic material is fully expressed (HOTCHKISS 1957). Streptomycin is added only after the newly transformed cells have been incubated for 2 hrs at 37°C. In uracil transformation only those cells will grow on minimal medium which have incorporated the wild-type allele. If cells are exhausted rapidly of their uracil reserves, the success of the incorporation will depend on the length of contact with complex medium. The following experiment was designed to determine the amount of time necessary to supplement cell reserves before expression of uracil independence. Strains 84 and 99, each exposed to wild-type DNA and treated with DNAase, were diluted ten-fold in complex medium and incubated at 37°C. Samples were diluted in and plated on uracil-free medium over a period of 105 min. The number of transformants to wild type in strain 99 remained constant over this period of time. Strain 84, however, showed an increase of 1.5 times in the number of transformants during 30 min exposure to complex medium. This led to the routine procedure of incubating transformed cells 45 to 60 min in complex medium before transfer to minimal plates.

Estimation of mutation rates: The mutation rate to uracil independence was estimated for only five strains using the null fraction method of LURIA and DELBRÜCK (1943). The method assumes, in this instance, that one wild-type cell can grow without inhibition in the presence of a large number of mutant cells. It was found that certain of the uracil-requiring strains inhibit the growth of wild-type cells when plated in the proportion of 10^6–10^7 to one, respectively. The estimation of the reversion rates is given in Table 2. Of this group, 47 and 50 inhibited growth of wild-type cells. (No inhibition was noted at the dilutions which were used for the enumeration of transformants.)

Transformation yield in fine structure analysis: In recombination experiments

TABLE 2

Reversion rates of five uracil mutants estimated by the null fraction method

Strain	Inhibition of prototroph by mutant	Reversion rate mutants/division
84	None	$5.8/10^9$
47	Inhibition	$6.7/10^{10}$
99	None	$< 5.7/10^{10}$
48	None	$5.0/10^9$
50	Inhibition	$< 2.0/10^{10}$

employing a mutant recipient *a* and a mutant DNA *b*, Index I was modified to give an index:

$$\frac{\text{Recombinants to UI}}{\text{Transformants to SR}} \quad \text{(Index II)} .$$

LACKS and HOTCHKISS (1960a) first introduced a denominator term into Index II to account for the "intrinsic" differences among markers. This is given explicitly as follows in Index III:

$$\frac{\dfrac{\text{Recombinants to UI of Recipient } a \times \text{DNA } b}{\text{Transformants to SR of Recipient } a \times \text{DNA } b}}{\dfrac{\text{Transformants to UI of Recipient } a \times \text{DNA } \textit{wild}}{\text{Transformants to SR of Recipient } a \times \text{DNA } \textit{wild}}}$$

where Recipient *a* = a uracil mutant which is streptomycin sensitive

DNA *b* = DNA from a uracil mutant with the streptomycin resistance marker

DNA wild = DNA from the uracil independent parental strain which has the streptomycin resistance marker.

LACKS and HOTCHKISS in their index compared the recombination of any two markers to yield wild type to the efficiency with which a positive allele was transferred to the recipient mutant strain from wild-type DNA. As pointed out by them, the resulting wild type depends not only on the exchange of the mutated allele in the recipient strain for a positive allele, but also on the omission of the mutant marker from the incoming DNA. This latter effect may also be marker specific. Since it is impossible in practice to select for transformants other than wild type in the uracil system, the index could be accepted as an approximation. The LACKS and HOTCHKISS index has been criticized by EPHRUSSI-TAYLOR, SICARD and KAMEN (1965) on the basis that the correction may still allow threefold differences in reciprocal recombination frequencies. More recently SICARD and EPHRUSSI-TAYLOR (1965) have used it if the cross is well controlled and if it considers only short distances.

Some comparisons were made between the values of Index II and Index III for reciprocal crosses. If all markers were incorporated with the same frequency,

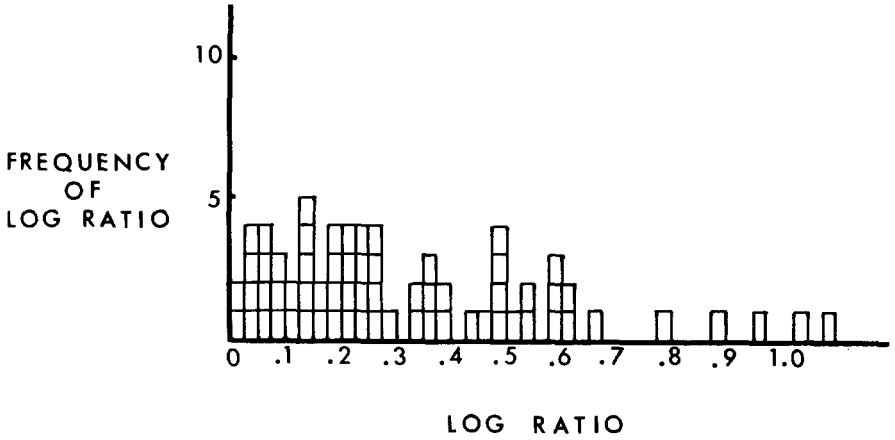

FIGURE 1.—Frequency histogram for the logs of the ratios of reciprocal crosses of 12 uracil mutants. Only one-half of the logs are given since the remaining half of the histogram is a mirror image of the first half.

