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Suppressors in Yeast

DONALD C. HAWTHORNE and URS LEUPOLD[1]

With 2 Figures

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1. Introduction

Suppressors are genes which suppress the expression of mutant phenotypes. The suppressors can be considered to be mutant genes themselves since they are generally not found in the wild type strains but are obtained by selecting for revertants of the mutant phenotype. A revertant arising by a mutation other than a base change in the original mutant codon carries a suppressor. If the new event occurred at another site within the original mutant gene,

1 Genetics Department, University of Washington, Seattle, Washington and Institute of General Microbiology, University of Bern, Switzerland.

it is called by some an intra-locus suppressor. However, in our view this is a misuse of the term suppressor which originated as a designation for those mutations occurring in some other gene, the suppressor gene, and which now must be defined as an external suppressor. The external or extra-locus suppressors can be further classified as being either functional suppressors or informational suppressors.

The functional suppressors act upon all the mutant alleles found at a given locus: they provide an alternate pathway or a by-pass of the mutant block. We shall not deal with them except to provide the following examples of their occurrence in yeast. Functional suppressors in *Saccharomyces cerevisiae* are the half revertant suppressors of the adenine-histidine blocks of *ade3* (ZIMMERMANN and SCHWAIER, 1963) and the isoleucine-valine blocks of *ilv2*, *ilv3*, and *ilv5* (KAKAR et al., 1964). An example where the mutant block is by-passed is seen with the *i⁻gal*, galactose constitutive, mutants obtained as suppressors of the *gal3*, slow galactose adaption, phenotype (DOUGLAS and PELROY, 1963). For other examples see MORTIMER and HAWTHORNE (1969).

The informational suppressors are allele-specific: they act upon only certain alleles at a given locus, but the same suppressor can act upon a wide variety of mutant phenotypes. The gene products of the informational suppressors intervene at some step in the translation of the mutant messenger-RNA so as to lead to the synthesis of a functional polypeptide. The most numerous suppressors of this category in yeast, the super-suppressors which have been described both in *Saccharomyces cerevisiae* (HAWTHORNE and MORTIMER, 1963) and in *Schizosaccharomyces pombe* (BARBEN, 1966; BARBEN and LEUPOLD, 1969), act upon nonsense mutants and are thought to be genes for tRNA's for the most part, although we shall provide some evidence that several suppressors may be coding for ribosomal components. Missense suppression has also been demonstrated in *S. cerevisiae* and again a mutant tRNA species is thought to be involved (GORMAN and GORMAN, 1971).

The precedence for the concept of informational suppression, the demonstration of nonsense mutants, and the identification of the nonsense codons, UAA, UAG, and UGA, has been set in experiments with *Escherichia coli* and its bacteriophage T4. For a summary of these developments, we can recommend two recent reviews (GAREN, 1968 and GORINI, 1970). *E. coli* was also the source of the cellular components for the *in vitro* studies resolving the amino acids codons. The resolution of the genetic code and its universality was a topic of a review in this series (JUKES, 1969). For the convenience of the reader, we will provide in the appendix a table of the code along with the proposed codon—anticodon pairing possibilities permitted by the wobble hypothesis (CRICK, 1966).

The appendix also includes a brief account of the life cycles of *S. cerevisiae* and *S. pombe* and the genetics pertinent to this study. For a fuller treatment of methods in yeast genetics, we again recommend two other reviews (MORTIMER and HAWTHORNE, 1969; LEUPOLD, 1970a).

2. The Characterization of the Super-suppressible Mutants in Saccharomyces cerevisiae and Schizosaccharomyces pombe

It is now accepted by those researchers working with bacteria and phage systems that the susceptibility of a mutant to amber (UAG), ochre (UAA), or umber (UGA, see appendix) suppressors defines it as a nonsense mutant. In yeast, does the criterion of suppressibility by the super-suppressors also define nonsense mutants? We can conclude that it does from the following considerations.

The suppressible alleles do not have the properties associated with missense mutants: as a rule, they are not leaky, temperature-sensitive, or osmotic-remedial. The original observations in *S. cerevisiae* were based on rather small samples, 23 suppressible alleles and 15 osmotic-remedial or temperature-sensitive alleles, distributed over 24 different loci (HAWTHORNE and FRIIS, 1964). However, for a single locus, a collection of 98 spontaneous *ade*8 mutants yielded 24 suppressible alleles versus 8 temperature-sensitive or osmotic-remedial alleles (ESPOSITO, 1968).

In *S. pombe*, the mutual exclusion of these mutant characteristics has prevailed at three loci for which extensive fine structure maps are available, *ade*7, *ade*6 and *ade*1 (LEUPOLD, 1958, 1961; GUTZ, 1961; LEUPOLD and GUTZ, 1965; RAMIREZ et al., 1963 and in preparation; CLARKE, 1965 a, b). Frequencies of suppressible versus temperature-sensitive or osmotic-remedial mutants were 23 vs. 9 among 203 *ade*7 mutants induced by UV and ICR-170 (BARBEN, 1966; MUNZ and LEUPOLD, 1970; LEUPOLD, unpublished), 8 vs. 10 among 125 *ade*6 mutants induced by UV and ICR-170 (LEUPOLD and GUTZ, 1965; BARBEN, 1966; MUNZ and LEUPOLD, 1970), and 31 vs. 40 among 365 *ade*1 mutants of spontaneous and UV or chemically induced origin (SEGAL, 1969; FLURY, 1970; FRIIS et al., 1971; SEGAL et al., 1973; RAMIREZ et al., in preparation).

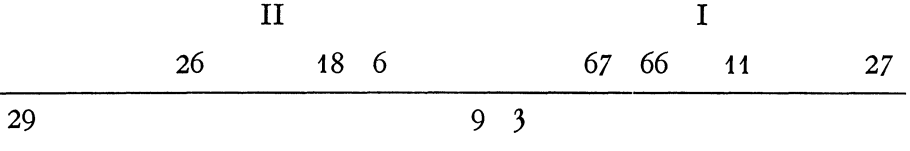
For genes where interallelic complementation can be demonstrated, the suppressible alleles, for the most part, are found in the non-complementing class. The few which do complement, do so only when combined with non-suppressible alleles. Moreover, in cases where the mutant sites have been placed on a fine structure map, the suppressible alleles are seen to complement only with mutants toward one side. This has been demonstrated with alleles of *trp*5 (MANNEY, 1964), *his*4 (FINK, 1966), and *leu*1 (NAKAI and MORTIMER, 1967, and MORTIMER, personal communication) in *S. cerevisiae* and with alleles of *ade*6 (BARBEN, 1966; MUNZ and LEUPOLD, 1970) and *ade*1 (BARBEN, 1966; FRIIS et al., 1971; SEGAL, 1969; SEGAL et al., 1973; FLURI et al., 1971 and in preparation) in *S. pombe*. The polarized complementation is interpreted to mean that the translation of the messenger-RNA stops at the site of the suppressible defect and that only missense sites covered by this peptide fragment will be complemented.

In *S. cerevisiae*, it has been possible to obtain physical-chemical evidence for chain termination with both *trp*5 and *his*4 suppressible alleles.

In the case of *trp*5, the gene for tryptophan synthetase, a single enzyme catalyzes three reactions:

1) Indole-3-glycerol phosphate $\rightleftharpoons$ Indole + glyceraldehyde-3-phosphate
2) Indole + L-serine $\rightarrow$ L-tryptophan
3) Indole-3-glycerol phosphate + L-serine $\rightarrow$ L-tryptophan + glyceraldehyde-3-phosphate

All three activities are missing in most *trp*5 mutants, but there are exceptional mutants where reaction (1) is retained (mutants 6, 18, and 26 with a phenotype of indole accumulation) or reaction (2) is retained (mutants 66 and 67 characterized by the ability to utilize indole in place of tryptophan for growth). These mutants are included in a simplified version of the map of the locus presented by MANNEY (1968).



From the clustering of mutants with these two phenotypes, it can be adduced that the catalytic site for reaction (1) is encoded in the right hand portion of the map, while reaction (2) is encoded in the left hand side of the map. The three suppressible alleles 3, 9, and 29 included on the map are able to complement missense mutants. Alleles 3 and 9 complement only allele 11, while allele 29 complements alleles 11, 66, and 67. All the missense alleles lie to the right of the nonsense partner, thus the direction of translation proceeds from the right to the left on the map.

The assay of crude extracts for reaction (1) showed that mutant 29 had about 10% and mutants 3 and 9 had between 0.5 and 1.0% of the wild type activity. (Mutant 29, although suppressible, is slightly leaky and is osmotic-remedial for growth on tryptophanless medium.) From the elution profiles for these enzymic activities from a Sephadex column, MANNEY (1968) concluded that the molecular weight of the active protein from mutants 3 and 9 was about 35000. The mutant 29 activity came off the column in the same fraction as the wild type enzyme which was calculated to be 160000 molecular weight.

The three enzyme activities coded by the *his*4 locus of *S. cerevisiae*, PR-AMP cyclohydrolase, PR-AMP pyrophospho-hydrolase, and histidinol dehydrogenase, are found in a complex which cannot be dissociated in the wild type strain. Since mutants lacking a single activity are isolated, the three activities can be assigned to three discrete regions, A, B, and C respectively, on the fine structure map of the locus. The mutants are also defined as A, B, or C from their pattern of complementation. The suppressible mutants are either C, BC or ABC in phenotype while mapping in the C, B, or A regions, respectively: thus, the translation is from left to right, A, then B, then C (FINK, 1966).

Extracts from a suppressible *his*4C mutant were compared to wild type extracts for the sedimentation of the PR-AMP cyclohydrolase and the PR-AMP pyrophospho-hydrolase active proteins by zonal centrifugation in a sucrose gradient. The estimates for the molecular weights were 45 000 for the mutant and 95 000 for the wild type proteins. A *his*4C missense mutant had these activities in a protein with the 95 000 molecular weight (SHAFFER et al., 1969).

A similar situation is encountered in the *ade*1 locus of *S. pombe* which has been shown to code for two enzyme activities involved in purine biosynthesis, GAR synthetase and AIR synthetase. Both activities appear to be associated with a single protein (FLURI et al., 1971 and in preparation). The *ade*1 locus is therefore believed to represent a single cistron coding for a bifunctional enzyme. Mutants lacking one or the other of the two enzyme activities are found to map in two discrete regions of the *ade*1 locus and to belong to two different complementation groups: Mutants which lack GAR synthetase activity map in the *ade*1A or left-hand region of the fine structure map of the *ade*1-locus. They are capable of complementing mutants which lack AIR synthetase activity and which map in the *ade*1B or right-hand region of the fine structure map. Mutants defective in both activities are incapable of interallelic complementation, and they can be located in either of the two regions.

All 23 suppressible mutants mapping in the *ade*1A region belong to the non-complementing type, whereas 5 out of 8 suppressible mutants mapping in the *ade*1B region are capable of complementing missense mutations in the *ade*1A region (FRIIS et al., 1971; SEGAL et al., 1973; RAMIREZ et al., in preparation). In agreement with their complementation behaviour, suppressible *ade*1A and *ade*1B mutants of the non-complementing type lack both activities. Suppressible *ade*1B mutants of the complementing type, on the other hand, lack AIR synthetase activity but retain GAR synthetase activity even though the specific activity is reduced if compared to that of the wild type enzyme (FLURI et al., in preparation). From these results, it can be concluded that translation proceeds from left (*ade*1A) to right (*ade*1B).

3. The Identification of the Nonsense Codons in Saccharomyces

a) A Direct Demonstration of UAA and UAG Mutants

Direct evidence for the identity of the nonsense codons in *S. cerevisiae* comes from the analysis of iso-1-cytochrome *c* mutants. SHERMAN et al. (1970) and STEWART et al. (1972) have exploited the fact that iso-1-cytochrome *c* is readily purified and the amino acid sequence can be determined. Two suppressible alleles, *cyc*1-2 and *cyc*1-9, result in no detectable iso-1-cytochrome *c* while back mutants of these alleles have normal levels of the protein. The revertant proteins can be wild type or differ from it by having glutamine, lysine, leucine, tyrosine or serine substituted in place of glutamic acid at position 2 (*cyc*1-9 revertants) or position 20 (*cyc*1-2 revertants). The codons for these amino acids, GAA and GAG (glutamic acid), CAA and CAG

(glutamine), AAA and AAG (lysine), UUA and UUG (leucine), UCA and UCG (serine) and UAU and UAC (tyrosine) differ by a single base from either the ochre, UAA, or amber, UAG, nonsense codons. However, since tryptophan (UGG) was never found to be substituted for the glutamic acid in the 45 revertants of *cyc*1-9 or the 33 revertants of *cyc*1-2, it was concluded that UAA was the nonsense codon carried by both mutants. Both alleles are suppressed by the same suppressors, class I, set 1 (GILMORE, 1967); although *cyc*1-9 is much less efficiently suppressed (GILMORE et al., 1971).

STEWART and SHERMAN (1972) also report that a mutant, *cyc*1-179 has been identified as an amber triplet at position 9 by the analysis of the revertant proteins. In this case tryptophan in addition to the above amino acids is found to be substituted for the wild type lysine residue. They also suggest that UGA is a nonsense codon in yeast since it does not appear to code for either cysteine or tryptophan: if it did, these amino acids would have been found in the revertants of the ochre mutants *cyc*1-2 and *cyc*1-9.

b) The Isolation of UAG and UGA Nonsense Mutants

With the realization that the super-suppressible alleles were nonsense mutants, a search for mutants bearing new nonsense codons, i.e. susceptible to a new set of suppressors, was undertaken in a group of mutants in which there is an enrichment for nonsense alleles. The complex locus *ade*5,7 provides a system in *Saccharomyces* which is comparable to the T4-rII deletion r1589 system for the detection of nonsense mutants (BENZER and CHAMPE, 1962). Mutants at this locus are classified as *ade*5, *ade*7, or *ade*5,7 by complementation tests. Diploids of *ade*5 × *ade*7 crosses give wild type growth on adenineless medium, while *ade*5,7 mutants do not complement *ade*5 or *ade*7 alleles (ROMAN, 1956). Fine structure mapping placed *ade*5 and *ade*7 mutants in separate regions and with few exceptions, the *ade*5,7 mutants were located among the *ade*5 sites (DORFMAN, 1964). On this basis it was proposed that the *ade*5 and *ade*7 regions represent two separate genes which encode a polycistronic messenger-RNA, and that translation starts with the *ade*5 cistron. Nonsense codons occurring in the *ade*5 cistron would prevent the translation of *ADE*7 and thus will be recognized as *ade*5,7.

The results of testing 105 mutants at this complex locus for their response to the original class of super-suppressors (Class I, Table 1) are consistent with the above prediction: 0 of 15 *ade*5 alleles, 6 of 47 *ade*7 alleles, and 16 of 43 *ade*5,7 alleles were suppressed (HAWTHORNE, unpublished).

The above tests for suppressibility were performed by isolating a suppressor in each mutant stock through the selection of double revertants for two known suppressible genes, *ura*4-1 (uracilless) and *leu*2-1 (leucineless), incorporated into the parental stock. To restore adenine prototrophy, the suppressor must not only recognize the nonsense codon in the mutant messenger-RNA but also substitute an amino acid which will lead to a functional polypeptide. A test requiring only the recognition of the nonsense

codon is achieved in diploids of constitution: *SUP*I (Class I) *ade7-1*/*ade5,7-x*, where *ade7-1* is a non-suppressible allele. Here it suffices that the suppressor simply mediates the translation of the nonsense codon to remove the impasse to the translation of the second cistron yielding the *ADE7* product; a wild type cistron for the *ADE5* product is provided by the *ade7-1* mutant.

The search for new nonsense codons was then directed toward those *ade5,7* alleles which were not suppressed in the diploid test. These *ade5,7* mutants were screened for their ability to give revertants spontaneously and in response to ethylmethanesulfonate, nitrous acid and ultraviolet regimens. Those which failed to give revertants from a sample totaling about 5×10^8 cells were considered to be deletions or reading frame shift mutants. On the other hand, the *ade5,7* mutants which gave 100–500 revertants were considered to be promising candidates for new nonsense codons. Revertant clones from these *ade5,7* mutants were backcrossed to wild type and tetrad analyses were undertaken to detect the presence of suppressors.

From the first four mutants examined in this manner, one allele *ade5,7-101* was reverted by suppressors of a new class. These suppressors did not act upon mutants from the original set of nonsense alleles, *leu2-1* *trp5-2* or *ura4-1*; however, they did act upon other mutant phenotypes *met8-1* (methionineless), *trp1-1*, *tyr6-1* and *tyr7-1* (tyrosine-phenylalanineless). (See Classes IX and X, Table 2.) The codon carried by *ade5,7-101* differs by a single base from the original nonsense codon: in the presence of a Class I suppressor, *SUP5*, it can mutate to an allele, *ade5,7-101′*, susceptible to the suppressor, *SUP5*. The fact that mutagens which favor the G+C→A+T transition, hydroxylamine and ethylmethanesulfonate, enhance the mutation *ade5,7-101* → *ade5,7-101′* indicates that *ade5,7-101′*, representing the original class of nonsense alleles, is an ochre (UAA) allele (HAWTHORNE, 1969b). The decision that the *ade5,7-101* mutant triplet is UAG rather than UGA rests on the observation that one can obtain, from wild type, suppressors of *ade5,7-101* (Class IX, Table 2) which are alleles of the Class I and II suppressors *SUP6*, *SUP7*, *SUP8* and *SUP11* (HAWTHORNE and MORTIMER, 1968, and HAWTHORNE unpublished). These suppressors are known to be substituting tyrosine in the translation of UAA (GILMORE et al., 1968, 1971). The tyrosyl-t-RNA genes can mutate to either ochre or amber, but not UGA, suppressors by a single base substitution in the anticodon triplet.

In the search for UGA mutants, a new series of 11 *ade5,7* mutants obtained with ethylmethanesulfonate treatment were tested for their susceptibility to either ochre or amber suppressors in the diploids of constitution *ade5,7-x*/*ade7-1*, *SUP*I/+ or *ade5,7-x*/*ade7-1*, *SUP*IX/+. Only 2 mutants responded to the ochre suppressors, while 6 responded to the amber suppressor. The remaining 3 *ade5,7* alleles reverted readily; and one, *ade5,7-143*, reverted by a suppressor. The new suppressor was allele specific: it did not act upon *ade5,7-101* or *ade5,7-101′*; nor did it act upon other amber alleles, *met8-1*, *trp-1*, *tyr6-1* and *tyr7-1*, nor upon ochre alleles, *ade2-1*, *his5-2*, *ilv1-1*, *leu2-1*, *lys1-1* or *ura4-1* (HAWTHORNE, unpublished).

The codon carried by *ade*5,7-143 differs from the ochre codon by a single base: in the presence of a Class I ochre suppressor, *ade*5,7-143 will mutate to *ade*5,7-143′, a susceptible allele. Again a G+C→A+T transition is involved since ethylmethanesulfonate enhances this mutation (HAWTHORNE, in preparation). Moreover, in the presence of the new suppressor, the ochre alleles *leu*2-1, *lys*1-1 and *his*5-2 will mutate, with UV irradiation, to the alleles *leu*2-1′, *lys*1-1′, and *his*5-2′ which are now susceptible to the new class of suppressors and no longer susceptible to Class I suppressors. If we accept the universality of the genetic code, we can assume the new nonsense class to be UGA.

4. The Isolation and Classification of the Super-suppressors in Saccharomyces

The discovery of super-suppressors in yeast was fortuitous. The construction of hybrids for some 20 genes, for chromosome mapping studies, had involved the selection of a revertant for *arg*4-2 in one hybrid and a revertant of *trp*5-2 in another. Both reversions were due to mutations of suppressors which proved to act upon other mutant alleles included in the cross: *ade*6-3 and *pet*3-1 in the first hybrid, and *his*4-1 and *leu*2-1 in the second hybrid. In a third case, a revertant for *ade*2-1 introduced a suppressor which also acted upon *lys*1-1. These suppressors were tested for their action spectra with regard to the genes being used in the linkage studies. They were found to have apparently identical spectra which included about a fourth of the 49 genes tested. Allele specificity was also demonstrated with these tests. Crosses between the suppressor stocks established that there were two different loci, S_a and S_b (HAWTHORNE and MORTIMER, 1963).