$$\text{Ratio} = \frac{\text{Index II for Cross Recipient}_a \times \text{DNA}_b}{\text{Index II for Cross Recipient}_b \times \text{DNA}_a}$$

then recombination would depend on distance between markers, and the ratio of reciprocal values would approach one. Ratios of indices of reciprocals for all pairs of recombinants among the 12 mutant strains were set up using in one case the Index II values and in the other Index III. A frequency histogram of the log ratios is shown for each index, Figures 1 and 2. Clearly the log ratios employing Index III are clustered within $\pm$ 0.5 log units while those for Index II are scattered and extend beyond $\pm$ 1.0 log units.

A statistical analysis was undertaken in order to determine whether the ratios of frequencies in reciprocal crosses using Index III were clearly different from those using Index II in which no allowance is made for differences in marker incorporation efficiency. For simplification only those ratios for the 8 closely linked markers of group I were included (see Figure 3). Ratios were selected so that in the following:

$$\frac{\text{Index: Recipient}_a \times \text{DNA}_b}{\text{Index: Recipient}_b \times \text{DNA}_a}$$

the *a* marker occurred on the right according to the map. This selection was done in order to include only one of the possible ratios for each pair of markers. The mean and the variance of the logarithms of the ratios were determined when the index employed was II or III. Results are shown in Table 3.

Using the significance test for the difference between the variances of two samples and a significance level of 0.01 (BURINGTON and MAY 1958), the two samples of ratios were found to be clearly different. From this analysis, a preference for Index III was indicated as a result of two factors: 1) the ratios of reciprocal crosses were distributed near the value of one using Index III; and 2) the variance of these ratios using Index III was seven times less than that using Index II.

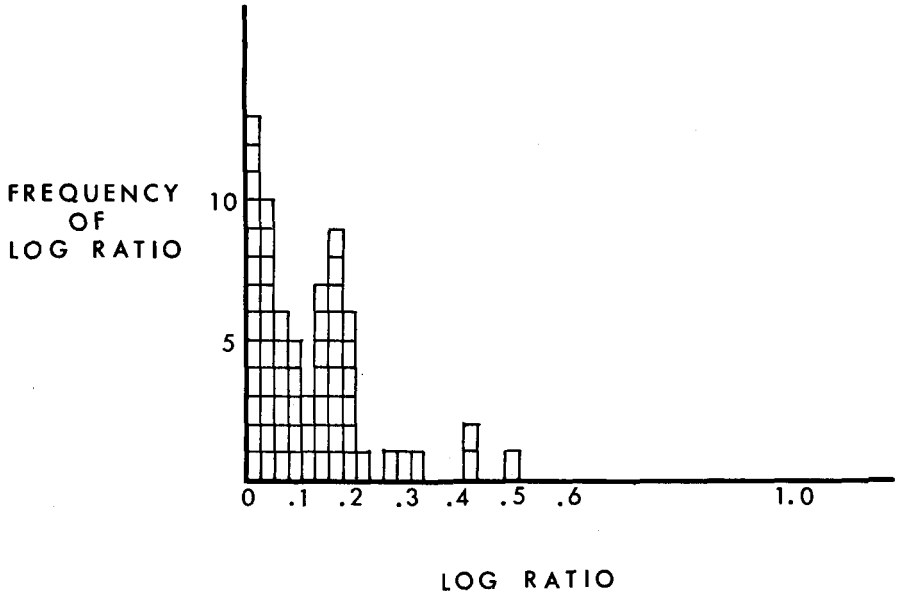

FIGURE 2.—Frequency histogram for the logs of the ratios of reciprocal crosses of 12 uracil mutants. Only one-half of the logs are given since the remaining half of the histogram is a mirror image of the first half.

$$\text{Ratio} = \frac{\text{Index III for Cross Recipient}_a \times \text{DNA}_b}{\text{Index III for Cross Recipient}_b \times \text{DNA}_a}$$

TABLE 3

Means and variances of the logarithms of the ratios of indices for reciprocal crosses

Index	Mean log ratio	Variance
II	—.016	.229
III	.011	.031

An arrangement for uracil markers: The arrangement of markers through the use of matrix fitting provided a means whereby all recombination values could be examined. Thus further evaluation of the transformation yield as Index II or as Index III could be made. The model for such an arrangement of markers in a matrix is as follows: Assuming a linear genetic structure, a group of mutations may be unambiguously ordered and indexed alphabetically *a, b, c . . . h, i. j,* etc. A distance between any pair of markers in this system is defined by an appropriate measure of the transformation frequency, T_{ab}, where one marker located in the recipient cell is designated by the subscript, *a,* and a second in the transforming DNA is designated by subscript, *b.* When this assembly of markers is arranged in its correct order in a matrix, the following relationships should hold:

$$T_{ij} < T_{ik}$$
$$T_{ij} < T_{hj} \ .$$

TABLE 4

A matrix arrangement for mutations using Index III values

Recipient strain	DNA donor strain 99*s*	50*s*	84*s*	96*s*	85*s*	51*s*	94*s*	47*s*
99	0	.006	.110	.127	.152	.349	.364	.558
50	.016	0	.132	.140	.173	.294	.310	.435
84	.120	.085	0	.030	.120	.195	.248	.513
96	.116	.114	.028	0	.073	.135	.214	.344
85	.099	.122	.048	.066	0	.064	.117	.264
51	.318	.285	.158	.173	.195	0	.074	.124
94	.359	.318	.272	.222	.209	.109	0	.181
47	.425	.429	.461	.371	.376	.192	.135	0

To fulfill the conditions of linearity, an increase above the zero diagonal must proceed in the direction of the arrows. To fullfill the conditions that reciprocals bear a direct relationship to each other, an increase below the zero diagonal must proceed in the direction of the arrows.

Once transformation yields are available for an unordered set of mutations, the defined measure of frequency can be calculated, and matrix fitting can proceed until an arrangement is attained which fulfills these conditions. The matrix will also include frequencies for reciprocal crosses. Since the roles of the parental chromosomes are not identical, there is no *a priori* reason to expect that the condition $T_{ij} = T_{ji}$ will be fulfilled. The search for a generally applicable measure of frequency which fulfills that condition is of interest because it may be expected to reflect the nature of the interaction.

Inserting Index II or Index III for the measure of frequency, two arrangements are shown in Table 4 and Table 5 for 8 of the uracil mutants. To fulfill the conditions of linearity there must be an increase in the indices above and to the right of the zero diagonal. Transformation yields of reciprocals must show an increase which proceeds down and to the left below the zero diagonal.

TABLE 5

A matrix arrangement for mutations using Index II values

Recipient strain	DNA donor strain 50*s*	99*s*	96*s*	85*s*	51*s*	94*s*	47*s*	84*s*
50	0	.003	.023	.029	.049	.052	.073	.022
99	.00065	0	.015	.018	.041	.043	.066	.013
96	.016	.017	0	.011	.019	.031	.049	.004
85	.026	.021	.014	0	.014	.025	.057	.010
51	.033	.036	.019	.022	0	.008	.014	.018
94	.071	.079	.049	.046	.024	0	.041	.060
47	.078	.077	.068	.069	.035	.025	0	.084
84	.085	.120	.030	.120	.195	.248	.513	0

To fulfill the conditions of linearity, an increase above the zero diagonal must proceed in the direction of the arrows. To fullfill the conditions that reciprocals bear a direct relationship to each other, an increase below the zero diagonal must proceed in the direction of the arrows.

TABLE 6

Index III values for crosses involving groups II and III

Recipient strain	DNA donor strain 47*s*†	48*s*	50*s*	51*s*	84*s*	85*s*	94*s*	95*s*	96*s*	97*s*	98*s*	99*s*	Wild type *s**
48	1.04	0	0.610	1.21	0.850	1.28	1.03	1.11	0.885	0.094	0.975	1.12	0.318*
95	1.07	0.586	0.980	0.963	0.845	0.991	0.725	0	1.43	0.941	0.026	1.48	0.310*
97	0.985	0.192	1.10	1.06	1.15	0.697	0.887	0.809	0.875	0	0.980	0.940	0.277*
98	1.01	1.39	1.13	1.21	0.785	1.09	1.23	0.022	1.35	1.36	0	0.851	0.308*
47	...	0.770	...	...	...	...	...	1.07	...	0.945	0.990	...	0.182*
50	...	0.953	...	...	...	...	...	1.17	...	0.677	1.55	...	0.167*
51	...	0.975	...	...	...	...	...	1.09	...	1.42	1.02	...	0.113*
84	...	1.04	...	...	...	...	...	0.895	...	0.960	1.12	...	1.00*
85	...	0.810	...	...	...	...	...	0.935	...	0.981	0.743	...	0.214*
94	...	1.07	...	...	...	...	...	1.06	...	1.39	1.21	...	0.220*
96	...	0.890	...	...	...	...	...	1.02	...	0.955	0.945	...	0.143*
99	...	0.982	...	...	...	...	...	1.00	...	1.25	0.940	...	0.118*

* Values are for Index I.
† *s* indicates presence of the streptomycin resistance marker.

The order of mutations such as that in Table 4 is the best that can be made using Index III. This arrangement is slightly better than one in which 50 precedes 99. In Table 5, where Index II has been used for the transformation yield, mutation 84 placed in the last column and last row is a misfit. It is impossible to place 84 anywhere in the matrix so that reciprocals will obey the expected relationships. That marker 84 does belong in this linkage group is shown subsequently. Further, all predictions for the results of three-factor crosses made on the basis of the order of Table 4 have been verified experimentally. In matrix fitting Index III was preferred for expressing transformation yield.