It soon became apparent that there were numerous suppressors which were able to act upon given genes from this set of suppressible alleles. KAKAR (1963) found five different suppressors specific for *iso*1-1. A centromere-linked suppressor S_d was used to assay the alleles of *trp*5 for suppressibility (MANNEY, 1964). Twelve allele-specific suppressors for *ade*1 and *ade*2 mutants were isolated by INGE-VECHTOMOV (1965).

INGE-VECHTOMOV (1965, 1966) in his study of super-suppressors was faced with the problem of developing a classification scheme with suppressible alleles for only a limited number of phenotypes: *ade*1, *ade*2, *rgh*1 (rough) and *rgh*3. His scheme is based on whether the suppressor is dominant (6 loci), semi-dominant (4 loci), or recessive (2 loci) in the suppression of *ade*1 alleles. Problems arise with a scheme based on the dominance of the suppressors in that this often depends on the mutant alleles chosen for the assays. In particular, heteroallelic, suppressible / non-suppressible, combinations may remain auxotrophic. For example, cases where an erstwhile dominant suppressor fails to restore prototrophy for *ade*2 heteroallelic combinations are cited by INGE-VECHTOMOV et al. (1966).

As a preliminary step in determining the number of suppressor loci, GLIMORE and MORTIMER (1966) and GILMORE (1967) used 5 mutant pheno-

Table 1. Super-suppressor classes in *Saccharomyces* from GILMORE and MORTIMER (1966)

Class	Alleles suppressed					Number of revertants
	*trp*5-48	*arg*4-17	*his*5-2	*lys*1-1	*ade*2-1	
I	+	+	+	+	+	41
II	+	+	+	+	−	4
III	+	+	+	−	−	21
IV	+	+	−	+	−	1
V	+	+	−	−	−	12
VI	+	−	+	−	−	1
VII	+	−	−	+	−	2
VIII	+	−	−	−	+	1

types to classify the suppressors by their spectra. Their scheme derived from the examination of 83 revertants for 2 or more requirements from the multiply marked stock subjected to ultra violet irradiation is given in Table 1. In this experiment the single revertant classes for the 5 mutants totalled 970. These were not crossed; so there is a possibility that there are additional suppressors which act upon only one of the mutants included in this set.

It could be assumed that the 8 different classes of suppressors would represent at least 8 different suppressor genes; however, it was known that several different suppressors could display the same action spectrum. Crosses to test allelism were undertaken with 20 Class I isolates and 6 loci were demonstrated. These 6 suppressors were also non-allelic with two suppressors on hand, S_b and S_d; thus suppressors at 8 different loci had the same phenotype as judged with these 5 mutants. Crosses between 10 Class III isolates gave a minimum of 5 different loci for this class of suppressors. Crosses between 6 Class I and 2 Class III suppressors showed that they were nonallelic as expected with the assumption that a difference in spectra itself was indicative of different loci being involved. On this basis, a minimum of 19 suppressor genes acting upon ochre alleles was predicted.

The same 5 suppressible alleles were used by MAGNI and PUGLISI (1966) to classify 5653 spontaneous revertants of which 5565 were double revertants. Again 8 classes of double revertants were observed, but only 3 are identical with the classes of GILMORE and MORTIMER (1966): there were 876 Class I, 4653 Class II, and 20 Class IV suppressors, while the new classes had from 1 to 8 representatives.

There are certain risks in accepting the phenotype seen in the original suppressor isolate; this was made evident in a study by HAWTHORNE (1969a). In stocks bearing 12 or 13 suppressible alleles (both ochre and amber), the initial revertants could be grouped in 16 to 18 phenotypes. However, crosses revealed that only 10 of these were valid despite the introduction of new suppressible alleles to give 20 mutants on which to base the scheme. The spurious phenotypes seen in the original scoring were due to the presence of two suppressors, suppressor plus modifier, or back mutant plus suppressor

Table 2. Classification of nonsense suppressors in *Saccharomyces*

Alleles	Suppressors													
	Ochre-specific							Omnipotent			Amber-specific			
	I *SUP2*	II *SUP11*	III *SUP15*	IV *SUP20*	IV′ *SUP25*	V *SUP30*	V′ *SUP31*	VI *SUP35*	VII *SUP40*	VIII *SUP45*	IX *SUP2-a*	X *SUP51*	XI *SUP61*	
ade5,7-63	±	−	±	±		−	−	−	−	−	−	−		
trp5-2	+	±	−	−	−	−	−	−	−	−	−	−	−	
ura4-1	+	+	−	−	−	+	−	−	−	−	−	−	−	
ade2-1	+	±	±	−	−	−	−	±	−	−	−	−	−	
can1-52	+	+	+	−	−	−	−	±	−	−	−	−		
tyr1-1	±	±	+	−	−	±	−	−	−	−	−	−		
lys1-1	+	+	+	±	−	±	−	−	−	−	−	−	−	
lys2-1	+	+	+	±	−	−	−	−	−	−	−	−	−	
his5-2	+	+	+	±	±	±	−	−	−	−	−	−	−	
ade6-3	+	+	+	±		−	−	±	−	−	−	−		
met4-1	+	+	+	±		−	−	±	±	−	−	−		
his4-1	+	+	+	±	±	−	−	±	±	±	−	−		
arg4-17	+	+			±						−	−		
iso1-1	+	+	+	+	−	±	±	+	+	+	−	−	−	
leu2-1	+	+	+	+	+	+	±	+	+	+	−	−	−	
trp5-48	+	+	+	+	+	+		+			−	−		
leu1-101	+			−				+	+		+	+	+	
met8-1	−	−		−	−	±	−	+	+	+	+	+	+	
tyr7-1	−	−	−	−	−	−	−	±	+	±	+	+	+	
tyr6-1	−	−	−	−	−	−	−	+	−	±	+	+	+	
trp1-1	−	−	−	−	−	−	−	−	+	±	+	−	+	
ade5,7-101	−	−	−	−	−	−	−	−	−	−	+	+	+	
lys2-101	−							−	−		+	+	−	
leu2-1u	−	−		−				+	+	+	−	−		
his5-2u	−	−		−				+		±	−	−		

For the nutritional requirements, + = good growth from replica prints by 2 days; ± = visible growth by 3 to 5 days; − = no signs of growth by 5 days. For canavanine resistance, the converse holds.

to give an excess of positive scores; and new mutations in the pathway for the requirements being tested resulted in false negative scores.

The classification scheme in Table 2 incorporates the most recent observations, but it is still based mainly on the classification presented by HAWTHORNE and MORTIMER (1968). New amber and UGA mutants are included to further characterize the suppressors of Classes VI through X. Classes VI, VII, and VIII can no longer be considered analogous to the bacterial ochre suppressors since these suppressors also act upon UGA alleles. There has been a subdivision of weak ochre-specific classes to conform with tests against new mutant alleles. The suppressors of Classes I and II correspond to the Class I suppressors in Table 1. Suppressor *SUP25*, Class IV′, is from the Class III suppressors of GILMORE (1967); there is no other correspondence between the schemes to be reported. This scheme in Table 2 also can be related to INGE-VECHTOMOV'S classification based on the dominance of the suppressors. The ochre-specific suppressors in Classes I and II are strong suppressors which are dominant in the suppression of most homozygous mutants. Suppressors in Classes III to V′ are progressively weaker and can be scored as dominant, semi-dominant, or recessive depending upon the particular mutant allele selected for the test. The omnipotent suppressors in Classes VI, VII, and VIII are recessive for all the mutant phenotypes suppressed. The amber suppressors in Classes IX, X, and XI are again dominant for most phenotypes.

One expects that with enough mutant alleles a classification scheme will distinguish between the suppressors substituting different amino acids as well as those recognizing different nonsense codons. A third factor, suppressor efficiency, can also contribute to the diversity of suppressor phenotypes. Thus with the ideal scheme, we can expect all suppressors in a given class to be identical, but we may find the same species, i.e. suppressors substituting the same amino acid, in two or more classes.

The one test as to whether or not a given class of suppressors is homogeneous is reassuring. GILMORE et al. (1968, 1971) have shown that the 7 suppressors of Class I, *SUP2* to *SUP8*, and the one suppressor of Class II, *SUP11*, all substitute tyrosine in the translation of UAA, the codon for position 20 in the mutant *cyc1-2* of iso-1-cytochrome-*c*. The yields of iso-1-cytochrome-*c* with the various suppressors varied from 4 to 12 percent of the normal wild type levels. No significance was attached to these differences since there were corresponding variations in the levels of iso-2-cytochrome-*c* which can be used as a measure of the state of adaption. The level of iso-1-cytochrome-*c* in the strain bearing *SUP11* was at 7 percent of the wild type level, approximately at mid-range of the levels observed for the 8 suppressors. Nevertheless, there is reason to believe that *SUP11* is less efficient than the other 7 suppressors because of its slightly abridged spectrum and the fact that it is recessive in the diploid of genotype *ade2-1*/*ade2-1*, *SUP11*/+, whereas the equivalent diploid heterozygous for a Class I suppressor is prototrophic for the adenine requirement.

5. Mapping the Suppressors in Saccharomyces

Even the most elaborate classification schemes are likely to succumb to the vagaries introduced by the modifiers of suppressor efficiency, the anti-suppressors, the allo-suppressors, and the cytoplasmic factor *psi*. These factors will be discussed later, but we can state now that it is often difficult to decide which is the unmodified phenotype of the suppressor when faced with different levels of expression in haploid segregants from the same cross. This happens most frequently with crosses involving suppressors in Classes III to V'. For this reason the map position of a suppressor is an essential aspect of its description.

The first extensive effort at mapping the suppressors involved 20 suppressors, 16 of which were placed on the linkage maps of *Saccharomyces* (HAWTHORNE and MORTIMER, 1968). There were several errors in the above study: *SUP*1 has been shown to be allelic with *SUP*5, *SUP*20 is allelic with *SUP*21, and *SUP*40 is an allele of *SUP*35. These errors have been corrected and new suppressors have been added to the map presented in Figure 1 which is based on the latest mapping efforts of MORTIMER and HAWTHORNE (1973). It might be noted that the 19 nonsense suppressors on the map are distributed over 11 chromosomes and 2 fragments, i.e. linkage groups not assigned to centromeres.

6. The Relationship of the Nonsense Mutants and Super-suppressors in Schizosaccharomyces

In *S. pombe*, classification of the nonsense codons and of their suppressors is still uncertain. From the overlapping patterns of allele-specific action shown by three known classes of super-suppressors it may be deduced, however, that the nonsense mutations sensitive to these suppressors represent two different types of nonsense codons. This is shown by Table 3 which combines data obtained by BARBEN (1966), HOFER (1969), U. LEUPOLD (unpublished) and P. THURIAUX (unpublished). Allele specific patterns of suppressor action are given for eight nonsense suppressors (representing six different suppressor loci) in combination with representative nonsense mutants mapping in the *ade*7, the *ade*6 and the *ade*1 locus.

At the *ade*7 locus, the table includes every nonsense mutant found in an analysis of 49 completely blocked mutants of UV induced origin which map at a minimum of 28 different mutant sites (out of a total of 152 UV-induced mutants shown to map at a minimum of 33 different sites by LEUPOLD, 1961; most of the mutants which were not tested for suppressor sensitivity are likely to be suppressor insensitive since they represent homoalleles of mutants which were demonstrated to be insensitive to the nonsense suppressors tested by BARBEN, 1966). Interallelic complementation has not been observed at the *ade*7 locus (LEUPOLD, 1961).

At the *ade*6 locus, Table 3 includes every nonsense mutant which was discovered in an analysis of 59 completely blocked mutants of UV induced

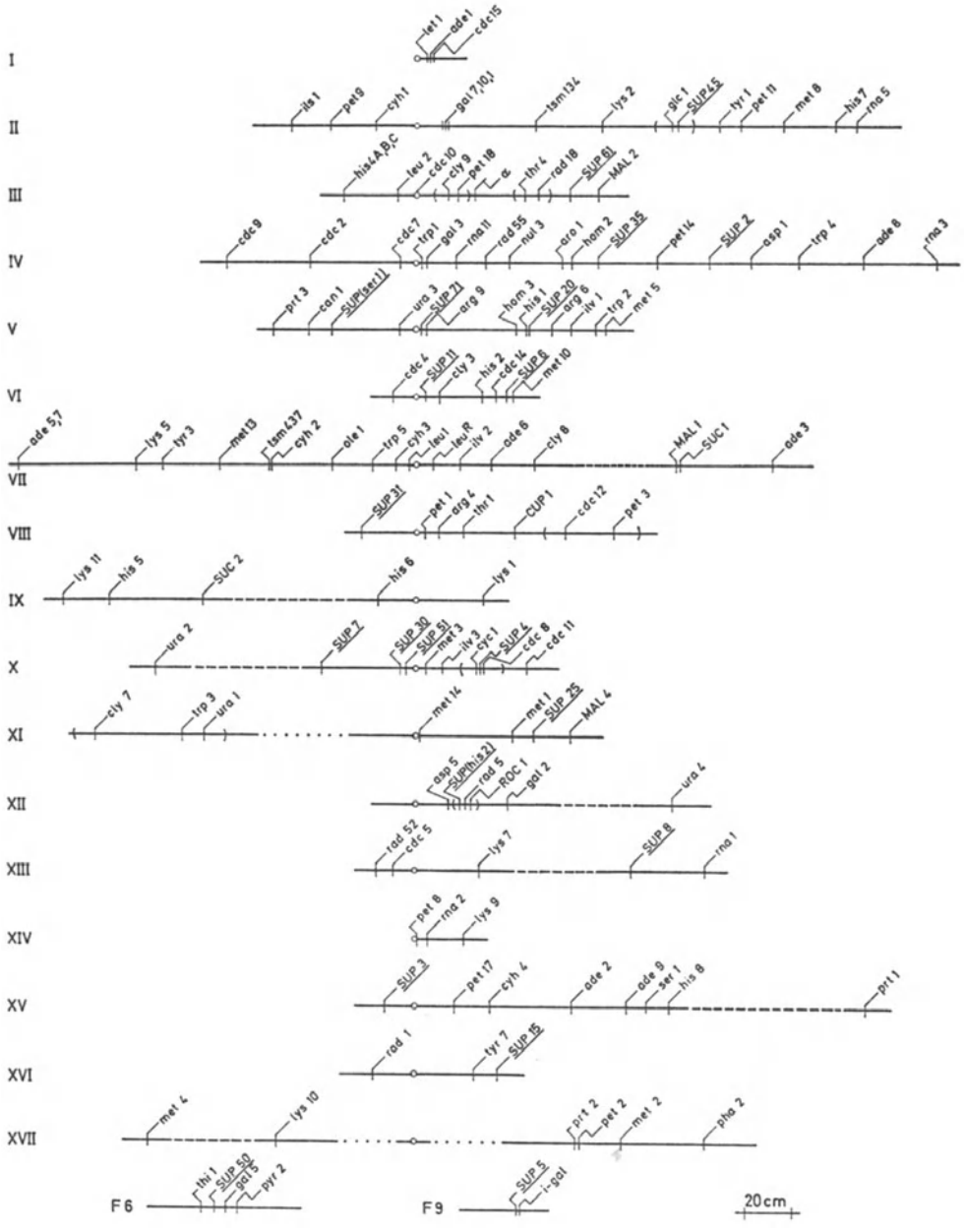


Fig. 1. Genetic Maps of *Saccharomyces*. The maps are taken from MORTIMER and HAWTHORNE (1973) with the permission of the editor of *Genetics*. Linkages established by tetrad or random spore analysis are represented by solid lines; those determined by mitotic and trisomic analysis are indicated by dashed and dotted lines, respectively. The sequence of genes within parentheses has not been determined relative to outside markers. Most of the gene symbols are described in the *Handbook of Biochemistry, 2nd Edition* (H. A. SOBER, ed., Chemical Rubber Co., Cleveland 1970)

origin representing 59 different mutant sites (out of 158 UV-induced mutants shown to map at a minimum of 68 different mutant sites by LEUPOLD and GUTZ, 1965; the suppressor sensitivity of homo-alleles was not tested in this

Table 3. Classification of nonsense suppressors in *Schizosaccharomyces*.

Patterns of allele-specific action of nonsense suppressors among UV induced nonsense mutants mapping in the *ade*7, *ade*6 and *ade*1 loci of *S. pombe*. Mutants are ordered according to their relative map location (from left to right; BARBEN, 1966) in the fine structure maps of *ade*7 (LEUPOLD, 1961), *ade*6 (LEUPOLD and GUTZ, 1965) and *ade*1 (RAMIREZ et al. 1963 and in preparation; CLARKE, 1965b). Mutants distinguishable by suppression but not by recombination are separated by smaller distances. Numbers of additional mutants which are indistinguishable by both suppression and recombination are indicated in brackets following the collection number of the mutant with which they are homoallelic. *ade*6 and *ade*1 mutants capable of inter-allelic complementation, in combination with suppressor insensitive missense mutants mapping towards their left, are indicated by an asterix. Additional nonsense mutants of chemically induced or spontaneous origin which are sensitive to one or both of the two nonsense suppressors *SUP*1-oa and *SUP*3-o, include 2 *ade*7 mutants (MUNZ and LEUPOLD, 1970), 1 *ade*6 mutant (non-complementing; GUTZ, 1971), 24 *ade*1 mutants (18 *ade*1A, non-complementing; 6 *ade*1B, 3 complementing and 3 non-complementing; FRIIS et al., 1971; SEGAL et al., 1973), and 2 *glu*1 mutants (BARBEN, 1966). *SUP*1-oa, *SUP*2-oa and *SUP*3-o correspond to suppressors *84f*, *413c* and *84h* of BARBEN (1966). As far as tested, suppressor sensitivity is indicated by "+", insensitivity as "−".

		Suppressors				
		1st class	2nd class		3rd class	
		I *SUP*1-oa *SUP*2-oa	II *SUP*3-o	II′ *SUP*8-o	III *SUP*3-a	III′ *SUP*8-a
*ade*7-	262 (+*1*)	+	−	−	+	−
	489	−	−	−	+	−
	695	+	+	+	−	−
	413 (+*3*)	+	−	−	+	+
	84 (+*1*)	+	+	+	−	−
	540	−	+	+	−	−
	608	+	−	−	−	−
	451 (+*7*)	+	−	−	+	+
	419	+	−	−	+	+
	461	+	+	+	−	−
	606	+	−	−	+	+
	572	+	−	−	−	−
		*SUP*1-oa	*SUP*3-o *SUP*9-o	*SUP*8-o *SUP*10-o		
*ade*6-	706	−	+	+		
	712	−	−	+		
	704	−	+	+		
	611	+	−	−		
	469	−	+	+		
	588*	+	−	−		
		*SUP*1-oa	*SUP*3-o *SUP*9-o	*SUP*8-o *SUP*10-o		
*ade*1A-	40	+	+	+		
	3 (+*2*)	+	−	−		
	H538	−	+	+		
1B-	25*	+	+	+		
	H259*	+	−	−		

locus). One mutant mapping at the right end of the fine structure map of the *ade6* locus, *ade6*-588, is capable of complementing a number of missense mutants mapping towards its left, thus defining the direction of translation as proceeding from left to right.

At the *ade1* locus, Table 3 includes all the nonsense mutants found in an analysis of 41 completely blocked mutants of UV and diethyl sulfate induced origin which map at a minimum of 30 different sites (out of 46 mutants shown to map at a minimum of 35 different sites by RAMIREZ et al., 1963). Two mutants which are located in the *ade1*B region towards the right end of the fine structure map are capable of complementing many missense mutants which map in the *ade1*A region towards their left, i.e., proximally with respect to the direction of translation which proceeds from left (*ade1*A) to right (*ade1*B).

Two inefficient suppressors of a first suppressor class, *SUP1* and *SUP2*, are capable of suppressing both of the different nonsense codons mentioned above. They may correspond to the bacterial ochre-amber suppressors which are known to suppress both ochre (UAA) and amber (UAG) but not umber (UGA) codons. Their suppressor active alleles may therefore tentatively be designated as *SUP1*-oa and *SUP2*-oa. They give identical patterns of allele specific action in the *ade7* locus, the only locus in which both suppressors have been tested. *SUP1*-oa, which was tested in all three loci, suppresses most of the mutants which are sensitive to suppressors of a second and a third class, except in the *ade6* locus where only two out of six nonsense mutants are found to be sensitive to *SUP1*-oa. This might be due to a highly multimeric structure of the *ade6* enzyme which does not permit inefficient suppressors to produce a sufficient concentration of functional enzyme, because of the trapping of completed polypeptide chains in aggregates consisting mainly of incomplete polypeptide subunits (BARBEN, 1966).

Four efficient suppressors of a second class, *SUP3*, *SUP8*, *SUP9* and *SUP10*, are capable of suppressing only one of the two nonsense codons. Judged from their efficiency, which appears to be higher in most mutant combinations than that of *SUP1* and *SUP2* when comparing growth rates of suppressed mutants in minimal medium (BARBEN, 1966, and unpublished observations), these suppressors might well correspond to the efficient types of ochre-specific suppressors such as those of class I (cf. Table 2) of *S. cerevisiae*. As a working hypothesis and in order to facilitate discussion, we will assume in the following that this is indeed the case. We will therefore designate their suppressor active alleles as *SUP3*-o, *SUP8*-o, *SUP9*-o and *SUP10*-o. The patterns of allele-specific action are identical in the *ade1* locus in which all four suppressors have been tested (BARBEN, 1966; HOFER, 1969; LEUPOLD, unpublished). This is also true for the *ade7* but not for the *ade6* locus (in the *ade7* locus the analysis has been confined to *SUP3*-o and *SUP8*-o so far) (BARBEN, 1966; P. THURIAUX and U. LEUPOLD, unpublished). One mutant in the *ade6* locus, *ade6*-712, is suppressed by *SUP8*-o and *SUP10*-o but not by *SUP3*-o and *SUP9*-o under standard conditions. However, strains combining

ade6-712 with *SUP3*-o or *SUP9*-o are osmotic remedial and, in heterozygous diploids of the type *ade6-712/ade6*-x *SUP3*-o/*sup3*$^+$ and *ade-712/ade6*-x *SUP9*-o/*sup9*$^+$, capable of interallelic complementation with many missense alleles at the *ade6* locus (A. STRAUSS, unpublished). In a few other cases of mutants which are sensitive to both pairs of suppressors under standard conditions, quantitative differences in the degree of suppression (as judged from prototrophic growth in minimal medium) are observed. This gives another method of distinguishing *SUP8*-o and *SUP10*-o from both *SUP3*-o and *SUP9*-o. It is therefore likely that *SUP8*-o and *SUP10*-o differ from *SUP3*-o and *SUP9*-o by inserting one of two different amino acids in response to the same nonsense codon and that in mutant *ade6-712*, only one of the two amino acids leads to a protein which is functional under normal conditions.

If the preliminary classification of the four suppressors of the second class as ochre-specific suppressors is correct, then a third class of suppressors involving two inefficient suppressors which are capable of suppressing a second type of nonsense codon only, is likely to correspond to the amber-specific suppressors of *S. cerevisiae*. Both of these suppressors, which we will designate tentatively as *SUP3*-a and *SUP8*-a in what follows, have been derived by mutation from efficient suppressors of the second class, *SUP3*-o and *SUP8*-o. They behave as alleles of the suppressors which they were derived from, but the original suppressors and their mutant alleles show mutually exclusive patterns of allele specific action when tested in combination with nonsense mutants in the *ade7* locus. With few exceptions, however, both patterns are contained in the overlapping pattern characterizing the suppressors of the first class, *SUP1* and *SUP2*. Again, minor differences distinguishing *SUP3*-a and *SUP8*-a (*ade7-262* and *ade7-489* are suppressed by *SUP3*-a but not by *SUP8*-a) suggest that the same nonsense codon is read as a different amino acid by *SUP3* and *SUP8*.

According to this tentative classification, nonsense mutants sensitive to the second class of suppressors are assumed to carry ochre mutations (UAA) whereas mutants sensitive to the third class of suppressors are postulated to carry amber mutations (UAG). It should be stressed, however, that this classification is preliminary only. The reverse classification (second class = *SUP*-a, third class = *SUP*-o) which was originally used (HOFER, 1969; LEUPOLD, 1970b) has certainly not yet been excluded. Amino acid sequence analyses of nonsense revertant proteins which could lead to an unambiguous classification have not yet been carried out in *S. pombe*, and the results of a preliminary analysis of the interconversion of the two nonsense codons and their suppressors following mutagenesis with UV and, more specifically, with ethylmethanesulfonate, are not yet conclusive enough to answer the question. They do show, however, that the two types of nonsense codons can be interconverted by a single base-pair substitution (A. AHMAD and U. LEUPOLD, unpublished). From a presumptive *ade7* amber mutant (*ade7-431*, cf. Table 3) carrying a second-class suppressor of the opposite specificity, *SUP3*-o, it has been possible to obtain not only prototrophs resulting from an alteration of

the suppressor specificity (*SUP3*-o → *SUP3*-a) or from a reversion of the nonsense allele to a functional allele (*ade7*-a → *ADE7*) but also prototrophs resulting from an adaptation of the nonsense allele to the suppressor present in the strain (*ade7*-a → *ade7*-o). When the new *ade7* nonsense allele found in the third type of prototrophs was separated from its suppressor, it was found to map at the same site as the original *ade7* nonsense mutation from which it was derived. The same three types of prototrophs resulting from the mutations *SUP8*-o → *SUP8*-a, *ade7*-a → *ADE7* and *ade7*-a → *ade7*-o have been obtained in a strain combining the presumptive amber allele *ade7*-431 with another second-class suppressor of opposite specificity, *SUP8*-o. Recent results obtained with a strain carrying a presumptive ochre mutation, *ade7*-84, in combination with a suppressor of the opposite specificity, *SUP8*-a, indicate that the interconversion of the two types of nonsense codons is also possible in the opposite direction, *ade7*-o → *ade7*-a.

The classification of the nonsense mutants sensitive to second and third class suppressors of *S. pombe* as ochre and amber mutants, respectively, should be considered as preliminary also for the following reason. Ochre and amber codons are likely candidates if it is assumed that ochre-specific suppression is due to a mutant tRNA carrying the anticodon IUA (derived from AUA by deamination of adenosine to inosine in the first anticodon position) and therefore capable of reading the two tyrosine codons UAU and UAC and the ochre codon UAA but not the amber codon UAG ("topaz" suppressors, BOCK, 1967). The interconversion of ochre-specific and amber-specific suppressors would then result from the transversion A+T ⇌ C+G in the anticodon region of a tRNA structural gene, leading to the corresponding base substitution *I*UA (ochre-specific) ⇌ *C*UA (amber-specific) in the first anticodon position of the mutant tRNA. Mutagenic data obtained in *S. cerevisiae* (HAWTHORNE, 1969a, b) are in agreement with this interpretation of ochre-specific suppression. An alternative possibility is that tRNA's carrying a derivative of 2-thiouridine ("S") in the first anticodon position (YOSHIDA et al., 1971) will read ochre (UAA) but not amber (UAG) when mutated to a suppressor active tRNA carrying the anticodon SUA ("sepia" suppressors, GILMORE et al., 1971). Ochre-specific suppressors of this type could be mutated not only to amber-specific suppressors reading UAG (because of a T+A→ C + G transition in the tRNA structural gene leading to the anticodon change *S*UA→*C*UA), but also to umber-specific suppressors reading UGA (because of a T+A→C + G transition leading to the anticodon change S*U*A→S*C*A).

The possibility can not yet be excluded therefore that the two types of nonsense codons demonstrated in *S. pombe* correspond to ochre and umber codons rather than to ochre and amber codons. In this case, however, the suppression of both types of nonsense codons by suppressors of class I is unlikely to be due to mutant tRNAs capable of reading both UAA and UGA since the two nonsense codons UAA and UGA differ in the second rather than in the third codon position. These suppressors may, however, correspond to the bacterial *ram* (ribosomal ambiguity) type of suppressors which suppress

all three nonsense codons UAA, UAG and UGA and which are known to affect ribosomal proteins rather than tRNA's (ROSSET and GORINI, 1969). This explanation is the more likely as those suppressors of *S. cerevisiae* which were originally believed to correspond to bacterial ochre-amber suppressors (HAWTHORNE and MORTIMER, 1968; HAWTHORNE, 1969a; MORTIMER and HAWTHORNE, 1969) have recently been shown to suppress the umber codon as well (HAWTHORNE, unpublished; see below). This possibility certainly also exists in *S. pombe* since two of the *ade7* mutants which are sensitive to suppressors of the first class (*ade7*-608 and *ade7*-572, cf. Table 3) are suppressed neither by the specific suppressors of the second class nor by those of the third class. It is conceivable therefore that the nonsense mutants sensitive to the first class suppressors *SUP1* and *SUP2* represent not only two but in fact all three codons that are known to serve as chain-terminating signals in protein synthesis. Preliminary attemps to isolate suppressors specific for the third nonsense codon have not yet been successful in *S. pombe*.

7. The Nature of the Codon Specific Suppressors in Yeast

In *E. coli*, amber, ochre, and UGA nonsense suppressors have been shown to be genes coding for tRNA's by the sequencing of suppressor associated tRNA's (GOODMAN et al., 1968; ALTMAN et al., 1971; HIRSH, 1971) or by demonstrating with *in vitro* systems that it is the tRNA's from the suppressor stocks which permit the translation of messenger RNA's containing nonsense codons (CAPPECHI and GUSSIN, 1965; ENGELHARDT et al., 1965).

Suppressor tRNA's from yeast are not utilized in the *E. coli* ribosome—f2 phage messenger system (KIGER and BRANTER, 1973) and a homologous *in vitro* system has not been perfected for yeast. However, by means of reversed phase column chromatography, altered tyrosyl-tRNA species can be found in the preparations from *S. cerevisiae* strains bearing the Class I ochre-specific suppressors or their amber-specific alleles (BRUENN and JACOBSON, 1972). Moreover, there exists a body of genetic data, from both mutation studies in *S. cerevisiae* and the fine structure mapping of suppressor loci in *S. pombe*, which indicates that the codon specific suppressors in yeast are also coding for tRNA's.

a) Mutation Studies with Saccharomyces

Let us consider the first group of suppressors to be studied in *S. cerevisiae*, the dominant suppressors *SUP2* to *SUP8* in Class I and *SUP11* in Class II (Table 2). MAGNI et al. (1966) report that the mutation of suppressors in Classes I and II is enhanced 10 to 15 fold with meiosis. The meiotic effect is taken as diagnostic of addition-deletion mutations (MAGNI and VON BORSTEL, 1962; MAGNI, 1963). Additions or deletions causing reading frame shifts in messages which are translated into proteins are unlikely to yield functional products unless a pre-existing frame shift was present. This would hardly be

the case at 8 different loci; thus MAGNI et al. (1966) argue that the suppressor genes do not code for proteins but for tRNA's.

MORTIMER (1969) undertook a study to determine whether the event leading to a suppressor mutation could occur at different sites or was always at the same site for a given suppressor gene. Within the limits of the assay system, the answer is that the same site is involved. The experimental diploids were constructed from crosses between different isolates of the suppressor *SUP6* and the control diploids were homoallelic for the parental suppressors. A suppressible ochre allele of *can1* was homozygous in the diploids to provide a selective system for isolating suppressor free spores. Both the experimental and control diploids gave the same yield of canavanine resistant spores, about $1\text{–}2/10^6$ spores. These rates are consistent with the meiotic induction of new mutational defects in either the canavanine locus or the suppressor locus.

The results obtained from the studies of the induction of the Class I and II suppressor mutations with chemical mutagens can be consistently interpreted with the assumption that the specific base substitutions occur in the triplet coding for the anticodon of the tRNA molecule. The mutations yielding the ochre-specific Class I and II suppressors are induced by ethylmethanesulfonate (HAWTHORNE, 1969a). This is precisely what is expected with genes coding for the tyrosyl-tRNA's. The normal tyrosyl-tRNA anticodon is GΨA (MADISON et al., 1966) coded for by the DNA triplet TAC/ATG. Ethylmethanesulfonate induces G+C → A+T transitions (KRIEG, 1963); thus the mutation to the triplet TAT/ATA is enhanced. From this triplet are transcribed the bases AUA which in turn are enzymically modified so as to recognize the ochre codon. This can be achieved by the deamination of the adenine in the first position to give inosine, i.e. the anticodon IΨA which will pair with UAA, UAC, and UAU, but not with UAG according to the wobble hypothesis (CRICK, 1966).

Amber suppressors (Class IX, Table 2) which are alleles of the Class I ochre-specific suppressors can be isolated directly from suppressor free strains or by the mutation of the ochre suppressor to amber specificity. Ethylmethanesulfonate does not enhance either mutation (HAWTHORNE, unpublished). Both the mutation from the wild-type tyrosyl-tRNA gene and the ochre suppressor tyrosyl-tRNA gene (the suppressor obtained with ethylmethanesulfonate mutagenesis) involve transversions to transcribe the amber anticodon CUA. In contrast, the amber suppressors will mutate to ochre specific suppressors in response to ethylmethanesulfonate (HAWTHORNE, unpublished). For example, a haploid bearing *SUP11*-a when plated on a medium to select ochre suppressors gave in the control plating of 2×10^7 cells only 1 revertant with the mutation *SUP11*-a → *SUP11* and 8 revertants *SUP11*-a+ *SUP*-I (Class I).

The plating of 1×10^7 cells surviving the ethylmethanesulfonate treatment gave 67 revertants which involved *SUP11*-a → *SUP11* and 47 revertants with both *SUP11*-a+*SUP*-I. The ochre specific suppressor gene derived from the amber suppressor allele by the ethylmethanesulfonate treatment

should be transcribing the anticodon UUA as the result of the G+C →A+T transition. The fact that the suppressor is still ochre-specific suggests that the uracil in the first anticodon position is modified as is the case with the first uracil of the anticodon of the glutamic acid tRNA which pairs with GAA but not GAG (SEKIYA et al., 1969). The substitution of sulfur in place of oxygen at the 2 position of the uracil prevents the pairing with G but not A (YOSHIDA et al., 1971).

HAWTHORNE (1969a) expected the ethylmethanesulfonate treatment to induce the mutation of ochre-amber suppressors derived from the glutamine (CAA) tRNA genes. Now, it can be seen from the above results that one should expect a second ochre-specific class to be enhanced with the application of this mutagen. There was no evidence for the enhancement of the other ochre-specific classes, III, IV, or V, in the above study. However, other experiments have revealed that Class III suppressor mutations sometimes are significantly enhanced with the ethylmethanesulfonate regimen. The recovery of Class III suppressors appears to be markedly dependent on the mutant allele and the medium used to select the revertants. (For a discussion of the role of the selective medium in the isolation of suppressors see QUIEROZ, 1973.) With the selection of tyrosineless revertants, *tyr*1-1, the recovery of Class III suppressors was particularly dramatic in one experiment: the control sample of 6×10^7 cells gave 9 Class I and 0 Class III suppressors, while the ethylmethanesulfonate sample with 1.5×10^7 viable cells gave 41 Class I and 34 Class III suppressors (HAWTHORNE, unpublished). Crosses involving 8 independent revertants have revealed only one suppressor, *SUP*15, in Class III.

The ethylmethanesulfonate regimen should enhance the recovery of two classes of amber suppressors: those derived from the glutamine (CAG) and tryptophan (UGG) tRNA genes. When the suppressors were selected in haploid strains as revertants of either *leu*1-101, *met*8-1, *ade*5,7-101, *trp*1-1, or *tyr*7-1 only two classes, IX and X, were recovered, and only the latter class, X, was enhanced with ethylmethanesulfonate mutagenesis. There was a 10 to 20-fold increase in suppressors over the control sample, even after approximately 80 percent kill with the treatment (3% EMS for 1 hr.).

Linkage studies and allelism tests indicate that at least 6 different suppressors belong to Class X. Only one of these genes is centromere linked, and it, *SUP*51, is the only one mapped: chromosome X, figure 1 (HAWTHORNE and MORTIMER, 1968). The possibility that two or more tRNA species might be included in Class X must be considered, but seems unlikely since all 6 suppressors present identical phenotypes with respect to the 7 amber alleles of the classification scheme. Alternative explanations for finding only 2 amber classes when a maximum of 7 is expected are: 1) that the potential amino acid substitutions will not yield active proteins for any of the 5 mutant alleles used in the selection step, or 2) that the pertinent tRNA genes are unique representatives of their species and thus their function cannot be lost through mutation without being lethal. The first explanation cannot be tested until we acquire additional amber alleles. The second explanation has been tested

by selecting suppressors in a diploid stock and confirmed to the extent that a haplo-lethal suppressor with a new spectrum, Class XI, was isolated.

When diploids bearing the Class XI suppressor, *SUP61*, are sporulated, they yield only 2 viable segregants per ascus and the suppressor is never found in these haploids. The lethal bearing spores generally will germinate and produce several buds and are capable of mating. By matings to these spores, the suppressor can be recovered. A triploid bearing *SUP61* and two wild type alleles was constructed and analyzed for two purposes: 1) to find the chromosome carrying *SUP61* by trisomic analysis, and 2) to determine the spectrum of *SUP61*. Aneuploid segregants carrying the suppressor were crossed successively with stocks bearing amber alleles as well as the various centromere markers. In this way the suppressor was located on chromosome III. It has been shown to be linked to mating type by tetrad analysis (see figure 1). The spectrum for Class XI (Table 2) represents the phenotype seen with the amber allele in single dose and the suppressor heterozygous, *SUP61*/+.

The mutation of the Class XI amber suppressor is induced with the ethylmethanesulfonate treatment. For example, a diploid homozygous for *trp*1-1, *tyr*6-1, and *met*8-1 plated on tryptophan-less medium gave, in the control sample (2.4×10^7 cells), 12 Class IX and 0 Class XI suppressors, while the ethylmethanesulfonate sample (1.3×10^7 cells) had 17 Class IX and 45 Class XI suppressors. The Class X suppressors are not selected on tryptophan-less medium.

The amber suppressors derived from the mutation of the glutamine and tryptophan tRNA genes are found in Classes X and XI. One species is unique; the other may have as many as 6 copies in the genome. It will be possible to decide which suppressors are derived from the tryptophan tRNA genes by demonstrating allelism with UGA suppressors.

Arginine, tryptophan, and cysteine tRNA genes are expected to mutate to UGA suppressors with ethylmethanesulfonate treatments. The mutagenesis regimen enhances the recovery of UGA suppressors in haploid stocks. However, with 4 UGA alleles, *ade*5,7-143, *his*5-2u, *leu*2-1u, and *lys*1-1u, only two classes of suppressors have been distinguished: a class obtained without treatment, represented by the original UGA suppressor, acts upon all the alleles, and the ethylmethanesulfonate induced suppressors which fail to act upon *ade*5,7-143. The only centromere-linked suppressor, *SUP71*, found in this latter class is not allelic with *SUP51*, the centromere-linked amber suppressor from Class X. It has been mapped on chromosome V, figure 1 (MORTIMER and HAWTHORNE, 1973).

The ethylmethanesulfonate treatment of diploids again enhances the recovery of suppressors with the same spectrum as seen with the haploid isolates. Thus far, six diploid isolates have been sporulated and dissected, but none of the suppressors were haplo-lethal.

The expectation that ethylmethanesulfonate should induce the cysteine tRNA genes to mutate to UGA suppressors rests on the analogy of the mutation of the tyrosyl-tRNA genes to ochre-specific suppressors. It has been

assumed that the anticodon of the cysteine tRNA is GCA which can pair with both cysteine codons, UGU and UGC, according to the "wobble hypothesis" (CRICK, 1966). The ethylmethanesulfonate induced G+C → A+T transition must occur at the first base of the anticodon triplet so that ACA is transcribed. Then enzymatic deamination of the first adenine of the anticodon gives ICA, capable of pairing with UGA, but also UGU and UGC. Therefore the ethylmethanesulfonate induced mutation of an unique cysteine tRNA gene should not give a haplo-lethal suppressor.

The question now arises as whether or not the mutation of a unique tryptophan tRNA gene to the UGA suppressor would result in a haplo-lethal. If the U in the first position of the suppressor anticodon, UCA, remained unmodified, it should still pair with the terminal G of the tryptophan codon, UGG, and no lethality would be seen. However, if the U were modified to S as postulated for the corresponding ochre-specific suppressor, then a haplo-lethal should be found.