Markers 48, 95, 97 and 98 were classified as unlinked to the other 8 markers and their recombination values are given in Table 6. The wild-type Index I is given for each recipient. Index II can be calculated from these values as the product of Index III and Index I of the recipient.

The geometric mean distances of reciprocal crosses computed from Index III values of Table 4 and Table 6 are shown in Figure 3. It can be seen here that there is good agreement between long distances and the sum of intervening small distances. There are certain departures from this general picture which will be discussed in a later section particularly with respect to the possible role of negative interference in this transformation system.

Use of double mutant 84–47 to demonstrate linkage in group I: The possibility of showing linkage of marker 84 to 47 within group I was realized with the isolation of the double mutant 84–47. If its transformation to wild type is effected by the independent (unlinked) incorporation of these markers, the frequency of transformation using wild-type DNA should be comparable to that using a combination of 84 and 47 DNA preparations, the product of the frequencies of each marker incorporated separately, 0.020×0.004. The observed frequency of transformation of markers from wild-type DNA was 0.010. These values were

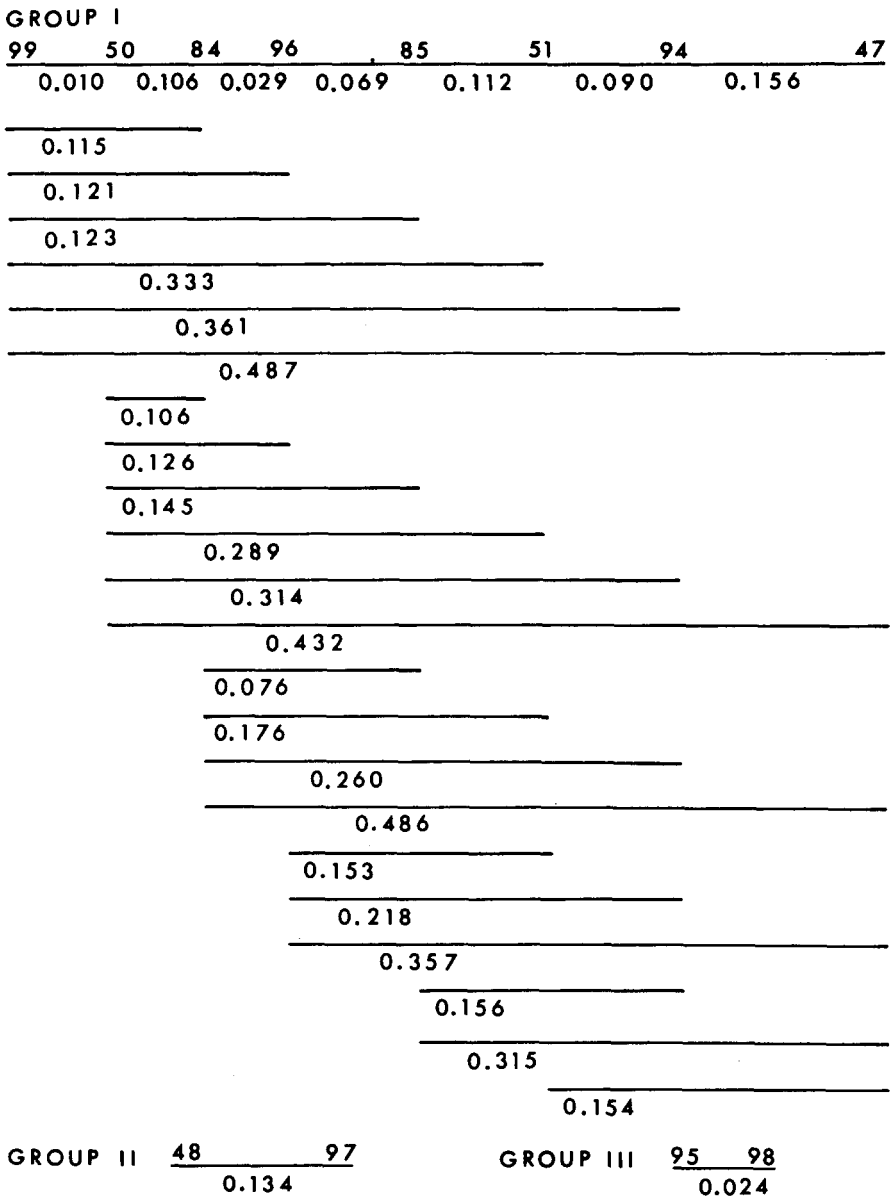

FIGURE 3.—Geometric mean distances for reciprocal crosses in three linkage groups of uracil dependent mutants.

taken from a single representative experiment where transformation frequency is equal to the number of transformants compared to the total colony forming units. While the diminished yield of double transformants relative to the more frequent single transformants seems qualitatively in accord with expectation for linked markers, the observation that the frequency of doubles is higher by a factor

of 2½ than the less frequent single is anomalous. Thus the probability of transformation of marker 47 by wild-type DNA is enhanced by its association with marker 84. IYER and RAVIN (1962) found a corresponding effect in the transformation of erythromycin markers. LACKS (1966) and GRAY and EPHRUSSI-TAYLOR (1967), on the other hand, have found that transformation in the double mutant is dominated by the lower index.