This possibility was the motive for an experiment to change the specificity of the amber haplo-lethal suppressor, *SUP61*, to an UGA suppressor. First, ochre-specific mutants of *SUP61* were selected after ethylmethanesulfonate mutagenesis of the heterozygous diploid. The ochre-specific allele of *SUP61* was haplo-lethal, as expected. Then following the ultraviolet irradiation of the new diploid, mutants showing a change from ochre to UGA specificity were isolated. The UGA suppressor obtained in this manner had the phenotype of the ethylmethanesulfonate induced UGA suppressors, it acted upon *his5*-2u, *leu2*-1u, and *lys1*-1u but not *ade5,7*-143, but it was still haplo-lethal. Thus the haplo-lethal suppressor either was derived originally from the glutamine (CAG) tRNA gene or if it was derived from the tryptophan tRNA gene, the anticodon of the tRNA is modified so that it no longer pairs with UGG.

b) The Fine Structure Mapping of Nonsense Suppressors in Schizosaccharomyces

Suppressors offer a unique possibility to study mutations affecting the function of transfer-RNA's. Besides mutations in the anticodon changing the coding specificity of the tRNA, mutations resulting in base substitutions outside the anticodon and affecting additional functional properties of the tRNA may be obtained. This is achieved by selecting for secondary mutations which inactivate the suppressor activity conferred upon the tRNA by the primary mutation in the anticodon. A number of partially inactivating secondary mutations in the su_{III} tyrosine suppressor tRNA gene of *E. coli* have been shown to produce mutant tRNA's differing from the original tRNA in each case by a single base change in one of the hydrogen-bonded regions or in the dihydrouracil loop of the molecule (ABELSON et al., 1970; SMITH et al., 1970). In some of these secondary mutants, fully suppressor active revertants have been isolated which represent tertiary mutations affecting an additional base of the tRNA (SMITH et al., 1970).

Genetically, secondary mutations inactivating the suppressor activity of a mutant tRNA by base changes outside the original mutation of the anticodon region may be recognized by their ability to recombine with the suppressor inactive wild type allele of the tRNA gene. One of the two types of recombinants expected from this type of cross will carry the suppressor active single mutant allele as a result of intragenic recombination events recombining the mutant configuration at the anticodon site with the wild type configuration at the site of the inactivating reversion. This type of recombinant can easily be selected for on the background of a suppressor sensitive nonsense mutation affecting the function of a protein which, under a given set of experimental conditions, is necessary for growth. The frequency of recombination restoring the active suppressor allele is expected to depend upon the distance between the anticodon site and the reversion site. Using this approach, GAREN et al. (1965) have been able to show that in the amber suppressor gene su_I of *E. coli*, there are several closely linked sites at which mutations inactivating the suppressor activity can occur.

Fine structure maps of the structural genes of tRNA's which are available in a suppressor active mutant form, may be derived not only from the frequencies of suppressor active recombinants observed in crosses of suppressor inactive revertants to the wild type allele of the suppressor, but also from those observed in pairwise crosses between suppressor inactive revertants. In *S. pombe*, this type of analysis has been applied so far to three specific nonsense suppressors of the efficient type, *SUP3*-o, *SUP8*-o and *SUP9*-o, which are believed to correspond to the ochre-specific suppressors of *S. cerevisiae*, and to one specific nonsense suppressor of the inefficient type, *SUP3*-a, which was derived from *SUP3*-o by mutation and which is believed to correspond to the amber-specific suppressors of *S. cerevisiae*.

Suppressor inactive reversions were induced in prototrophic strains carrying a suppressor active allele of a given suppressor in combination with a suppressor sensitive nonsense allele of an adenine gene (*ade6*-704 in the case of *SUP3*-o; *ade6*-706 in the case of *SUP8*-o; *ade1*-40 in the case of *SUP9*-o; and *ade7*-413 in the case of *SUP3*-a), using nitrous acid (in the case of *SUP3*-o, *SUP8*-o and *SUP3*-a) or N-methyl-N'-nitro-N-nitrosoguanidine (in the case of *SUP9*-o) as mutagenic agents. The genetic constitution of the adenine dependent mutants derived from these prototrophic strains was determined in appropriate backcrosses, and those which mapped in the suppressor locus (rather than in an adenine locus or in one of at least 14 different modifier loci *sin1*—*sin8* which exhibit an antisuppressor activity in their mutant form; HOFER, 1969; M. MINET and P. THURIAUX, unpublished) were retained for further analysis. A few of the suppressor inactive revertants which were thus isolated proved to be conditional lethals of the temperature sensitive or osmotic remedial type when combined with a nonsense mutation in an adenine locus (cf. Fig. 2).

Twenty-five auxotrophic revertant (r) strains of constitution *ade6*-o *sup3*-o,r and an auxotrophic strain of constitution *ade6*-o *sup3*$^+$ carrying the

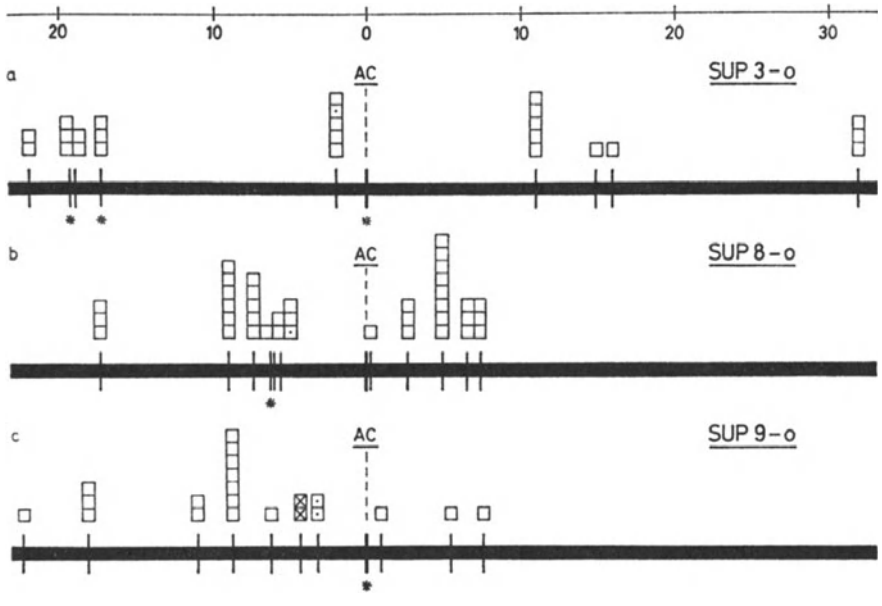


Fig. 2a—c. Fine structure maps of three nonsense suppressors of *Schizosaccharomyces pombe:* a) *SUP*3-o, b) *SUP*8-o, c) *SUP*9-o. The three nonsense suppressors are presumed to be mutant forms of structural genes for tRNA's. Their fine structure maps show the location of the original mutation which led to the suppressor active allele and which is believed to be located in the anticodon region (AC), in relation to the location of secondary suppressor inactive reversions which were derived from the suppressor active allele by mutagenesis with nitrous acid (*SUP*3-o, *SUP*8-o) or nitrosoguanidine (*SUP*9-o). Mapping is based on the formation of intragenic prototrophic recombinants carrying the suppressor active allele (constitution *ade*-o *SUP*-o) in pairwise crosses between auxotrophic strains carrying suppressor inactive revertant alleles (*ade*-o *sup*-o,r1 × *ade*-o *sup*-o,r2) or the suppressor inactive wild type allele of the suppressor locus (*ade*-o *sup*$^+$ × *ade*-o *sup*-o,r) in combination with a suppressor sensitive nonsense mutation in an adenine locus. Map distances are given as prototrophic recombinants per 10^6 progeny spores. Mutant sites which yield increased recombination frequencies as a result of specific marker effects are marked with an asterix (cf. text). Revertant alleles of the suppressor locus which lead to conditional lethality when combined with a suppressor sensitive nonsense mutation in an adenine locus are indicated as ⊠ (temperature sensitive) or ⊡ (osmotic remedial)

suppressor inactive wild type allele at the suppressor locus were then crossed in all pairwise combinations, and the frequencies of prototrophic progeny spores of constitution *ade*6-o *SUP*3-o resulting from intragenic recombination were determined as described previously (LEUPOLD, 1970a). They permit the construction of a fine structure map of the tRNA structural gene which is presumed to have given rise to the suppressor active allele *SUP*3-o (LEUPOLD, 1970b; HUBSCHMID and LEUPOLD, in preparation). In this map which is based on meiotic recombination, the reversions which have led to the 25 suppressor inactive revertant alleles are found to map at nine different sites (Fig. 2a). The revertant sites are located on both sides of the original mutation which had led to the suppressor active allele and which is presumed to have

affected the anticodon region of the tRNA structural gene. They define a chromosomal region stretching over a map length of 54 intragenic meiotic map units, 1 map unit corresponding to 1 prototrophic recombinant per 10^6 ascospores.

Compared with the meiotic map length of protein structural genes coding for enzymes involved in purine biosynthesis in *S. pombe*, this map length is what might be expected of a structural gene coding for a tRNA which is about 80 nucleotides long, if allowance is made for the fact that recombination frequencies per unit length of DNA may vary between different regions of the genome. With the exception of the *ade*8 locus which is the structural gene of adenylosuccinase and which appears to be located in a region of unusually low recombination frequencies, all adenine loci which have been mapped so far exhibit a map length which is several times longer than that of the *SUP*3 locus (*ade*8: 35 map units, ANGEHRN, 1964; MEGNET and GILES, 1964; *ade*6: 203 map units, LEUPOLD and GUTZ, 1965; LEUPOLD, unpublished; *ade*2: 206 map units, TREICHLER, 1964; *ade*1: 266 map units, RAMIREZ et al., in preparation; *ade*7: 519 map units, LEUPOLD, 1958, 1961; *ade*4: 658 map units, MATZINGER, unpublished; *ade*9: 1730 map units, ADONDI and HESLOT, 1970).

The map length of the fine structure maps of *SUP*9-o and *SUP*8-o is even smaller than that of *SUP*3-o, suggesting that these nonsense suppressors may have been mapped less exhaustively or that they may be located in chromosome regions exhibiting lower frequencies of recombination per unit length of DNA. Pairwise crosses between 21 auxotrophic revertant strains of constitution *ade*1-o *sup*9-o,r and an auxotrophic strain of constitution *ade*1-o *sup*9$^+$ yielded prototroph frequencies which permitted the construction of a map exhibiting 10 revertant sites in addition to the anticodon site and covering a map distance of 30 intragenic meiotic map units (Fig. 2b; HOFER, 1969; HOFER and LEUPOLD, in preparation). A similar map, which shows 10 revertant sites and the anticodon site distributed over a chromosomal region of 25 units map length, has been constructed on the basis of the results of pairwise crosses between 38 revertant strains of constitution *ade*6-o *sup*8-o,r and a strain of constitution *ade*6-o *sup*8$^+$ (Fig. 2c; HAESLER, 1971; HAESLER and LEUPOLD, in preparation).

In both the *SUP*8-o and the *SUP*9-o map, the site of the original mutation presumed to be located in the anticodon region of a tRNA structural gene is located more eccentrically with respect to the ends of the fine structure map than in the *SUP*3-o map. If only structural gene mutations and no promotor mutations or mutations defective in tRNA maturation have contributed to the revertants isolated, and if base substitutions in many regions including those near the 5′- and 3′- ends of the tRNA can abolish its function as shown for su_{II} tyrosine suppressor tRNA of *E. coli* (SMITH et al., 1970), then an exhaustive map of a tRNA structural gene should show the original mutant site approximately in the middle of the map.

This assumes that the original mutant site is indeed located within and not outside of the anticodon region as is the case in a tryptophan suppressor

tRNA of *E. coli* capable of suppressing UGA mutants (HIRSH, 1971). In the specific suppressors *SUP3*-o and *SUP8*-o of *S. pombe*, however, the original mutation leading to the suppressor active allele is very likely to have affected the anticodon region of a tRNA structural gene since both suppressors could be further mutated to suppressor active alleles exhibiting the opposite allele specifity, *SUP3*-a and *SUP8*-a. There is every reason to believe that this mutation could also be demonstrated in *SUP9*-o where this has not yet been attempted. We conclude therefore that the asymmetric location of the original mutant site in the fine structure maps of *SUP8*-o and *SUP9*-o is probably a reflection of the present stage of the mapping which may indeed have been less exhaustive in these loci than in *SUP3*-o.

This may also be the explanation for the observation that map expansion (FINCHAM and HOLLIDAY, 1970), which starts with map distances corresponding to about one half of the total map distance in the fine structure map of *SUP3*-o (LEUPOLD, 1970b, discussed by FINCHAM and HOLLIDAY, 1970; HUBSCHMID and LEUPOLD, in preparation), is barely noticeable in the fine structure maps of *SUP9*-o (HOFER, 1969; HOFER and LEUPOLD, in preparation) and *SUP8*-o (HAESLER, 1971; HAESLER and LEUPOLD, in preparation).

Locating some of the mutant sites (designated by an asterix in the maps of Fig. 2) presents peculiar difficulties which are due to specific marker effects upon recombination. This is true, for instance, of one revertant site in the map of *SUP8*-o, r139. It maps near a normal site, r77, with which it gives only 0.5 prototrophic recombinants per 10^6 ascospores. In agreement with this map location, r139 shows increasing recombination frequencies with other revertant sites located at increasing distances towards its left and towards its right. Compared with the recombination frequencies given by its nearest neighbour, r77, however, these frequencies are several times larger, the factor of increase being between 13× and 28× with revertant sites to the left and between 6× and 8× with revertant sites to the right (HAESLER, 1971; HAESLER and LEUPOLD, in preparation).

Similar marker effects are exhibited by two revertant sites of the *SUP3*-o map, r57 and r10, which are located near the left end of the fine structure map in the immediate neighbourhood of a revertant site r30 which shows normal mapping behaviour. The factor of increase characterizing the recombination frequencies given by r57 are smaller than those observed with r139 in *SUP8*-o (between 2× and 10× when compared to those given by the nearest neighbour, r30) whereas those characterizing r10 are even larger (between 15× and 49× when compared to those given by the nearest neighbour, r30). On the basis of the large recombination frequencies yielded with all other revertant sites except r30, the two exceptional sites r10 and r57 had originally been mapped outside the present map towards the left, thus producing a longer map which showed much stronger map expansion (LEUPOLD, 1970b, discussed by FINCHAM and HOLLIDAY, 1970). It was only after their close linkage with r30 (and a revertant homoallelic with r30) was found that their exceptional nature was recognized (HUBSCHMID and LEUPOLD, in preparation).

It is interesting to note that similar marker effects upon recombination are shown by the anticodon sites of *SUP*3-o and *SUP*9-o. This contrasts with the recombinational behaviour of the anticodon site of *SUP*8-o which fits more or less additively into the fine structure map defined by the revertant sites (except site r139 which was mentioned above). Thus, the recombination frequencies yielded by the anticodon site of *SUP*9-o are in agreement with a location near a revertant site r85 with which it gives only 4 prototrophs per 10^6 ascospores. But although the recombination frequencies yielded by the anticodon site with other revertant sites show the expected increase with increasing distances towards both sides of this location, they are larger than the frequencies given by r85 by factors varying between $3\times$ and $15\times$. Similarly, the anticodon site of *SUP*3-o can be located in the neighbourhood of a revertant site r8 with which it gives 11 prototrophic recombinants per 10^6 ascospores, but the recombination frequencies which it gives with more distant revertant sites exceed those given by its neighbouring site r8 by factors of between $2\times$ and $11\times$.

The difference in the marker effect shown by the anticodon site of *SUP*3-o and *SUP*9-o on one hand (marker effect present) and *SUP*8-o on the other hand (marker effect absent) is striking. It is conceivable that this difference reflects a difference in the wild type anticodon of the tRNA's involved. Thus, if ochre-specific suppression is due to a mutant anticodon *A*UA which is deaminated to *I*UA as discussed in an earlier paragraph (Bock, 1967), then *SUP*8-o might for instance represent the mutant form of the structural gene for a tyrosine tRNA with the anticodon *G*UA (or rather GΨA, Madison et al., 1966). It would therefore require a G+C → A+T transition for its mutation to the suppressor active form. *SUP*3-o and *SUP*9-o, on the other hand, could represent mutant alleles of structural genes for serine tRNA's with the anticodon I*G*A (Zachau et al., 1966). In this case, they would require a G+C → T+A transversion for the mutation to their suppressor active form. The reverse relationship (*SUP*3-o and *SUP*9-o = tyrosine, *SUP*8-o = serine) would of course also be possible. In two-point crosses involving the anticodon site and a revertant site, this would lead to different base pair inconsistencies whenever the anticodon site would become included in hybrid DNA, and in combination with surrounding base sequences (Norkin, 1970), these differences might well lead to a specific marker effect in one type of tRNA structural gene (*SUP*3-o and *SUP*9-o) but not in the other (*SUP*8-o). The conclusion that the amino acid inserted by the mutant tRNA's of *SUP*3-o and *SUP*9-o may differ from that inserted by the mutant tRNA of *SUP*8-o, is indeed supported by the finding that the patterns of allele specific action, which are identical in the *ade*1 and *ade*7 locus, are found to differ with respect to one mutant in the *ade*6 locus (*ade*6-712). As discussed in an earlier paragraph, this mutant is suppressed by *SUP*8-o and *SUP*10-o but not by *SUP*3-o and *SUP*9-o (Barben, 1966; P. Thuriaux and U. Leupold, unpublished; cf. Table 3).

In the case of *SUP*3, an additional mutation in the anticodon region which transforms the original suppressor active allele *SUP*3-o into an allele ex-

hibiting the opposite specificity, *SUP3*-a, abolishes the marker effect at the anticodon site. This has been shown by constructing a second fine-structure map of *SUP3* on the basis of the prototroph frequencies yielded by pairwise crosses of 13 spontaneous and 2 nitrous acid induced auxotrophic revertants derived from *SUP3*-a (constitution *ade7*-a *sup3*-a,r) and an auxotrophic strain carrying the suppressor inactive wild type allele at the suppressor locus (constitution *ade7*-a *sup3*$^+$). In this map which locates the anticodon site within a region defined by five revertant sites, the recombination frequencies shown by the anticodon site in crosses with revertant sites, fit more or less additively into the recombination frequencies determined in revertant × revertant crosses (HUBSCHMID, 1972; HUBSCHMID and LEUPOLD, in preparation). The *SUP3*-a map still remains to be correlated with the *SUP8*-o map.

If for the sake of argument we assume that *SUP3*-o produces an ochre-specific serine suppressor tRNA with the mutant anticodon I*U*A instead of the wild type anticodon I*G*A, then the second mutation which leads to the amber-specific mutant anticodon *C*UA and which abolishes the marker effect would concern a neighbouring base pair in the anticodon region of the structural gene of the tRNA. In the case of a tyrosine tRNA, both the first mutation transforming the wild type anticodon *G*UA into the ochre-specific anticodon *I*UA, and the second mutation turning this into the amber-specific anticodon *C*UA, would affect the same base-pair within the anticodon region of the structural gene of the tRNA. In either case, the modification of the local nucleotide sequence which results from the second base-pair substitution might suffice to abolish the marker effect which is produced by the nucleotide sequence resulting from the first base-pair substitution.

In tetrad analyses of single-point crosses involving either selected revertant sites (*sup*-o,r × *SUP*-o) or the anticodon site (*sup*$^+$ × *SUP*-o, or *sup*$^+$ × *SUP*-a) in *SUP3*-o, *SUP3*-a, *SUP8*-o and *SUP9*-o, the conversion frequencies shown by exceptional markers (i.e. markers which exhibit strong marker effects in two-point crosses) do not differ significantly from those given by markers which behave normally in random spore analyses of two-point crosses. Furthermore, conversion is equally frequent from mutant to wild type and from wild type to mutant, independently of whether an exceptional marker or a normal marker is involved (A. AHMAD and D. ZBAEREN, in preparation).

This distinguishes the exceptional markers studied in these tetrad analyses (revertant site *sup3*-o,r10 and the anticodon sites of *SUP3*-o and *SUP9*-oa) from the exceptional mutant site *ade6*-M26 described by GUTZ (1971). *ade6*-M26, a nonsense mutant sensitive to suppressors of the first (*SUP1*-a) and second class (*SUP3*-o), shows specific marker effects not only in random spore analyses and tetrad analyses of two-point crosses (e.g., increased recombination frequencies) but also in tetrad analyses of single-point crosses (i.e., an increased total conversion frequency and more frequent conversion from mutant to wild type than from wild type to mutant). No other nonsense mutant isolated in the *ade1*, *ade6* and *ade7* loci shows the drastic increase in

recombination frequency exhibited by *ade*6-M26 when crossed with other mutants of the *ade*6 locus (between 3× and 21× when compared with the recombination frequencies given by the nearest normal marker, *ade*6-M375).

The marker effects observed in the suppressor loci are specific for meiotic recombination. This has been shown for *SUP*3-o and *SUP*9-o by intragenic mapping procedures based on the induction of mitotic recombination in heterozygous diploids of heteroallelic constitution (WYSSLING, 1972; WYSSLING and LEUPOLD, in preparation). Methylmethanesulfonate was used for inducing mitotic recombination, using the methods described by SNOW and KORCH (1970).

In *SUP*9-o, mitotic mapping has so far been confined to the anticodon site and the two extreme revertant sites r49 and r104 which are located at the left and the right end of the meiotic map, respectively. In the mitotic map, genetic distances between these three sites are found to be additive (r49× anticodon = 4.7 map units, anticodon×r104 = 2.6 map units, r49×r104 = 6.9 map units; 1 map unit = 1 prototroph/10^8 survivors/min[2] of treatment with MMS, under the conditions described by SNOW and KORCH, 1970). This contrasts clearly with the meiotic situation where the sum of the recombination frequencies measured in the anticodon×revertant crosses exceeds by far the recombination frequency determined in the revertant×revertant cross (r49×anticodon = 258 map units, anticodon×r104 = 51 map units, r49× r104 = 36 map units; 1 map unit = 1 prototroph/10^6 ascospores).

In *SUP*3-o, eight of the nine known revertant sites have been mapped mitotically, in addition to the anticodon site. With one minor exception, the order of the sites is the same as in the meiotic map. Genetic distances are found to be more or less additive over the whole length of the map. This shows again that the specific marker effects exhibited by the revertant sites r10 and r57 and by the anticodon site disappear upon mitotic mapping based on induced mitotic recombination. The same is true of the general marker effect underlying map expansion (FINCHAM and HOLLIDAY, 1970): Map expansion has been observed in the meiotic but not in the mitotic map of this suppressor locus.

Comparison of the relative genetic length of the mitotic MMS maps of *SUP*3-o (12 map units) and *SUP*9-o (7 map units) with that of *ade*7 which is the structural gene of Saicar synthetase (FISHER, 1969) and therefore of a protein (168 map units; WYSSLING, 1972; WYSSLING and LEUPOLD, in preparation) adds additional support to the conclusion, suggested already by the comparison of the meiotic map length of these and other loci, that the specific nonsense suppressors of the second class (*SUP*3-o, *SUP*8-o, *SUP*9-o, *SUP*10-o) and their alleles of the third class (*SUP*3-a, *SUP*8-a) are mutant forms of transfer ribonucleic acids rather than of protein components of the protein synthesizing machinery. Fine structure mapping may also help to decide whether the same is true of the nonspecific nonsense suppressors of the first class (*SUP*1-oa, *SUP*2-oa).

8. Omnipotent Suppressors

Two loci in *Saccharomyces* appear to be the site of nonsense suppressors which are not coding for tRNA's. The suppressors at these loci are omnipotent in the sense that they act upon all three classes of nonsense alleles. However, these suppressors, *SUP35* (chromosome IV) and *SUP45* (chromosome II), are weak suppressors with rather restricted spectra, especially in regards to the ochre alleles suppressed. Suppressors with slightly different spectra are found at the same locus: for example, *SUP35* (Class VI) and *SUP40* (Class VII) are alleles, and *SUP45* is also allelic with a suppressor having a more restricted spectrum. Only small samples (10–12) of tetrads were analyzed from the suppressor × suppressor crosses, but since all the omnipotent suppressors are recessive, allelism is confirmed with the expression of the suppressed phenotypes in the diploids.

The omnipotent suppressors at both loci are induced by both ethylmethanesulfonate and nitrous acid regimens (HAWTHORNE 1969a, and unpublished). Neither amber nor ochre-specific suppressors are induced by nitrous acid. Nitrous acid does induce the back mutation of ochre alleles; therefore it is promoting a base substitution in the nonsense triplet. The transition A+T→ G+C or the transversions A+T→ T+A and A+T→ C+G will give sense codons. We can deduce that the nitrous acid regimen causes only the A+T→ G+C transition, for if it promoted the transversions, then it should have enhanced the mutation of amber and ochre-specific suppressors. Thus nitrous acid and ethylmethanesulfonate are acting upon different bases to give the omnipotent suppressor mutation. This conclusion cannot be reconciled with mutations involving the anticodon triplet of a tRNA gene. Possibly mutations outside the anticodon triplet could reduce the specificity of the anticodon—codon pairing to enable a sense anticodon to pair with a nonsense codon (HIRSH, 1971). However, if this were the case, one might expect alleles of these suppressors to show specificity for a given nonsense codon, but none have been found among the several hundred suppressors examined.

The observation that the suppressors are recessive is another facet of the argument against their coding for tRNA's. Even if the suppressor product were a tRNA which was ineffective in competition with chain termination, we should expect to see a dosage effect in the diploid in that the presence of suppressors at the two different loci would likely be equivalent to homozygosity at one suppressor locus.

Since the tRNA genes are unlikely prospects for the source of the omnipotent suppressors, other components in the translation apparatus need to be considered. The mutation of genes coding for ribosomal proteins or release factors (CAPECHHI, 1967) would be more compatible with the above observations, particularly the recessive nature of the mutant allele.

The precedent for a ribosomal nonsense suppressor is seen with the *ram* (ribosomal ambiguity) mutants of *E. coli* (ROSSET and GORINI, 1969). All three nonsense codons are suppressed in *E. coli* strains bearing *ram*.

9. Missense Suppressors

Informational suppression was first conceived in the context of missense suppression (YANOFSKY and ST. LAWRENCE, 1960). In a classic demonstration, the "finger printing" of the cross-reacting material from a tryptophan synthetase mutant, A36, in *E. coli* revealed that arginine had replaced a glycine residue of the wild type protein. In the presence of an allele specific suppressor, *su36*, a small amount of the wild type peptide containing glycine is found along with the mutant peptide containing arginine (BRODY and YANOFSKY, 1963). Through the use of *in vitro* protein synthesizing systems, the essential component contributed by the suppressor bearing stock was shown to be a tRNA (CARBON et al., 1966).

A case of missense suppression in *S. cerevisiae* has been described by GORMAN and GORMAN (1971). The mutant allele, *his2*-1, is osmotic-remedial and does not respond to ochre-specific or amber suppressors. Two dominant suppressors, *SUP*-H1 and *SUP*-H2, act upon *his2*-1 but not upon 4 other *his2* alleles. The suppressors do not act upon known ochre and amber alleles nor upon any other mutant tested. Twelve presumptive missense mutants were included in the tests against the suppressors. The failure to find other susceptible alleles from a small sample of missense mutants is in keeping with the likelihood that missense suppression will require a particular amino acid substitution to restore an active site or promote the correct folding of the protein.

The two suppressors are neither linked to *his2* nor to each other. One of them, *SUP*-H1, is centromere-linked, and so it possibly is the suppressor of *his2*-1 located on chromosome XII by MORTIMER and HAWTHORNE (1973).

In *S. pombe*, missense suppressors have not been studied in any detail. Two classes of suppressors described by BARBEN (1966) are likely to belong to this category, however. The allele specific patterns of action of these suppressors are very restricted and do not overlap with those of the known nonsense suppressors. Two of these suppressors, *SUP*4 and *SUP*5, suppress 3 heteroallelic *ade7* mutants (*ade7*-519, *ade7*-680 and *ade7*-541) out of 49 completely blocked mutants tested. Two suppressors of a second class, *SUP*6 and *SUP*7, suppress only one of the 49 mutants (*ade7*-465). No mutant sites sensitive to any of these suppressors are known in the *ade*6 locus (59 completely blocked mutants tested) and the *ade*1 locus (28 completely blocked mutants tested: the suppression of *ade*1-H259 by *SUP*6 and *SUP*7 described by BARBEN could not be confirmed in later experiments; LEUPOLD, unpublished).

10. Adverse Effects of Suppressors

Suppressors are selected under conditions where they are essential for the growth of the cell; however when the cells are returned to a complete medium their presence may become disadvantageous. In *S. cerevisiae* a suppressor's effect on growth rate has been quantitated in only a few cases and unfortunately

the various combinations of mutant versus wild type allele and suppressor versus wild type allele were not presented in a strictly isogenic background. In general, the influence of a single suppressor appears to be nominal, ranging from a slight stimulation for an ochre-specific suppressor, *SUP*1 (*iso*1-1) (KAKAR, 1963) to a slight inhibition for the missense suppressors, *SUP*-H1 and *SUP*-H2 (GORMAN and GORMAN, 1971). Exceptions to this generalization are the Class IX amber suppressors, *SUP*2-a through *SUP*8-a, which in conjunction with a cytoplasmic factor confer a petite phenotype (CLAISSE and HAWTHORNE, unpublished), and the Class I and II ochre suppressors, *SUP*2 through *SUP*8 and *SUP*11, which when combined with the cytoplasmic factor *psi*+ are lethal (COX, 1971). These cases will be presented in detail in the next section dealing with the modifiers of suppressors.

Both recessive suppressors isolated by INGE-VECHTOMOV (1965), s_{48} and s_{80}, cause the haploid stocks to have a prolonged lag phase in their growth curves on complete medium and also result in lesser yields (SMIRNOV et al., 1968). Because of the recessiveness of the suppressors, the authors suggest that they are not coding for tRNA's. Most likely they are alleles of *SUP*35 and *SUP*45, the omnipotent suppressors.

Certain isolates of omnipotent suppressors at either locus, *SUP*35 or *SUP*45, cause a marked increase in the frequency of inviable spores, and those suppressor bearing spores that do germinate grow more slowly than their suppressorless sister spores. The combination of *SUP*35 and *SUP*45 in a haploid segregant appears no more deleterious than the presence of the single suppressor with the more drastic effects.

The two *his*2-1 missense suppressors, *SUP*-H1 and *SUP*-H2, together in the same haploid are definitely more deleterious than either alone. This is evident from a comparison of the generation times on a complete medium for *his*2-1 cells with no suppressor, with *SUP*-H1, with *SUP*-H2, and with both *SUP*-H1 and *SUP*-H2, which are 120, 130, 128, and 165 minutes respectively (GORMAN and GORMAN, 1971).

The segregation of two Class I ochre-specific suppressors to the same spore results in poor viability or minute spore colonies containing morphologically aberrant cells (GILMORE, 1967). GILMORE did not find these symptoms of the "two suppressor effect" with haploids containing two of his Class III suppressors or when a Class I suppressor was combined with a Class III suppressor. There is no pronounced enhancement of ill effects with the following assortment of suppressors to the same haploid spore: two amber suppressors from Class X; a Class I ochre and a Class IX amber; a Class III ochre and a Class X amber and an UGA suppressor, *SUP*71; a Class I ochre, a Class X amber, and an UGA suppressor.

The absence of a compounding of ill effects with the two omnipotent suppressors in the same haploid segregant suggests that their products function in sequence so that one suffices to evoke the limitation to growth. The poor growth of a haploid strain bearing two missense suppressors could be due either to excessive mistranslation of the codon in question or a reduction

in the supply of the normal tRNA from which the suppressors were derived. The explanation advanced for the deleterious effect of the two Class I ochre suppressors in the same haploid is that there is excessive translation of the "stop" codons for the normal messengers (GILMORE, 1967). It cannot be a deprivation of tRNA's needed for the translation of the tyrosine codons UAU and UAC since most Class I suppressors will have the anticodon IΨA capable of recognizing these codons. The fact that other combinations of the specific nonsense suppressors are not unduly harmful would indicate that UAG and UGA have a much lesser role than UAA in the normal chain termination.

Of the nonsense suppressors known in *S. pombe*, only the ochre-amber suppressors of class I cause a measurable increase of the generation time of strains grown in an enriched yeast extract-glucose medium (M. MINET, and P. THURIAUX, unpublished). In stock cultures, however, both the ochre-amber suppressors of the first class (*SUP*1-oa and *SUP*2-oa) and the amber-specific suppressors of the third class (*SUP*3-a and *SUP*8-a) show adverse effects. Both classes of suppressors cause a selective pressure in favour of mutants which have lost suppressor activity, either because of secondary mutations in the suppressor locus or because of mutations in modifier loci. Stock cultures of strains carrying ochre-specific suppressors of the second class (*SUP*3-o, *SUP*8-o, *SUP*9-o and *SUP*10-o) are much more stable in this respect.

Tetrad analyses of crosses between strains which carry the ochre-amber suppressors *SUP*1-oa and *SUP*2-oa have shown that the double mutant combination *SUP*1 *SUP*2 is lethal. No lethality has been observed, however, in crosses involving an ochre-amber suppressor (*SUP*1-oa) and an ochre-specific suppressor (*SUP*3-o) (BARBEN, 1966). In the six possible pairwise crosses between strains carrying two different ochre-specific suppressors of the second class, *SUP*3-o, *SUP*8-o, *SUP*9-o and *SUP*10-o, only the double mutant combination *SUP*3-o *SUP*9-o proves to be lethal (M. MINET and P. THURIAUX, unpublished). As has been discussed above in section 6 and 7b, it is likely that *SUP*3-o and *SUP*9-o differ from *SUP*8-o and *SUP*10-o by reading the same nonsense codon as two different amino acids. *SUP*3-o and *SUP*9-o may well correspond to the ochre-specific suppressors of class I of *S. cerevisiae* which show the same lethal effects in double mutant combinations (GILMORE, 1967) and which are known to substitute tyrosine in the translation of UAA (GILMORE et al., 1968, 1971).

The presumptive missense suppressors *SUP*6 and *SUP*7 (cf. section 9) have also been found to have lethal effects when combined in progeny spores of double mutant constitution *SUP*6 *SUP*7 (M. MINET and P. THURIAUX, unpublished).

11. Modifiers of Suppressors

The deleterious nature of the suppressors is seen in the spontaneous appearance of genetic modifiers restricting their expression. In *S. cerevisiae*, these anti-suppressors have been obtained with missense suppressors (GORMAN

and GORMAN, 1971), ochre suppressors (COX, 1965; SOIDLA and INGE-VECHTOMOV, 1966), and amber suppressors (HAWTHORNE, 1967).

The nature of the first modifier to be described, the cytoplasmically inherited determinant *psi* discovered by COX (1965), remains something of an enigma so its characterization will be reported in some detail. The suppressor in question, *SUQ5*, was obtained in the selection of adenine prototrophs in a heteroallelic *ade2-1/ade2-c* diploid. The tetrad analysis of the spores from the revertant diploid established that *SUQ5* is specific for the ochre allele *ade2-1*: a spore with *ade2-1* and *SUQ5* gives a white adenine independent colony, while a spore with *ade2-c* and *SUQ5* gives a red adenine dependent colony. The discovery of the *psi* factor followed from the investigation of rare red/white sectored spore clones. When cells from the red sector were crossed to cells from a red *ade2-1*, suppressorless stock, the resultant diploid was white and adenine independent and yielded asci with 2 white adenine independent segregants and 2 red adenine dependent segregants. The backcross of cells from the red sector to cells from a white *ade2-1 SUQ5* haploid gave a white adenine independent diploid yielding tetrads of 4 white adenine independent spore clones. These results indicate that the cells of the red sector have the genotype *ade2-1 SUQ5*. To explain the failure of *SUQ5* to suppress *ade2-1*, it was necessary to postulate that another component *psi+* was required for the expression of *SUQ5*. The cells of the red sector are *psi−*, and in the first test cross above, the *ade2-1* suppressorless stock contributed *psi+*. Since *SUQ5* is expressed in the diploid, *psi+* is dominant. Moreover, the failure of *psi−* to reappear in the spore clones from either cross indicates that *psi* is not a nuclear gene.

Three red sectored colonies of spontaneous origin were examined by COX (1965). All 3 behaved as described above and intercrosses of cells from the red sectors gave red adenine auxotrophic diploids. Thus these 3 cases appear identical in that there was a loss of the *psi+* factor.

YOUNG and COX (1971) sought mutants in the *SUQ5 psi+* system by looking for multiple auxotrophs in a haploid strain bearing *SUQ5 psi+* and the suppressible alleles *ade2-1 met tyr*. The examination of 8359 colonies arising from ultraviolet irradiated cells gave 18 red adenineless, methionineless, and tyrosineless colonies. Three of these colonies were *psi−*. When crossed to a red *ade2-1 SUQ5 psi−* haploid, they gave diploids that were red and adenine dependent and which yielded only adenineless spores. Crosses of these 3 mutants to a white *ade2-1 SUQ5 psi+* haploid gave white diploids yielding only white prototrophic spores. One of the 18 colonies was defective in the *SUQ5* gene: when crossed to either the red *ade2-1 SUQ5 psi−* haploid or the white *ade2-1 SUQ5 psi+* haploid, it gave a white prototrophic diploid which yielded asci with 2:2 segregations for the adenine requirement. The remaining 14 colonies did not fall into either category. A representative "U-16" had the following properties: when crossed to the red *ade2-1 SUQ5 psi−* haploid, it gave a red diploid yielding only red adenineless spores; crossed to a white *ade2-1 SUQ5 psi+* haploid, it gave a pink adenine dependent diploid which

yielded in 4 of 8 asci, spores showing 1:3 or 2:2 segregations for white prototrophic versus red adenineless phenotypes.

At this point, this behaviour of U-16 could be explained by a suppressive *psi*— factor, i.e. a *psi*— which supplants *psi*+, or by a dominant nuclear gene which prevents the expression and replication of *psi*+. The latter interpretation was favored when backcrosses to white *ade*2-1 *SUQ*5 *psi*+ haploids with the 4 adenineless red clones from one of the 0:4 asci from the preceding diploid gave in two cases hybrids displaying a pattern for the tetrad analyses similar to that of the above pink diploid. The other two hybrids were white and yielded tetrads with mostly white prototrophic segregants, an indication that the red parents were not bearing a suppressive *psi*—.

This nuclear gene *R* which inhibits the expression of *psi*+ causes a progressive loss of the *psi*+ factors in the vegetative descendants from *ade*2-1 *SUQ*5 *R psi*—/*ade*2-1 *SUQ*5 *r psi*+ zygotes. This was demonstrated by an experiment in which zygotes were sporulated directly or isolated to give rise to diploids which were propagated 2, 3, or 4 days before inoculation of the sporulation medium. The percent of red (*psi*—) spore clones rose from 52% for the zygotes to 61%, 71%, and 74% for 2, 3 and 4 days of growth.

Another manifestation of *psi*+ is seen in the lethality resulting from the combination of *psi*+ and a Class I or II ochre-specific suppressor (Cox, 1971). This was first seen in the tetrad analysis of another revertant of the heteroallelic *ade*2-1/*ade*2-c diploid. Only two spores per ascus gave viable colonies, and they were always adenine dependent. Since both the *ade*2-1 and *ade*2-c alleles were recovered, it was proposed that a suppressor, *SUQ*2, was responsible for the prototrophy of the diploid and the lethality in the spores. Spores with the lethal gene were rescued by mating them to *ade*2-1 vegetative cells, and in this way, the suppressor was shown to be present. Successive spore to cell matings were undertaken to establish that *SUQ*2 acted upon the ochre alleles *his*5-2 and *lys*1-1 as well as *ade*2-1.

The *psi*+ factor was implicated in the lethality of *SUQ*2 in haploids as a result of the investigation of a rare tetrad (1/200) with 3 viable spores, one of which was *ade*2-1$^+$, i.e. bearing *SUQ*2. Crosses with this *ade*2-1$^+$ segregant gave on the one hand diploids which again segregated a lethal and the two viable spores were suppressorless, and on the other hand were diploids which yielded 4 viable spores, with two bearing a suppressor. In one of the latter crosses, the test parent was known to be *psi*—; it carried *SUQ*5 which was not being expressed. Since *SUQ*5 was assorted to the haploid spores of this diploid and still not expressed in these tetrads, the diploid and thus the *ade*2-1$^+$ *SUQ*2 parent must also have been *psi*—. The other diploids from which the lethal segregated were from crosses to *psi*+ haploids.

The tetrad analyses of the crosses involving *SUQ*2 established that this suppressor was centromere-linked, 10 percent second-division segregation, and linked to *his*2 on chromosome VI. With this location, *SUQ*2 could be *SUP*11. Although allelism tests were not made, *SUP*11 behaves as *SUQ*2 in crosses to *psi*+ haploids: the diploids give tetrads segregating a lethal and the two

viable spores are suppressorless. In the control crosses to *psi*− haploids, the diploids give asci with four viable spores and *SUP*11 is recovered in two of the spores.