Three-factor crosses: Using the double mutant 84–47, certain predictions for three-factor crosses were possible from the arrangement of uracil markers in the matrix of Table 4. Mutants 50 and 99, located outside of the 84–47 region, could be expected to yield a recombination index with the double mutant approximating that obtained for the index of each of these markers with the single marker 84. It was assumed that reciprocal crosses of 50 or 99 with strain 84–47 would necessitate only 2 crossovers. In the case of markers between 84 and 47, when 84 and 47 are in the recipient cells, 4 crossovers are necessary to obtain wild-type recombinants avoiding the inclusion of the marker in the mutant DNA; when 84 and 47 are in the DNA then only 2 crossovers are necessary to obtain wild type but the exclusion of 2 linked markers from the recombinant chromosome is also imperative. Markers between 84 and 47 could, therefore, be expected to recombine in this system at a frequency much lower than that of 50 or 99. Experimental values for the recombination of all mutant DNA preparations with recipient 84–47 are given in Table 7. Values for the reciprocal crosses have also been given.

These results are in agreement with expectation. Both 50 and 99 recombined with strain 84–47 at frequencies ranging from 0.095 to 0.145. Markers 51, 85, 94 and 96 recombined with those of strain 84–47 at frequencies of 0.007 to 0.064. High index values were obtained with the unlinked 48, 95, 97 and 98 markers in agreement with previous recombination values in which two-factor crosses were examined.

TABLE 7

Index III values using the double mutant 84–47 as recipient and as source of transforming DNA

DNA donor strain	Recipient 84–47 index value	Recipient strain	DNA donor strain 84–47*s* index value
47*s*	0	47	0
48*s*	1.13	48	1.27
50*s*	0.123	50	0.095
51*s*	0.041	51	0.025
84*s*	0	84	0
85*s*	0.026	85	0.030
94*s*	0.064	94	0.021
95*s*	1.10	95	0.715
96*s*	0.011	96	0.007
97*s*	0.860	97	1.20
98*s*	1.19	98	0.975
99*s*	0.136	99	0.145
wild type *s*	0.534*	84–47	0

* Index I for this transformation only.

A case for negative interference in transformation: Negative interference has been defined by CHASE and DOERMANN (1958) as the positive correlation of a recombination event in one region with a recombination event in a nearby region. In their system, T4 bacteriophage, this correlation increased as map intervals decreased.

Where data on three (or more) factor crosses are not available, a somewhat less conspicuous result of negative interference may be seen by examining the additivity of recombination frequencies between closely linked markers. The above random prevalence of clustered crossovers will result in an apparent map expansion at short distances. Negative interference in transformation could be looked for by comparing the recombination yields from the crosses in two short regions to the yield in one long region, the two short regions being exactly delimited by the long region. For markers in the order *a*, *b*, and *c*, the wild-type transformants resulting from the cross $\text{Recipient}_a \times \text{DNA}_b$ and the cross $\text{Recipient}_c \times \text{DNA}_b$ are to be compared with the wild-type transformants from the cross $\text{Recipient}_a \times \text{DNA}_c$. Two events are assumed to be necessary in each cross to yield wild type. Where no interference is present, the sum of the recombinants of the first two crosses should equal the number of recombinants from the third cross. If as in negative interference there is a high correlation of the second recombination event with the first in the above crosses, and this correlation is greater with the increasing closeness of markers, then it might be expected that the first two crosses yield together more recombinants than the third cross.

A similar system for demonstrating negative interference was described in the T4 system (EDGAR, *et al.* 1962) where recombination was assayed by the growth of r^+ phages on strains of lysogenic *E. coli* (λ). In this system distances between two *r* types were compared to the sum of intervening short distances. All distances were measured by the numbers of wild-type recombinants arising from two-factor crosses relative to total phage yield.

A plot of map data from Figure 3 according to the phage model first suggested the possibility that we were dealing with a region of high negative interference in the uracil transformation system. In Figure 4, the frequency of recombination of outside markers is plotted *versus* the sum of small distances (the sum of recombination frequencies). The number of small distances in this sum is given. There is an increase in the divergence of points from the theoretical 45 degree line where one long distance is exactly equal to the sum of all intervening short distances.