To see if *psi*+ interacted with other ochre-specific suppressors, *psi*+ stocks were crossed to haploids bearing the Class I suppressors *SUP*2, *SUP*3, *SUP*4, and *SUP*5 and Gilmore's Class III suppressors S_s (*SUP*25) and S_t. The combination of *psi*+ and a Class I suppressor was extremely deleterious, sometimes even lethal in the diploid. Some zygotes from these crosses lysed immediately or gave rise to only a few cells before the onset of lysis. Other zygotes gave rise to minute colonies of slow growing cells; however, none of the clones were able to sporulate. The combining of *psi*+ with *SUP*25 or S_t did not cause lethality in either diploids or haploids, or modify the expression of these suppressors.

No lethality was seen when *psi*+ was combined with the amber-specific allele of *SU*Q2. The mutation of *SU*Q2 to *SU*Q2-a was obtained in *psi*− haploids. The crosses of *SU*Q2-a isolates to *psi*+ stocks gave diploids yielding asci with four viable spores (COX, 1971).

In summary, *psi*+ is essential for the expression of the ochre suppressor *SU*Q5. The combination of *psi*+ with *SUP*11, *SUP*2, *SUP*3, *SUP*4, *SUP*5, and presumably the remainder of the Class I ochre-specific suppressors is lethal. If *psi*+ interacted with the ochre suppressors *SUP*25 or S_t, the effects went undetected; the same is true for the combination of *psi*+ and the amber suppressor *SUP*11-a (*SU*Q2-a). With this information, it is still not clear whether *psi*+ only affects translation by a particular set of ochre suppressors, perhaps those with the anticodon IUA or IΨA, or if the effect is a generalized increase of translation efficiency by all suppressors. The lethality of the combination of *psi*+ with *SUP*11 or a Class I suppressor is probably due to excessive stop codon translation; we need not expect *psi*+ to have the same lethal effect in combination with *SUP*11-a with the assumption that UAG is seldom the normal stop codon.

The presence of *psi*+ in a cell has no apparent effect on phenotype other than through its interaction with *SU*Q5, *SUP*11, or a Class I suppressor. While it is clear that *psi*+ is nonessential, it is not known whether *psi*+ or *psi*− is the natural state. Both *psi*+ and *psi*− strains are found in laboratory collections where there has been no intentional selection for either state.

The source of the genetic information imparted by *psi*+ is another unknown. It is not the mitochondrial DNA. YOUNG and COX (1972) have shown that *psi*+ is inherited independently of the mitochondrial gene *ery*R (erythromycin resistance) and the *rho*+ factor conferring respiratory sufficiency in crosses to suppressive *rho*− stocks. Conversely, the nuclear gene *R*, which prevents the expression and replication of *psi*+, has no effect on the expression or transmission of *rho*+ or *ery*R. An alternative source of a cytoplasmically transmitted gene would be a plasmid such as the carrier of the killer factor (BERRY and BEVAN, 1972).

SOIDLA and INGE-VECHTOMOV (1966) discovered a naturally occurring genic modifier of their nonsense suppressor S_5 with the tetrad analysis of crosses involving S_5 and the suppressible alleles *ade*2-37 and *ade*2-105. The action of S_5 on the alleles *ade*2-37 and *ade*2-105 is incomplete in that the colonies are still a pale pink. The presence of the antisuppressor intensifies the coloration. Even in the absence of the suppressor, the antisuppressor is manifested by restricting the complementation pattern of *ade*2-105 and non-suppressible alleles of *ade*2. Since no phenotypes other than the adenine dependence were discussed, it is not clear whether the modifier is interacting with S_5 and the nonsense alleles of *ade*2 or if it is involved in the regulation of the adenine pathway.

An antisuppressor for the missense suppressors *SUP*-H1 and *SUP*-H2 arose spontaneously with the maintenance of the suppressor bearing stocks on the YEP standard medium of yeast extract, peptone, and glucose (GORMAN and GORMAN, 1971). When the stored *his*2-1 *SUP*-H1 or *his*2-1 *SUP*-H2 stocks were sampled, they were found to have a high proportion of cells unable to grow on histidineless medium. This phenomenon was attributed to the physiological state of the cells since most could give rise to clones, on YEP, which eventually regained the prototrophic phenotype. However, from 0.1 to 5 percent of the sample gave stable auxotrophic clones and in all cases these clones were also petites.

The petite condition itself did not impose the histidine requirement. Petites were induced in the prototrophic sister clones without the induction of the histidineless phenotype.

Crosses with the auxotrophic petite isolates to *his*2-1 suppressorless haploids gave prototrophic diploids whether or not the diploids were grande or petite. Therefore the suppressor is still present and the modifier is recessive. The tetrad analysis of the asci from a grande diploid gave 7 (2:2), 4 (1:3), and 10 (0:4) segregations (+:−) for the histidine phenotype. If a single gene is responsible for the reappearance of the histidine requirement, then it is segregating independently of the suppressor. Evidence for the involvement of a single gene in the petite auxotrophic parent, strain "L-1", was obtained from a cross giving a diploid homozygous for the suppressor, *SUP*-H3 (an allele of *SUP*-H1), and *his*2-1. Only 2:2 segregations were seen in 19 asci from this diploid. To show that the auxotrophy was not the result of a second site mutation in the *his*2-1 allele, a cross of "L-1" to a *HIS*2 *SUP*-H3 haploid was analyzed: the 8 (4:0), 10 (3:1), and 9 (2:2) segregations indicate the modifier gene *sin*1 (suppressor interacting) is not linked to *his*2.

It should be noted that the petite auxotrophic parent "L-1" was a cytoplasmic (*rho*−) petite and so the spore clones from the above hybrids were grandes. Thus the *rho*− or respiratory deficiency is not necessary for the expression of *sin*1. To account for the fact that the original auxotrophic isolates were all petites, we can only assume the petite condition is conducive for either the mutation or selection of *sin*1.

Strong selective pressure for antisuppressors is created by the presence of a Class IX amber suppressor, *SUP*2-a through *SUP*8-a, in either haploid or diploid cells. These suppressors are dominant in the imposition of a pseudopetite phenotype. The suppressor bearing strains cannot use glycerol as an energy source and the diploids do not sporulate; however, they have a nearly normal cytochrome profile, although cytochrome-*c* may be somewhat in excess. After a prolonged lag period, they are able to adapt to ethanol or lactate, and their aerobic growth on glucose results in yields closer to those of the suppressorless grande parent than a *rho*$^-$ isolate of the parent (CLAISSE and HAWTHORNE, unpublished).

From a glycerol medium plate, grande revertants of a pseudopetite haploid (2098-4A''-2-3) bearing *SUP*5-a were picked and examined for the suppressible phenotypes. One revertant displaying a partial loss of the suppressor activity, *ade*5,7(+) *trp*1(+)*tyr*6(+) → *ade*5,7(−) *trp*1(−) *tyr*6(+), was crossed to a suppressorless *ade*5,7-101 *trp*1-1 *tyr*6-1 haploid. The diploid was adenine and tryptophan dependent but tyrosine independent. However, adenine independent spores occurred in the asci with a distribution indicative of the segregation of complementary genes for adenine independence: 2 (2:2), 11 (1:3), and 5 (0:4) +:− ratios. This result was interpreted as the segregation of a dominant antisuppressor, *SIN*2, unlinked to the suppressor *SUP*5-a (HAWTHORNE, unpublished).

Crosses with *SUP*5-a *SIN*2 strains to a diverse selection of stocks with no history of *SUP5*-a or *SIN*2 revealed that *SIN*2 was present in 5 of the 8 strains sampled. The explanation for this rather surprising result is that *SIN*2 was already present in 2098-4A''-2-3 and the mutational event selected on the glycerol plate was a mutation of *SUP*5-a making it susceptible to *SIN*2. This conclusion was reached after demonstrating that *SIN*2 had no effect on the original ochre suppressor *SUP*5, and on newly isolated amber suppressors *SUP*5-a, *SUP*3-a, *SUP*7-a, and *SUP*8-a. It has been possible to select mutants of *SUP*7-a and *SUP*8-a which are sensitive to *SIN*2 and whose spectra are restored when *SIN*2 is crossed out (HAWTHORNE, unpublished).

Restoration of the full expression of the mutated *SUP*5-a′ in the presence of *SIN*2 can be achieved by a mutation of yet another class of modifiers, *sal* the "allo-suppressors" (HAWTHORNE, 1967). The allo-suppressors also enhance the expression of ochre suppressors: the weak ochre-specific suppressor *SUP*20 (Class IV) in the presence of an allo-suppressor will now have *tyr*1-1 in its spectrum as well as acting more efficiently upon the alleles *met*4-1 and *ade*6-3. Moreover, the allo-suppressors in combination with a Class I ochre-specific suppressor will suppress amber alleles fairly efficiently: for example, the combination of *SUP*5 *sal*1 with *trp*1-1 will result in good growth of a haploid on tryptophanless medium (about 50 percent wild type yield) by 3 days. The allo-suppressors by themselves are weak suppressors; the linkage data indicate they could be alleles of the omnipotent suppressors *SUP*35 and *SUP*45. However, they have more restricted spectra than the omnipotent suppressors which are isolated directly, in that only *leu*2-1 and *iso*1-1 are

suppressed and then very poorly. The allo-suppressors are recessive by themselves or in their interaction with *SUP*5-a', *SUP*5, or *SUP*20.

In *S. pombe*, mutations in modifier genes causing a reduction of the suppressor activity have regularly been obtained, along with secondary mutations in the suppressor locus, when auxotrophic revertants of constitution *sup*-o,r, *sup*-a,r or *sup*-oa,r where selected from prototrophic strains containing a nonsense suppressor on the background of a suppressor sensitive adenine mutant (HOFER, 1969; HAESLER, 1971; HUBSCHMID, 1972; M. MINET and P. THURIAUX, unpublished). In the case of the inefficient suppressors *SUP*3-a and *SUP*1-oa, they represented the overwhelming majority of the mutants obtained. At present, fourteen gene loci *sin*1—*sin*14 which in their mutant form interfere with suppressor activity have been identified (HOFER, 1969; M. MINET and P. THURIAUX, unpublished; *sin*1, *sin*2 and *sin*3 correspond to *mod*1, *mod*2 and *mod*5 of HOFER, 1969; *sin*4, *sin*5, *sin*6 and *sin*7 to *mod*-15, *mod*-71, *mod*-111 and *mod*-117 of HUBSCHMID, 1972; and *sin*8 to *mod*-68 of HAESLER, 1971; two presumptive modifiers of HOFER, *mod*3 and *mod*5 now have been identified as mutations in adenine loci).

The gene products coded for by these modifiers are unknown. However, *sin*1, at least, is likely to code for a protein since nine independent mutations in this locus have been found to map at eight different sites within a chromosomal region which has the map length of a protein structural gene (292 meiotic map units, one map unit corresponding to 1 prototrophic recombinant per 10^6 ascospores). In diploid combinations of heterozygous constitution, these mutants fail to show interallelic complementation (HOFER, 1969).

When separated from the accompanying suppressors, these modifiers do not exhibit drastic phenotypic effects. Thus, in medium containing yeast extract and glucose, the growth rate of strains carrying *sin*1 is indistinguishable from that of wild type strains. However, with strains carrying either *sin*2, *sin*3 or *sin*14, the generation time is approximately doubled.

The fourteen antisuppressors of *S. pombe* have been analyzed for the suppressor specificity of their modifying action, using growth on minimal medium of suppressor sensitive adenine mutants carrying both a suppressor and an antisuppressor as a criterion to distinguish between active and inactive combinations (HOFER, 1969; M. MINET and P. THURIAUX, unpublished.) One of the modifiers which were tested in combination with ochre-specific suppressors has been found to differentiate between *SUP*3-o+*SUP*9-o on the one hand and *SUP*8-o+*SUP*10-o on the other hand: modifier *sin*1 which was isolated on the background of the ochre suppressor *SUP*9-o (HOFER, 1969), shows an antisuppressor activity in combination with *SUP*3-o and *SUP*9-o but not with *SUP*8-o and *SUP*10-o. Most modifiers like *sin*8 which was isolated on the background of *SUP*8-o (HAESLER, 1971), have an antisuppressor activity on *SUP*8-o and *SUP*10-o as well as on *SUP*3-o and *SUP*9-o. As far as has been tested, the antisuppressing activity of these modifiers is the same in combination with the amber-specific alleles *SUP*3-a and *SUP*8-a as it is with the ochre-specific alleles *SUP*3-o and *SUP*8-o.

This classification of the four specific nonsense suppressors into two pairs of suppressors differing in their sensitivity to the modifying action of anti-suppressors parallels the classification of the same suppressors (i) on the basis of their allele specific action (cf. sec. 6), (ii) on the presence or absence of a marker effect at the anticodon site of their fine structure maps (cf. sec. 7b) and (iii) on the presence or absence of a lethal effect in their double mutant combinations (cf. sec. 10). It lends further support to the conclusion that *sup*3$^+$ and *sup*9$^+$ code for isoaccepting tRNAs transferring one amino acid whereas *sup*8$^+$ and *sup*10$^+$ code for isoaccepting tRNAs transferring a different amino acid.

All modifiers except *sin*8 restrict the expression of the ochre-amber suppressor *SUP*1-oa, but with the exception of *sin*5 *sin*6 and *sin*8, they have no effect on the activity of the presumptive missense suppressors *SUP*6 and *SUP*7.

12. Concluding Remarks

The studies of the nonsense mutants and their suppressors in yeast have provided a modest test of the universality of the genetic code. The three nonsense codons, UAA, UAG, and UGA, discovered in *E. coli* also are found in yeast. In yeast there are suppressors specific for each of the nonsense codons, but thus far, suppressors analogous to the bacterial ochre, i.e. capable of suppressing both UAA and UAG but not UGA alleles, have not been found. This difference between the prokaryotic and eukaryotic organisms with regard to the patterns of suppressor specificity is still compatible with the predictions of the wobble hypothesis for codon-anticodon pairing, if we allow for the greater extent of the enzymic modification of the bases seen in the anticodons of the yeast tRNA's.

One contradiction to this statement that there are only ochre-specific and no ochre-amber suppressors in yeast can be discovered in Table 2. There it can be seen that Class I ochre-specific suppressors are acting upon a presumptive amber allele, *leu*1-101. This paradox is being investigated; at this time we can only speculate that it might be an extreme manifestation of the role of the context of the UAG triplet in the message which permits the pairing of G with I or perhaps S (see appendix).

The importance of the reading context of the nonsense codon on the extent of its translation by suppressors (SALSER, 1969; YAHATA et al., 1970) is demonstrated with more certainty in the comparison of suppressor efficiency in acting upon the two ochre alleles *cyc*1-2 and *cyc*1-9 (GILMORE et al., 1971). In the translation of *cyc*1-9 with *SUP*7, only 1 percent of the wild type level of iso-1-cytochrome *c* is achieved, while the translation of *cyc*1-2 with *SUP*7 gives 9 percent of the wild type level.

The elucidation of the roles of the modifiers in enhancing or restricting suppressor efficiency is one of the goals of our investigations. We suggest that

one modifier, the allo-suppressor, acts by increasing the ambiguity of the codon-anticodon pairing, because of the observation that the combination of a Class I ochre-specific suppressor with an allo-suppressor acts upon amber alleles. It remains to be seen whether or not any of the anti-suppressors in yeast act through the restriction of ribosome ambiguity in a manner analogous to the *str*A mutants of *E. coli* (STRIGINI and GORINI, 1970). Because of the more extensive enzymic modifications of the yeast tRNA's, one anticipates a greater diversity of modifiers than what is found with *E. coli*.

Yeasts also differ from *E. coli* in having a greater redundancy of the tRNA genes. From DNA-RNA hybridization experiments, SCHWEIZER et al. (1969) estimate the presence of 320 to 400 tRNA genes in the genome of *S. serevisiae*. The selection of nonsense suppressors in *Saccharomyces* has provided evidence for a redundancy as high as 8 for the tyrosyl tRNA genes. Yet there is a unique species, either a tryptophan or a glutamine tRNA gene, giving rise to the suppressor in Class XI. Unless there is a differential regulation of the transcription of the various tRNA genes, the state of redundancy of each species must, through evolution, in some measure reflect the occurrence of the corresponding amino acid in the proteins. In the assay of total yeast protein from several brewery yeasts (*S. carlsbergensis*?), tryptophan, tyrosine, and glutamine+glutamic acid occur in ratios of approximately 1:3:10, respectively (EDDY, 1958). Data collected by REECK (1970) on the amino acid composition of 6 enzymes from baker's yeast (*S. cerevisiae*) give an average ratio of 1:2.6:7.9 for these same amino acids. On this basis we would predict the unique species of Class XI will prove to be the tryptophan tRNA gene.

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13. Appendix

a) The Genetic Code

The culmination of the endeavors of various investigators towards the resolution of the genetic code is documented in the *Cold Spring Harbor Symposia on Quantitative Biology Vol. 31* (1966). We have copied in Table 4, the display of the code most useful for visualizing the mutation of tRNA genes to the specific suppressors for the ochre, amber, and umber codons. We apologize for using laboratory jargon for the nonsense codons, but it does simplify discussions. In this connection, we prefer "umber" (BOCK, 1967) rather than "opal" as a nickname for the third nonsense codon since this permits the convenience of a single letter specification in the gene symbols of nonsense alleles, for example *leu*2-1u.

The codon-anticodon pairings in Table 5 are the possibilities for pairing the third base of the codon with the first base of the anticodon. (This may seem a little awkward to a classical geneticist, but it is presented this way

Table 4. The genetic code

UUU Phenylalanine	UCU Serine	UAU Tyrosine	UGU Cysteine
UUC Phenylalanine	UCC Serine	UAC Tyrosine	UGC Cysteine
UUA Leucine	UCA Serine	UAA Ochre nonsense	UGA Umber nonsense
UUG Leucine	UCG Serine	UAG Amber nonsense	UGG Tryptophan
CUU Leucine	CCU Proline	CAU Histidine	CGU Arginine
CUC Leucine	CCC Proline	CAC Histidine	CGC Arginine
CUA Leucine	CCA Proline	CAA Glutamine	CGA Arginine
CUG Leucine	CCG Proline	CAG Glutamine	CGG Arginine
AUU Isoleucine	ACU Threonine	AAU Asparagine	AGU Serine
AUC Isoleucine	ACC Threonine	AAC Asparagine	AGC Serine
AUA Isoleucine	ACA Threonine	AAA Lysine	AGA Arginine
AUG Methionine	ACG Threonine	AAG Lysine	AGG Arginine
GUU Valine	GCU Alanine	GAU Aspartic acid	GGU Glycine
GUC Valine	GCC Alanine	GAC Aspartic acid	GGC Glycine
GUA Valine	GCA Alanine	GAA Glutamic acid	GGA Glycine
GUG Valine	GCG Alanine	GAG Glutamic acid	GGG Glycine

Table 5. Codon-anticodon pairing possibilities

First base of anticodon	Third base of codon
U	A
	G
C	G
A	U
G	U
	C
I	U
	C
	A
S	A

to comply with the convention of writing polynucleotides from the 5′-terminal to the 3′-terminal, left to right.) The arguments for these possibilities are discussed by CRICK (1966) in the wobble hypothesis and by YOSHIDA et al. (1970). These possibilities should be considered to apply to the normal state of ribosomal restriction; even so, from the sequencing of the UGA-suppressor tRNA, a tryptophan-tRNA of *E. coli*, by HIRSH (1971), it was demonstrated that a C, the first base of the anticodon, as a consequence of a base substitution 10 bases removed, pairs with A in the codon.

b) Life Cycles of Yeast

Heterothallic strains have been employed for the study of suppressors in both *S. cerevisiae* and *S. pombe*. In each yeast, the haploid stocks are of either

of two mating types, designated α or *a* in *S. cerevisiae* and + or − in *S. pombe*. The mixing of stocks of opposite mating type leads to cell fusions and zygote formation. In *S. cerevisiae*, the zygotes give rise to stable diploid clones which can be propagated vegetatively and generally a special regimen is required for meiosis and sporulation. In *S. pombe*, meiosis occurs in the zygote. With both yeasts, an ascus with 4 spores containing the 4 products of a meiosis is the normal situation. When the spores are isolated by micromanipulation or free spore collections, they will give rise to stable haploid clones.

Generally the suppressors are isolated in haploid stocks. If the stock is mutant for a number of suppressible alleles, the multiple revertants can be directly classified as due to suppressors (GILMORE and MORTIMER, 1966). Even with a single suppressible allele, suppressor mutations sometimes can be distinguished from back mutants by the colony morphology. For example, when one selects for revertants of *trp*1-1, one finds two predominant colony types: 1) a large colony appearing within 2 days that feeds the background mutant cells, and 2) a colony arising a day or two later that does not feed the background cells (PARKS and DOUGLAS, 1957). The colonies of the first type arise from back mutants, while the second class are derived from suppressor mutations.

If there are no convenient phenotypes for distinguishing suppressor and back mutants, the revertant isolates must be backcrossed to a wild type stock; the diploids sporulated; and the spore clones examined for the reappearance of the mutant phenotype. In any case, the suppressor isolates should be crossed to the mutant stock to test for dominance or recessiveness of the suppressor and to verify that a single gene is responsible for the revertant phenotype by the observation of 2:2 segregations of the parental phenotypes in the 4 spore clones of an ascus.

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Cell Interaction in Conjugation of Ciliates

A. MIYAKE[1]

With 14 Figures

Table of Contents

I. Introduction

Ciliates are eukaryotic unicellular organisms characterized by, (1) cilia on the cell surface, and (2) two types of nuclei which have differentiated into the "germinal" micronucleus and the "somatic" macronucleus in a single cell.

Ciliate cells are separated from each other during most of the life cycle, but under certain conditions two cells temporarily unite and undergo a series of developmental processes including meiosis, fertilization, and reconstructions of nuclear and cortical systems. Since the outline of this phenomenon of conjugation was revealed by BÜTSCHLI (1876), a great number of investigators have been attracted by the mystery and beauty of this sexual process. Accumulated results were occasionally reviewed (CALKINS, 1933; SONNEBORN, 1947; WICHTERMAN, 1953; METZ, 1954; WENRICH, 1954; GRELL, 1962, 1967, 1968; HANSON, 1967; HIWATASHI, 1969; RAIKOV, 1969, 1972).

One of the old yet unsolved problems is that of the conjugation-initiating mechanism. The internal and external conditions which influence the occurrence of conjugation were extensively studied, but the results were rather controversial until mating types were discovered by SONNEBORN (1937). It is now generally believed that ciliates conjugate when cells of complementary mating types meet under appropriate conditions, the most important of which are, (1) cells should be deprived of food after a period of rapid growth, and

1 Max-Planck-Institut für Molekulare Genetik, Berlin-Dahlem, Germany.

(2) cells should be in the mature period of the life cycle. Mating types may be transmitted unchanged through many cell generations. Thus, cell clones of complementary mating types, which were separately grown, may be mixed to induce a burst of conjugation (SONNEBORN, 1950, 1970). However, even under optimal conditions there is always a time lag between the mixing of cells and the appearance of the first conjugant pair. In many ciliates this time lag is about one hour. It is during this time lag that cells of complementary mating types interact with each other and as result of this interaction they gain a capacity to unite into conjugant pairs. Therefore, the heart of the problem of the conjugation-initiating mechanism is in this cell interaction of about one hour.

This "preconjugant cell interaction" is worthy of intensive investigation not only because its understanding is essential for the study of ciliate conjugation but also because this system appears to provide one of the simplest examples of the cell interaction as a trigger for developmental processes. The system is simple because, (1) there are only two types of cells involved, (2) the cells are separate from each other, (3) the interaction induces clear-cut results such as cell union and meiosis, (4) these changes are induced in a relatively short, predictable time, and (5) the cell interaction can be experimentally induced at any time. The system is regarded as a trigger for developmental processes because the preconjugant interaction induces the formation of bicellular conjugant pairs, which leads to a series of processes such as meiosis, fertilization, and degeneration of cortical structures, and eventually induces the total reorganization of the cell.

The preconjugant interaction is followed by the "conjugant interaction" which begins as cells form a conjugant pair. Very important in this interaction is the exchange of genetic information. But the conjugant interaction is also important for the regulation of the developmental processes in conjugation. For the investigation of this regulation, the study of the preconjugant interaction is essential, because the preconjugant interaction not only induces the conjugant pair but it also participates in the induction of nuclear changes in conjugation as will be described below. It is hoped therefore that this article, which mainly deals with the preconjugant interaction, may also serve to clarify the mechanism of developmental processes in conjugant pairs.

II. Outline of Conjugation

The whole process of ciliate conjugation is briefly presented here as a background for later discussions. As an example, conjugation of *Paramecium aurelia* (HERTWIG, 1889; MAUPAS, 1889; DILLER, 1936; SONNEBORN, 1947; JURAND and SELMAN, 1969), a species with one macronucleus and two micronuclei, is described, but it is essentially the same in other ciliates.

Preconjugant Cell Interaction. When cells of complementary mating types meet under appropriate conditions, they form conjugant pairs after about an hour of preconjugant cell interaction which includes "mating reaction"

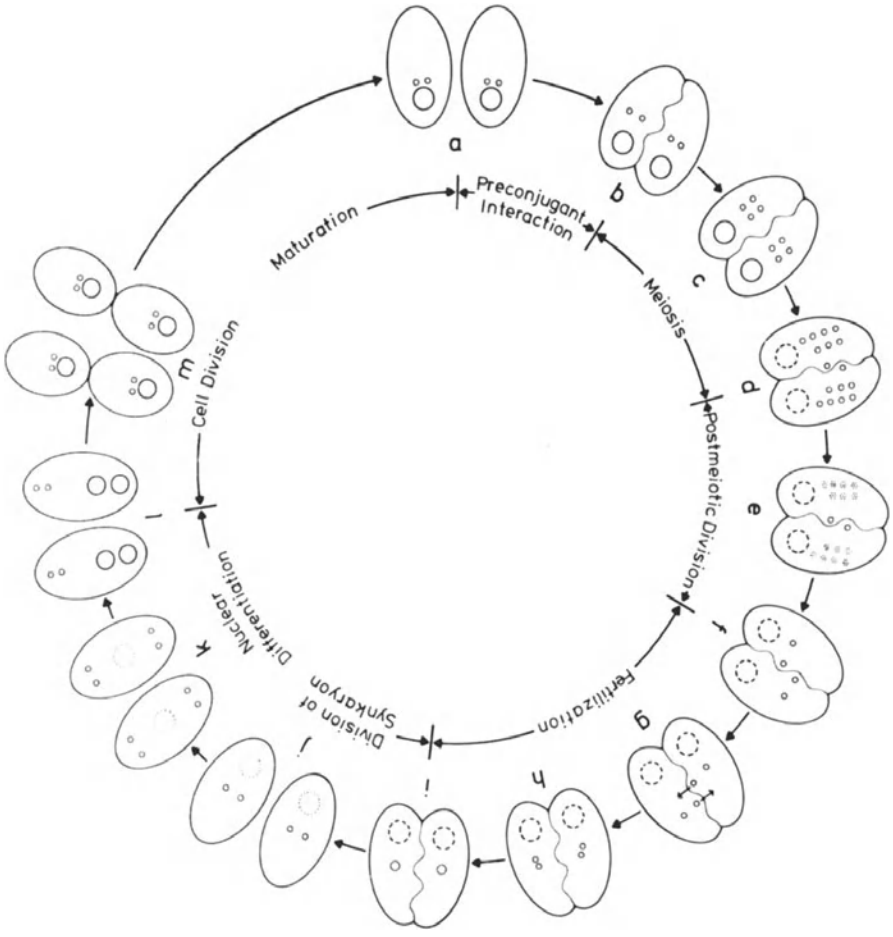


Fig. 1. Diagrammatic illustration of conjugation in *Paramecium aurelia*. The larger circle and the smaller circle in each cell represent the macronucleus and the micronucleus respectively

(Fig. 1, a–b). A characteristic of the mating reaction is agglutination which is mediated by cilia, but in conjugant pairs cells unite by a direct contact of the pellicle.

Meiotic Divisions and Fertilization. As a conjugant pair is formed, both micronuclei of each cell undergo meiosis. By the two successive meiotic divisions eight haploid nuclei are produced (Fig. 1, b–d), seven of which degenerate (e). The remaining one divides once again to produce a "stationary" and a "migratory" nucleus (f). The latter moves into the partner cell and fuses there with the stationary nucleus to form a synkaryon (f–i). It takes about 6 hours at 25°C to complete these processes.

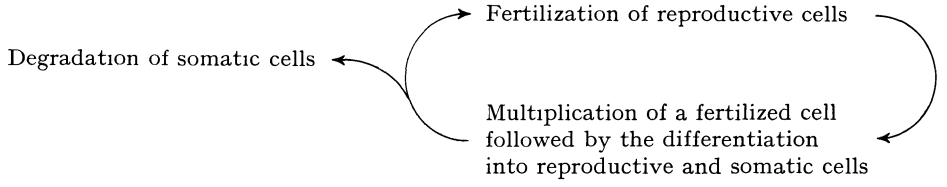
Development of a New Nuclear System. The synkaryon divides twice producing four nuclei (Fig. 1, i–k) two of which develop into macronuclear anlagen and the other two into micronuclei (l). Meanwhile conjugants separate (i–j) and the old macronucleus disintegrates and disappears. In Fig. 1, this is symbolically illustrated by the fading out of the macronucleus (d–k).

In the postconjugant cell division the two macronuclear anlagen are segregated in daughter cells while the micronuclei divide (m). When the macronuclear anlagen grow into the macronuclei, the original condition of the nuclear system, i.e., one macronucleus and two micronuclei in a single cell, is restored. This takes place about one day after the beginning of a cell interaction.

Reconstruction of Cortical Structures. During the period when the cells unite in pairs the oral structure mostly disappears together with the cilia and trichocysts on the oral side of the cells. These structures regenerate later.

Sexual Maturation. Reorganized cells become mature, i.e., the mating-type character is expressed, after a maximum of about 35 cell divisions (SONNEBORN, 1957). When such cells encounter cells of complementary mating type under appropriate conditions, the process of conjugation starts all over again.

At this point it may be interesting to note that there is a similarity between the life cycle of ciliates and that of multicellular eukaryotes. The essential features of the life cycle of the latter may be represented in the following scheme:



This is exactly what occurs in ciliates if the word *cell* is replaced by *nucleus*. If this similarity is valid, ciliate conjugation may have much potential value for investigating the basic mechanism of the life cycle of multicellular eukaryotes.

III. Preconjugant Cell Interaction

The preconjugant cell interaction is defined here as the interaction between cells of complementary mating types which leads to the formation of conjugant pairs. As it occurs between complementary mating types, each mating type must have a specific substance or substances which participate in the interaction. These substances may be called "gamones" by somewhat enlarging the original definition (HARTMANN and SCHARTAU, 1939)—Gamone sind spezifische Stoffe, welche die chemische Wechselwirkung zwischen weiblichen und männlichen Geschlechtszellen bedingen und zu deren Vereinigung führen—by applying this term to any specific substances that induce chemical interaction between cells complementary for fertilization and lead them to unite. Such an extension has already been used (WIESE, 1961; MIYAKE and BEYER, 1973). In some ciliates, gamones are found in the medium in which they live, but in some others they are found only on the cell surface. In the former, the preconjugant cell interaction may start without cell contact while in the latter a direct cell contact is needed. These two types of preconjugant cell interaction will be separately presented and the relationship between them will be discussed later.

A. Interaction by Soluble Gamones

The cell-free fluids have mating-type specific effects on conjugation in *Euplotes patella* (KIMBALL, 1939; KATASHIMA, 1961), *E. eurystomus* (KATASHIMA, 1959), *Blepharisma intermedium* (MIYAKE, 1968; MIYAKE and BEYER, 1973) and *Tokophrya* (SONNEBORN, personal communication). Since our present knowledge about excreted gamones mainly depends upon the studies on *B. intermedium*, they will be presented first.

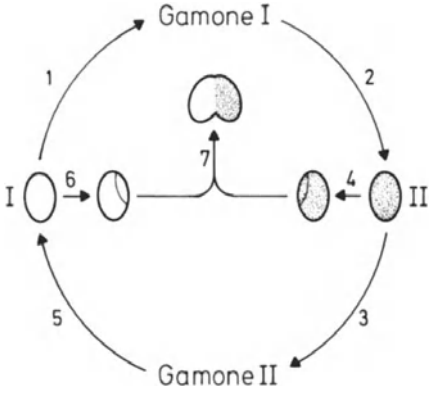


Fig. 2. Diagrammatic illustration of cell interaction in the initiation of conjugation in *Blepharisma intermedium*. I: Mating type I cell; II: Mating type II cell. (Modified from MIYAKE and BEYER, 1973)

Complementary mating types I and II of *B. intermedium* can be kept unchanged through many cell generations. Under such a condition conjugation occurs only when two mating types are mixed. When they are mixed no striking reaction is seen at the beginning but gradually cells become adhesive and after about 2 hours conjugant pairs are formed. The cell interaction in this system was found to consist of 7 steps as shown in Fig. 2. Type I cells autonomously excrete gamone I into the medium (step 1). This gamone reacts with a hypothetical receptor in type II cells (step 2) and specifically transforms them so that they are able to form a cell union (step 4) and at the same time induces them to excrete gamone II into the medium (step 3). This gamone reacts with a hypothetical receptor in type I cells (step 5) and specifically transforms them so that they are able to form a cell union (step 6). During the transformation, cells gradually become adhesive and form loose cell aggregates. When transformed type I and type II cells meet, they unite to form conjugant pairs (step 7) (Fig. 3 a).

If only type I cells are treated by gamone II, they start uniting into pairs (Fig. 3 b) within 2 hours. Similarly type II cells treated by gamone I start uniting into pairs (Fig. 3 c) within 2 hours. In the mixture of two mating types both heterotypic (I–II) and homotypic (I–I and II–II) pairs may be formed. All three kinds of pairs look like conjugant pairs but further processes of conjugation occur only in heterotypic pairs. Homotypic pairs persist for

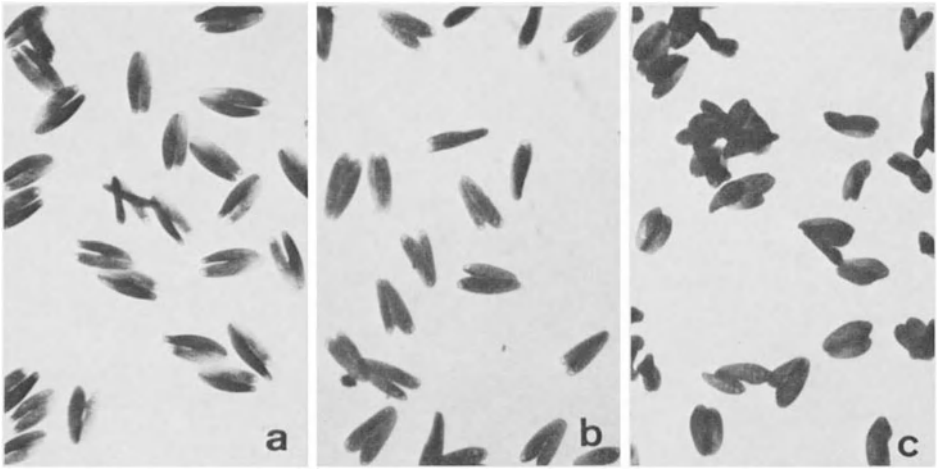


Fig. 3a–c. Cells of *Blepharisma intermedium* in pairing. ×25. a Heterotypic pairs between mating types I and II. The darker looking cell of a pair is mating type II, 20 hrs after mixing the two mating types. b Homotypic pairs of mating type I induced by a cell-free fluid of mating type II with gamone II activity, 24 hrs after beginning the treatment. c Homotypic pairs of mating type II induced by a cell-free fluid of mating type I with gamone I activity, 2 hrs after beginning the treatment. (Modified from MIYAKE, 1968)

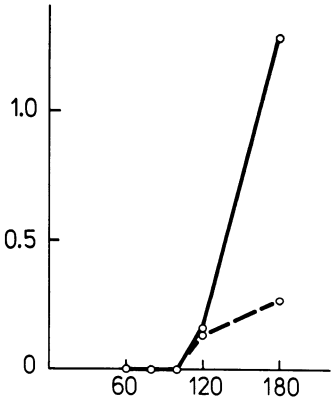


Fig. 4. Gamone II induction by gamone I in mating type II cells of *Blepharisma intermedium* (non-*augex*, 5×10^3 cells/ml). Abscissa: Time (min) after addition of gamone I (10^4 units/ml). Ordinate: Extracellular (units/ml $\times 10^2$) and intracellular (units/cell $\times 10^{-2}$) gamone II activity. —— extracellular activity; - - - intracellular activity. 25°C. (MIYAKE and BEYER, 1973)

days if enough gamone of the complementary mating type is present but no nuclear changes characteristic of conjugation are observed in these pairs. If they are washed free of gamones, they separate within about two hours, leaving cells which are much like those before the pair formation.

An example of the gamone II induction by gamone I (Fig. 2, steps 2–3) is shown in Fig. 4. 10^6 units of gamone I were added to 5×10^5 type II cells suspended in 100 ml. The unit of the gamone activity is the smallest amount of gamone that can induce the pair formation in about 500 cells suspended in

1 ml. Both intra- and extracellular gamone II activities were first detected 120 minutes after beginning the gamone I treatment, indicating that these cells started producing gamone II in 120 minutes under the presence of gamone I and that the produced gamone II is immediately excreted. Some cultures of type II autonomously produce and excrete gamone II (*augex* form). In such cultures, gamone I enhances the production and excretion of gamone II.

Type I cells always excrete gamone I except when they grow very rapidly, but the excretion rate changes according to the nutritive condition of the cell. If gamone I is not excreted at the maximal rate (8×10^{-2} units/hr/cell), its excretion can be enhanced by gamone II. Thus, the reaction chain consisting of steps 1, 2, 3 and 5 of Fig. 2 is a positive feedback cycle. When cells of complementary types meet under these conditions, they plunge headlong into conjugation stimulating each other by ever increasing excretion of gamones.

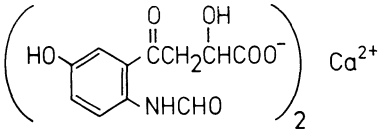


Fig. 5. Blepharismone, gamone II of *Blepharisma intermedium*

Gamone II (blepharismone[2]) has been puridied, crystallized and identified as calcium-3-(2′-formylamino-5′-hydroxybenzoyl)lactate (Fig. 5) (KUBOTA et al., 1973). This gamone at a concentration of 0.001 μg/ml can induce stet pair formation in type I cells suspended at a density of 500 cells/ml. Gamone I has been partially purified and appears to be a protein of about 20000 molecular weight (MIYAKE and BEYER, 1973). Recently, this gamone was purified further by CM and DEAE cellulose chromatography. This preparation, which as shown by acrylamide gel electrophoresis contained only a glycoprotein, induced pair formation at a concentration of 0.00006 μg/ml in type II cells suspended at a density of 500 cells/ml (MIYAKE and BEYER, unpublished).

Investigations on each step of the system of preconjugant cell interaction as indicated in Fig. 2 by using pure or nearly pure gamones are now underway. Some of the results obtained are briefly described below.

Step 3: A lag of about 2 hours between the beginning of gamone I treatment and the gamone II production by type II cells (Fig. 4) suggests that gamone II synthesizing machinery is built up anew after cells are treated by gamone I. When cells were washed after a 30 minutes of gamone I treatment, the gamone II was nevertheless produced although the production was much

2 Blepharismone is the first gamone in Protozoa of which the molecular structure has been revealed. It was first named as blepharismin by KUBOTA et al. (1973). However, the same name was used by GIESE for the red pigment of *Blepharisma* in his book on this ciliate (GIESE, A. C.: *Blepharisma*. Stanford Univ. Press 1973) which appeared almost at the same time. To avoid the future confusion, gamone II is renamed as blepharismone with the agreement of the authors of the paper (KUBOTA, TOKOROYAMA, TSUKUDA, KOYAMA and MIYAKE, personal communication).

less than in the control cells which were not washed. When cells were washed after one hour of the treatment, the gamone II production was the same as the control, at least for a period of up to 4 hours. Thus the building up of the gamone II producing system appears to continue after gamone I is removed, and once built up, it normally functions, at least for several hours. The molecular structure of gamone II indicates that it is biogenetically involved in tryptophan metabolism (KUBOTA et al., 1973). Indeed, preliminary experiments demonstrated that type II cells incorporate ^{14}C-tryptophan into gamone II, strongly suggesting that this is really the case.