Figure 5 shows a plot differing from the EDGAR type in that the logarithm of one long map distance C is compared to the logarithm of the sum of each independent pair of distances exactly comprising C. This gives a less restricted view of recombination events which in the EDGAR plot are limited to a comparison of long distances with a sum of small distances all of which may be influenced by high negative interference. The plot in Figure 5 is of the relationship $C = (A + B)$ where C represents the distance between any two markers of Group I and $(A + B)$ represents any two consecutive distances delimited by C. The logarithm of the

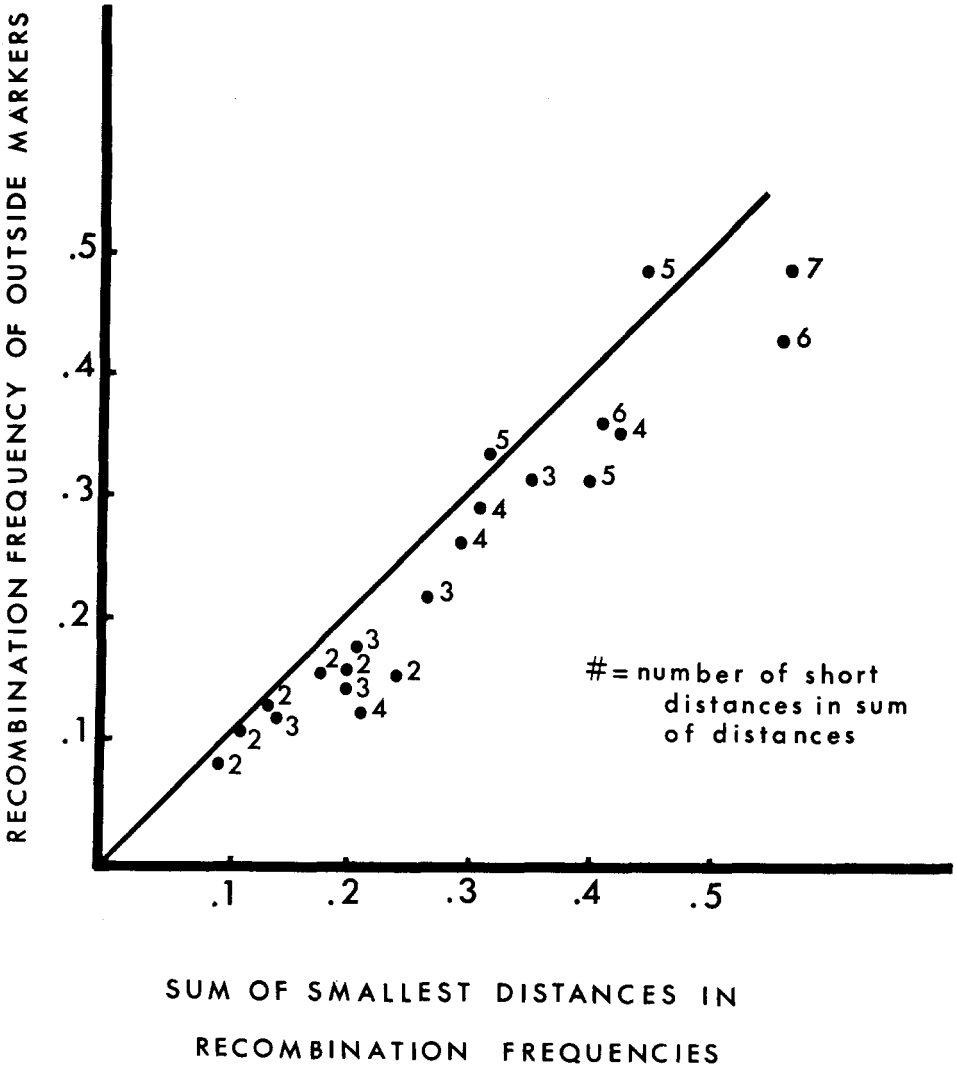

FIGURE 4.—The recombination frequency of outside markers versus the sum of shortest distances according to EDGAR *et al.* (1962).

geometric mean distances for C (from Figure 3) and the logarithm of the sum (A + B) are plotted as abscissa and as ordinate, respectively. At the left end of the curve it is apparent that all points are above the proportionality line C = (A + B); i.e., (A + B) > C. For low values of C, i.e., for values less than 0.2 Index III units (log = −0.7), any two consecutive distances delimited by C are greater than C. Were all the points not simply accidentally located above the line of proportionality, it might be strongly contended that the phenomenon of negative interference was operative in this transformation system. The data were, therefore, analyzed to determine the significance of the occurrence of these points.

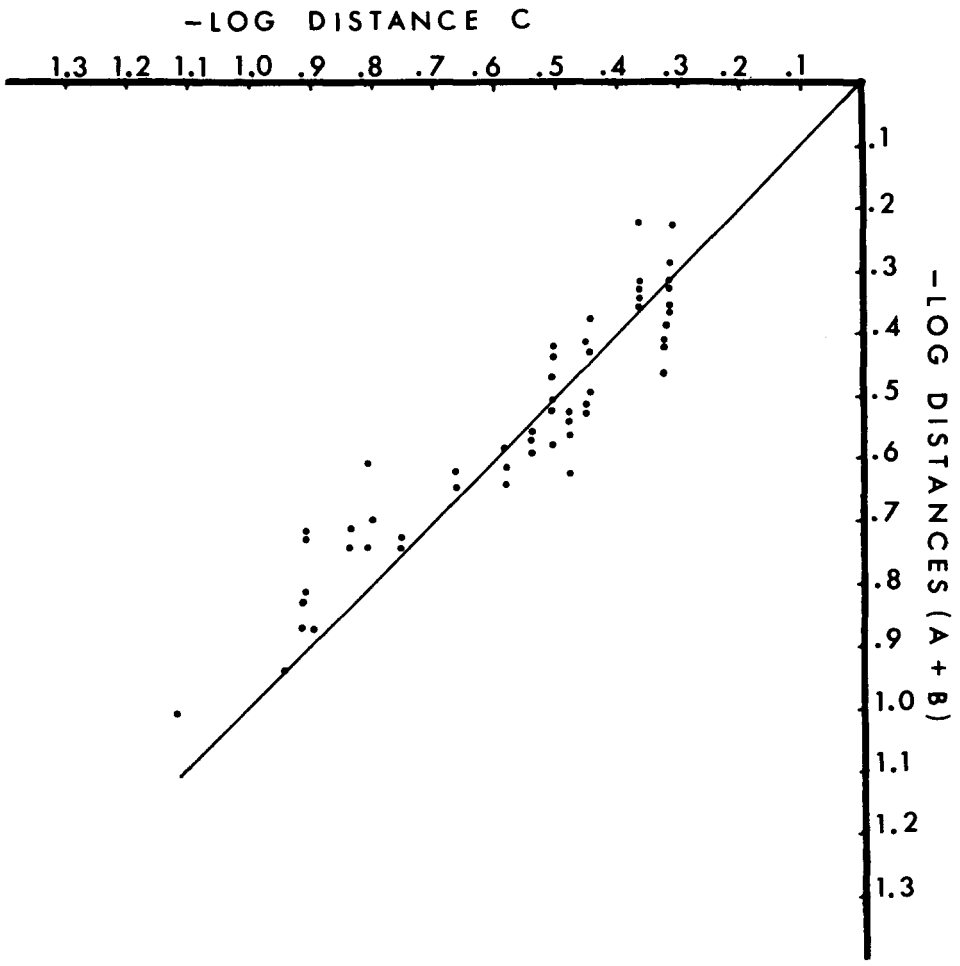

FIGURE 5.—Distances C versus (A+B) using the log of the geometric mean distance. The solid line is equal to the relationship when log C = log (A+B).

The data were divided into two sets, points below (Set I) and those above (Set II) the value of 0.2 Index III units for C. The question was asked as to how often would all of the points of Set I occur above the line of proportionality if given the variance of the set of points greater than C = 0.2 Index III units. For each set of points using log values, the mean deviation from the line of proportionality and the variance were determined. These results are summarized in Table 8.

As can be seen from the data, the variance of the two sets was quite close. Using the variance of Set II, however, the difference between means was determined. As a result of the significance test for the difference between means (BURINGTON and MAY 1958), it was shown that in less than one in 1000 times would we expect Set I with a mean of 0.095 and a variance of 0.0041 to be accidentally distributed as it is in Figure 5. Therefore, we conclude that it is extremely likely that nega-

TABLE 8

Mean deviations and variances of (A + B) distances from the line of proportionality

	Mean deviation	Variance
Set I		
C distances < 0.2	0.095	0.0038
Set II		
C distances > 0.2	—0.014	0.0041

tive interference is operative in regions less than approximately 0.2 Index III units. On our map this could include a region of at least 5 markers, for example the region 99 to 85.

While the conventional interpretation of negative interference as the reflection of a mechanism of recombination involving a short region of effective pairing is formally applicable to these data, as indicated below, its relevance to the actual biological mechanism is not clear, and other hypotheses that might generate similar quantitative effects should also be considered.

Although negative interference is conventionally defined in terms of crossings involving three or more factors, where the observation of a recombination between two adjacent markers is found to increase the probability of a recombination between one of these and another nearby marker, the present data do not provide this kind of evidence. Where we have been able to carry out three-factor crosses, the markers are too widely separated to show measurable interference. It has been shown (BERNSTEIN 1962; STAHL, EDGAR and STEINBERG 1964) that both the results of three factor crosses and the excess map distance obtained by the summation of low recombination frequencies can be accounted for by the assumption of a mapping function relating the probability of observing a net recombination to distances between markers and certain adjustable parameters that presumably reflect the biological properties of the recombination mechanism. The form of the mapping function is determined by the specific assumptions concerning the processes taking place in the switch region.

The data of Figure 5 have been compared with the least intricate function tested by STAHL, *et al.* (1964). This is identified as the modified BERNSTEIN model which gives the following equation:

$$P_D = \alpha D + 2(1-\alpha)\ K\ (1-e^{D/K})$$

Where P is the probability of recombination between two markers separated by a distance, D, which is proportional to the number of intervening nucleotides; K is a biological length along the chromosome corresponding to the mean distance between an exchange and a second exchange returning the sequence to the starting strand; and α is an adjustable parameter that was not originally included by BERNSTEIN (1962).

A simple way to compare our data with the mapping function is to consider the ratio of the sum of the probabilities of recombination in two adjacent regions of length D_1 and D_2 to the probability of recombination in a region of lengths $D_1 + D_2$. The value of α is arbitrarily taken as 0.3 corresponding to the best value

for fitting T4 frequencies and K is an adjustable parameter which specifies the mean distance to a second exchange. Without presenting a detailed comparison, the data and the model are found to be compatible if it is assumed that K can have a value of map distance of 0.011 in the units of recombination Index III.

It is of interest to compare this value of K, noting mainly its order of magnitude rather than the precise value, with an estimate of map distances in terms of nucleotides. Using a similarly derived map recombination index, LACKS (1966) estimates a value of 5×10^{-4} recombination index units per nucleotide. The mean distance between an exchange with transforming DNA and return would then be $0.011/5 \times 10^{-4}$ or, roughly, 20 nucleotides. It is difficult to reconcile so short a distance with the much longer segments of DNA, several thousands of nucleotides found by FOX and ALLEN (1964) to be integrated into the host chromosome from transforming DNA input.

A different approach to the question of interference is provided by the clear indications in the data that recombination frequency is determined in part by the specific local character of the difference between the nucleotide sequences of the marker and wild type. Such indications have also been found by others in other loci (IYER and RAVIN 1962; LACKS 1966; and GRAY and EPHRUSSI-TAYLOR 1967). With recombination between two relatively close markers, the process that responds to marker specificity may occur at both sites and be responsible for interaction between them. Obviously the relevant distance is the separation of the markers, rather than the end to end lengths of the incorporated segment. Again, using LACKS' calibration factor, the relevant distances between markers are in the vicinity of 100 nucleotides. This distance is somewhat too great to lend plausibility to the interaction between two sharply localized enzymatic processes or two anomalies in DNA structure. If, on the other hand, there is an excision mechanism that is triggered by a fault in base pairing at the marker site, and this could move down the chromosome an appreciable distance, as presumably is the case of the UV repair process, then interaction at this separation can be imagined.

Attention has been given to the study of GRAY and EPHRUSSI-TAYLOR (1967) particularly with respect to their three-factor crosses which can be compared to the ones reported here. In both cases, differences in the efficiency of transformation of mutants have been accounted for by the use of the LACKS and HOTCHKISS index (Index III). Both have used the high level streptomycin resistance marker of DR. R. D. HOTCHKISS as a reference. The markers in the double mutants of GRAY and EPHRUSSI-TAYLOR are separated by distances of 0.1 to as much as 0.6 index units with the majority of distances greater than 0.2. The geometric mean distance between the uracil markers 84 and 47 is 0.485. In 12 crosses given in Table 9, the mean ratio of Observed : Expected is 1.02. These values compare closely with those of GRAY and EPHRUSSI-TAYLOR. The results indicate that there is no observable interference using the mutant 84–47. However, the map distances between these markers are so large that negative interference, as detected in close two-factor crosses, would not be expected. Technically the strain consisting of mutations 84 and 47 was relatively simple to synthesize. Double mutants of much

TABLE 9

Comparison of the observed indices of three-factor crosses with values expected from two-factor crosses

Recipient	Donor DNA	Observed	Expected	Observed/Expected
84–47	99*s*	0.136	0.120	1.13
84–47	50*s*	0.123	0.085	1.45
99	84–47*s*	0.145	0.110	1.32
50	84–47*s*	0.095	0.132	0.72
84–47	51*s*	0.041	0.037	1.11
84–47	85*s*	0.026	0.045	0.58
84–47	94*s*	0.064	0.033	1.94
84–47	96*s*	0.011	0.011	1.00
51	84–47*s*	0.025	0.020	1.25
85	84–47*s*	0.030	0.013	0.59
94	84–47*s*	0.021	0.049	0.43
96	84–47*s*	0.007	0.010	0.70

closer span and of greater value in a study of interference would have been impossible to synthesize in the system of selection utilized here.

The index III values in Table 7 for the cross in which the double mutant is present in the recipient are greater by a factor averaging 1.8 than the indices for the reciprocal crosses where the two mutant markers are present in the donor DNA. This may seem somewhat surprising, since the former would be expected to require four exchanges between the donor and recipient DNA, while the latter would require only two. It can be inferred from the experimental result that the probability of having one or an odd number of exchanges between the outermost markers and the nearest ends of the donor DNA must be very nearly the same as the probability of an even number of exchanges; that is to say, the end of the donor is effectively unlinked to the nearest marker. Secondly, the frequency of transformation of a doubly mutant cell to wild type with wild-type DNA, which enters into the denominator of the calculation of the index III value, is diminished by the significant probability of a recombination including only one or the other of the two markers.

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SUMMARY

A number of auxotrophic mutants of pneumococcus have been isolated using a modification of a previously described medium. Twelve of these mutants requiring uracil were studied in recombination experiments. The transformation yield in genetic crosses was dependent upon the efficiency of transformation of the recipient mutant. When allowance was made for recipient variation in a recombination index, reciprocals showed a close relationship to each other and a linear

arrangement of linked markers was possible. The comparison of long map distances with the sum of intervening short distances indicated that negative interference was operative in this transformation system in regions less than 0.2 index units. Reasonably good additivity among recombination indices was found and was improved by correction for negative interference.

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