Steps 4 and 6: The induction of homotypic pairs by a gamone depends more strictly on the continuous presence of the gamones. If cells are washed after 1 hour of the gamone treatment, no pairs will be formed. If cells are washed after the pair formation, pairs separate within 2 hours. It is interesting that the maintenance of cell contact needs a continuous stimulus by a specific compound.

Step 5: Induction of homotypic pairs in type I cells by gamone II is inhibited by 2 mM L-5-hydroxytryptophan, L-tryptophan, D-5-hydroxytryptophan, and D-tryptophan. The activity of 0.64 μg/ml gamone II solution with these inhibitors was 1/16·1/8·1/4·1/2 of the control respectively. The inhibition by L-5-hydroxytryptophan and L-tryptophan was proportional to the concentration within the range of 0.25–4.00 mM and 0.50–4.00 mM respectively. Glycine, L-alanine, L-serine, L-valine, L-threonine, L-proline, L-histidine and L-phenylalanine have no inhibiting effect at these concentrations. The result suggests that 5-hydroxytryptophan and tryptophan compete with gamone II on the receptor site in type I cells.

Direct interactions between gamones I and II are unlikely to occur because the gamone II activity was not appreciably changed by the presence of 10^6 units/ml of gamone I. Similarly, the gamone I activity was not appreciably changed by the presence of 0.8×10^4 units/ml of gamone II.

In other species of *Blepharisma*, the system of preconjugant cell interaction has not yet been extensively studied. However, it has been demonstrated that the cell-free fluid of some other species of *Blepharisma* shows similar activity to the gamones of *B. intermedium* and that some species respond to gamone II by pair formation. Thus, the strain Niigata of *B. japonicum* described by INABA et al. (1958) and INABA (1965) excretes gamone I activity and responds to gamone II by pairing. Strains McMan (*B. americanum* type), SES and 622 (both *B. sinuosum* type) of the Hirshfield collection at New York University respond to gamone II by pairing. The strain Nara described by INABA et al. (1958) which resembles *B. tropicum* (INABA, 1965) excretes gamone II activity (MIYAKE, 1968) as well as strain Berlin which was identified as *B. americanum* by WILFERT (1972) and strain Mont (*B. americanum* type) of the Hirshfield collection. These results suggest that a system similar to that of *B. intermedium* will be found also in other species of this genus.

In *E. patella* (KIMBALL, 1939) conjugant pairs are formed in a similar way to *B. intermedium*. When complementary mating types are mixed, cells swim

freely at least for 90 minutes of "waiting period" and then they become comparatively quiet and more or less aggregate at the bottom of the container. Pairs of cells then begin to unite. Their cell-free fluid contains a factor which can specifically induce conjugation in cells of other mating types. One variety of this species was found to have six mating types. Conjugation occurred when they were mixed two at a time, in all possible combinations, except when cells of the same mating type were mixed. It was concluded by KIMBALL that each mating type excretes into the medium one or two of the three kinds of factors 1, 2 and 3, and these factors can induce conjugation in any type of cell which does not excrete the same factor into the medium. For example, the cell-free fluid of a mating type which excretes factor 1 can induce conjugation in mating types which excrete 2, 3 or 2+3 but not in mating types which excrete 1, 1+2 or 1+3, etc. In the other stocks of this species such mating-type-specific conjugation-inducing effects of cell-free fluid was not detected, but cell-free fluid of one mating type was able to shorten the "refractory period" in the complementary type (KATASHIMA, 1961). In *E. eurystomus*, cell-free fluid of one mating type was reported to specifically *inhibit* the formation of homotypic pairs of the complementary mating type (KATASHIMA, 1959).

If, in *Tokophrya*, a sessile ciliate, complementary mating types are placed within a certain distance of each other, they orient and stretch toward each other, indicating that they communicate by fluid factors (SONNEBORN, personal communication).

Although the chemical nature of the fluid factors in *Euplotes* and *Tokophrya* have not been investigated, these results suggest that preconjugant interaction by excreted gamones could be of wide occurrence in ciliates.

B. Interaction by Cell-Bound Gamones

In some ciliates including *Paramecium aurelia* (SONNEBORN, 1937), *P. caudatum* (HIWATASHI, 1949; VIVIER, 1960), *P. bursaria* (LARISON and SIEGEL, 1961), *P. multimicronucleatum* (MIYAKE, unpublished), *Euplotes crassus* (HECKMANN, 1964), *E. vanus* (HECKMANN, 1963), *E. minuta* (NOBILI, 1966), *Oxytricha bifaria* (SIEGEL, 1956) and *Tetrahymena pyriformis* (NANNEY, personal communication) cell-free fluid was found to have no mating-type-specific effect on the induction of conjugation suggesting that these ciliates do not excrete gamones into the medium. If gamones are not present in the medium they should be on the cell surface. Since our present knowledge about cell-bound gamones mainly depends upon studies on *Paramecium*, they will be presented first.

If gamones are only on the cell surface and if only two types of cells participate in the interaction, an inevitable conclusion is that the interaction should begin with a direct contact between these two types of cells. In fact, in all the four species of *Paramecium* mentioned above, a conspicuous cell agglutination begins within a few seconds of mixing cells of complementary

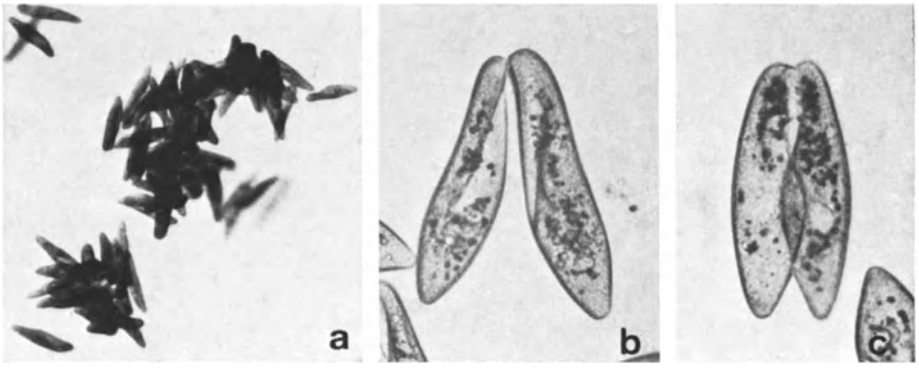


Fig. 6a–c. Three kinds of cell union in the process of conjugant-pair formation in *Paramecium*. a Mating reaction in *P. multimicronucleatum*. ×30. b Holdfast union in *P. caudatum*. ×150. c Fully formed conjugant pair including paroral union in *P. caudatum*. ×150

mating types under appropriate conditions. This agglutination of cells which is called the mating reaction is mating-type-specific and occurs only between cells of complementary mating types (SONNEBORN, 1937; JENNINGS, 1938; HIWATASHI, 1951; LARISON and SIEGEL, 1961). However, a cell which has first agglutinated with a cell of the complementary mating type can temporarily stick to a cell of the same mating type (SONNEBORN, 1937, 1942). Direct observations of agglutinating cells indicate that the mating reaction is mediated by cilia (SONNEBORN, 1937; JENNINGS, 1939). This is confirmed by the demonstration that detached cilia of one mating type specifically adhere to cells of the complementary mating type (METZ, 1954; COHEN and SIEGEL, 1963; MIYAKE, 1964; BYRNE, 1972). The ability to undergo the mating reaction appears to be limited to the oral side of the cell (HIWATASHI, 1961; COHEN and SIEGEL, 1963; MIYAKE, 1964). Since two reacting cells rarely cover the whole of this area, more than two cells of the complementary mating type can adhere to a single cell resulting in the formation of clumps consisting of two to hundreds of cells (Fig. 6a).

After the mating reaction has occurred for an uninterrupted period of 60 minutes cells start uniting in pairs at their anterior regions. Prior to this, cilia are seen degenerating at this region in many cells (MIYAKE, 1966). When cells of such pairs are artificially separated, no cilia are found in the area of contact (HIWATASHI, 1955). Therefore it appears that after a specified period during the mating reaction, cilia at the anterior region of the cell degenerate and the new union is formed by a direct contact of deciliated surfaces. In this union which is called "holdfast union" (METZ, 1947) the area of contact is very small (Fig. 6b). However, this region soon enlarges mainly towards the posterior part of the cell resulting in fully formed conjugant pairs (Fig. 6c). At this stage cell union is particularly firm at the paroral region and the union of this region is called the "paroral cone union" (METZ, 1948). The enlargement is accompanied by the disappearance of neighbouring cilia and trichocysts

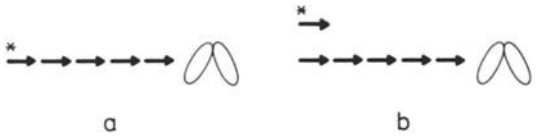


Fig. 7a and b. Diagrammatic illustration of the two alternative relationships of the mating reaction (arrow with an asterisk) to the reaction steps essential for the induction of conjugant union. The mating reaction is in "series" (a) or in "parallel" (b) with the essential steps

(HIWATASHI, 1955). Conjugant pairs thus formed no longer undergo the mating reaction (METZ, 1948).

Cell attachment in the holdfast union is different from that in the mating reaction in many respects. In addition to the distinct morphological difference mentioned above, these unions are affected differently by some enzymes. For example: 4 mg per 100 ml trypsin strongly inhibits the mating reaction but does not affect the occurrence of holdfast union as will be described in more detail below (MIYAKE, 1969); 5 mg per 100 ml lipase strongly inhibits the occurrence of holdfast union (MIYAKE, 1969) while it inhibits the mating reaction only weakly. A mutant stock CM of *P. aurelia* can undergo the mating reaction but never forms the holdfast union (SONNEBORN, 1942; METZ and FOLEY, 1949). Also, the holdfast union is, contrary to the mating reaction, not mating-type-specific. This was first demonstrated in *P. caudatum* by marking cells of complementary mating types in different colours by the vital staining technique (HIWATASHI, 1951). In this experiment, the mating reaction occurred only between cells of different colours, but the holdfast union was formed not only between cells of different colours but also between cells of the same colour. Similar results were obtained in *P. bursaria* by marking mating types by the presence or absence of symbiotic *Chlorella* (LARISON and SIEGEL, 1961). Mating type nonspecificity of the holdfast union was also demonstrated by the fact that this union can be induced between cells of the same mating type by, (1) killed cells or detached cilia of the complementary mating type, and (2) conjugation-inducing chemical agents, as described below. Clearly then the holdfast union is an entirely different kind of cell union from the mating reaction.

Since the holdfast union is formed *after* an uninterrupted occurrence of the mating reaction for about 60 minutes, cells must be somehow transformed during this period so that they are able to form the holdfast union. A question may be raised whether the mating reaction itself gives the information to the cell to turn on the machinery which leads the cell to form holdfast union (Fig. 7a), or whether the function of the mating reaction is only to facilitate a mating-type-specific cell association. In the second alternative, the other reaction which is essential for the turning on the mechanism of holdfast-union formation must occur in parallel to the mating reaction (Fig. 7b). At present there is no strong evidence for this "*parallel-reaction* hypothesis", but there are at least three instances in which the mating reaction is separable from the

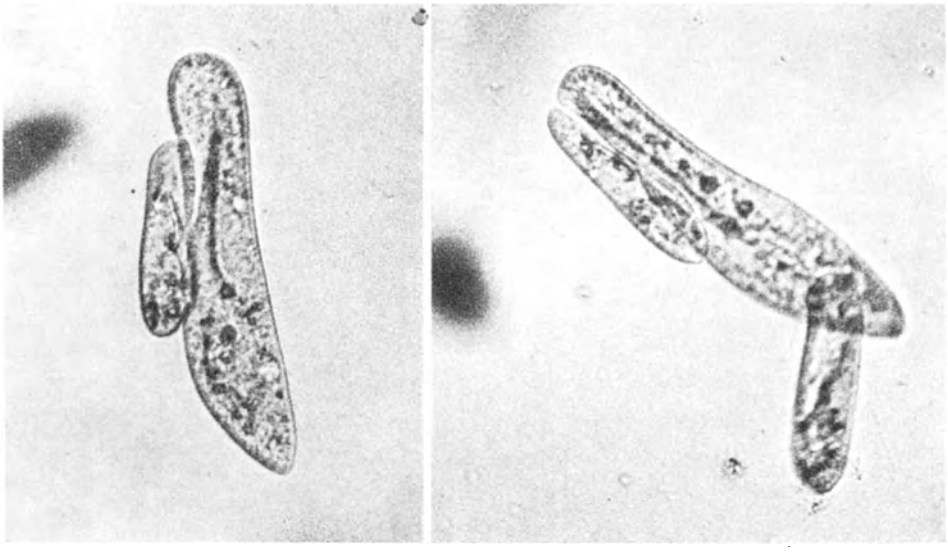


Fig. 8. Interspecific conjugation induced by chemical agents (KCl: 18 mM+acriflavine: 0.35 mg per 100 ml + Ca-poor conditions) between *Paramecium aurelia* (smaller cells) and *Paramecium multi-micronucleatum*. ×200. (MIYAKE, 1968a)

formation of conjugant pairs and hence may be worthy of discussing in relation to this hypothesis. These instances are: (1) Induction of conjugation by chemical agents, (2) conjugation induced by some "killer" stocks, and (3) CM stocks.

Induction of conjugation by chemical agents has been so far successful in 5 species of the "aurelia group" of *Paramecium* (MIYAKE, 1956, 1958, 1968a, 1970; HIWATASHI, 1959, 1970; CRONKITE, 1972). In *P. multimicronucleatum*, for example, cells are grown in a Ca-poor culture medium, concentrated to $4–8\times10^3$ cells/ml and mixed with a KCl+acriflavine solution so as to make the final concentrations of these chemicals 8–30 mM and 0.4–0.8 mg per 100 ml respectively. KCl may be replaced by some other K-salts, $MgCl_2$, or heparin. Acriflavine may be replaced by acridine yellow or phosphine. Acriflavine is not needed in *P. caudatum* if cells are washed with a Ca-poor salt solution, and under these conditions acetamide, urea or methylurea promote the conjugation inducing effect of these agents. In this "chemical induction" the holdfast union is formed about 60 minutes after the beginning of the chemical treatment and soon afterwards typical conjugant pairs are formed. The percentage of conjugated cells may be as high as 90 percent. Exconjugants can establish viable progeny after normal reorganization. In these respects chemically-induced conjugation is identical to conjugation induced by mating types. However, the chemical induction strikingly differs from the induction by mating types in 4 respects: (1) It is not mating-type-specific and can induce conjugation between cells of the same mating type as well as between cells of different species (Fig. 8), (2) it induces holdfast

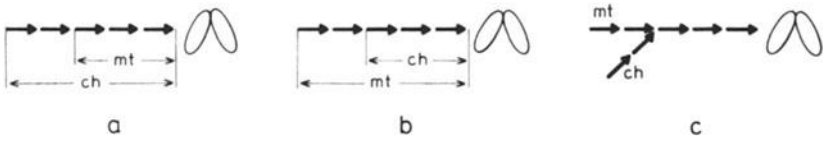


Fig. 9. Diagrammatic illustration of the three possible relationships between the mating-type-induced conjugation (*mt*) and the chemically-induced conjugation (*ch*)

union without any conspicuous agglutination of cells comparable to the mating reaction, (3) it is completely inhibited by 1 mM $CaCl_2$ which has little effect on conjugation by mating types, while (4) it is not inhibited by 4 mg per 100 ml trypsin which completely inhibits the induction of conjugation by mating types.

There are three possible relationships between the chemically-induced conjugation (*ch*) and the mating-type-induced conjugation (*mt*): (1) The essential mechanisms of *mt* are all included in *ch* (Fig. 9a), (2) the essential mechanisms of *ch* are all included in *mt* (Fig. 9b), and (3) the first part of the conjugation-inducing mechanism is specific to each induction (Fig. 9c). (1) and (2) are eliminated by the finding of a specific inhibitor for each of *mt* and *ch*, i.e., trypsin and Ca respectively, because one of the inductions which is included in the other cannot have a specific inhibitor. In (3), the presence of a specific inhibitor for both *mt* and *ch* is compatible, since each of them has a mechanism which the other has not. Therefore (3) is the valid relationship. As an outcome of this conclusion, the chemical induction cannot support the *parallel-reaction* hypothesis. The absence of the mating reaction in the chemical induction does not say anything about the essentiality of this reaction in the mating-type-induced conjugation, because chemicals and mating types begin the induction of conjugation with a mechanism characteristic to each of them.

Cell-free fluid of some killer stocks induce conjugation-like unions in certain sensitive stocks (Chen, 1945; Sonneborn, 1947; Preer, 1948). This effect of killer fluid is not mating-type-specific. The fact that the Ca-poor condition favours this induction (Miyake, unpublished) suggests a similarity to the chemical induction. However, the study on the inducing effect of these killers is still so preliminary that further investigation is needed to discuss it in detail in relation to the *parallel-reaction* hypothesis.

As mentioned above, stock CM of *P. aurelia* can undergo a mating reaction but never form conjugant pairs. When CM cells of complementary mating types are mixed, a strong mating reaction continues for many hours but not a single conjugant pair is formed. Thus, the mating reaction is not sufficient for the occurrence of conjugation. One may here assume that what CM cells lack could be the hypothetical *parallel reaction*. However, CM cells can induce conjugation in normal cells of the opposite mating type; when CM cells and normal cells react, only the latter form conjugant pairs. Still one might assume that CM cells possess the inducer molecule of the *parallel reaction* but lack the receptor molecule and therefore they can induce conjugation in normal

cells but they themselves cannot be induced to conjugate. However, this assumption is unlikely because the chemical induction is not effective in CM cells (MIYAKE, 1968a), indicating that the defect of CM cells should be somewhere in the pathway included in the chemical induction. Since the latter has already been shown not to include the *parallel reaction*, the missing step of CM cells is not likely to be the *parallel reaction*.

Since even the above three instances cannot support the *parallel reaction* hypothesis, it is tentatively concluded that in the conjugation by mating types the mating reaction itself is the reaction which directly turns on the machinery which leads the cell to the formation of conjugant pairs (Fig. 7a).

"Mating-type substances" form the material basis of the mating reaction (METZ, 1947). Attempts to obtain these cell-bound gamones in soluble form have so far failed. However, cells can be killed without destroying the ability to undergo the mating reaction (METZ, 1947, 1954; HIWATASHI, 1949a, 1950). These killed cells specifically stick to living cells of the opposite mating type and induce pseudoselfing conjugation in *P. aurelia* and *P. calkinsi*, or conjugation in *P. caudatum*. Similarly, detached cilia not only stick to living cells of the opposite mating type but also induce conjugation between them in *P. multimicronucleatum* (MIYAKE, 1964), in *P. caudatum* (FUKUSHI and HIWATASHI, 1970), and in *P. aurelia* (BYRNE, 1972; CRONKITE, 1972). These killed cells and detached cilia were thoroughly washed and used as a partially isolated mating-type substance to study its chemical nature.

Extensive studies on the effect of various agents on these preparations of mating-type substances which have been summarized by METZ (1954) and HIWATASHI (1969) indicate that they are proteins. This result is supported by the study on the effect of antibiotics on the biosynthesis of mating-type substances (COHEN, 1965).

Recently a successful production of mating-type-specific antibody was reported (SASAKI et al., 1972). Detached cilia of mating type VI of *P. caudatum* were repeatedly washed with salt-alcohol to remove most of the "immobilization antigens" and then injected intramuscularly into guinea pigs with Freund's incomplete adjuvant. The antiserum obtained in this way was reported to specifically inhibit the ability to undergo mating reaction in type VI cells.

The problem of how the mating reaction induces the conjugant pairs has not yet been investigated as intensively as its importance warrants. Although mating-type substances have not yet been isolated, there are some possible ways to probe into this mechanism. In one such attempt, the induction of conjugant pairs by the mating reaction was artificially stopped at different times with and without the re-induction (MIYAKE, 1969) as will be described below.

The mating reaction between living cells and detached cilia of *P. multimicronucleatum* is irreversibly stopped within one or two minutes by 4 mg per 100 ml trypsin. If trypsin was added within the first three quarters of the time needed to induce holdfast union, holdfast pairs never appeared. However,

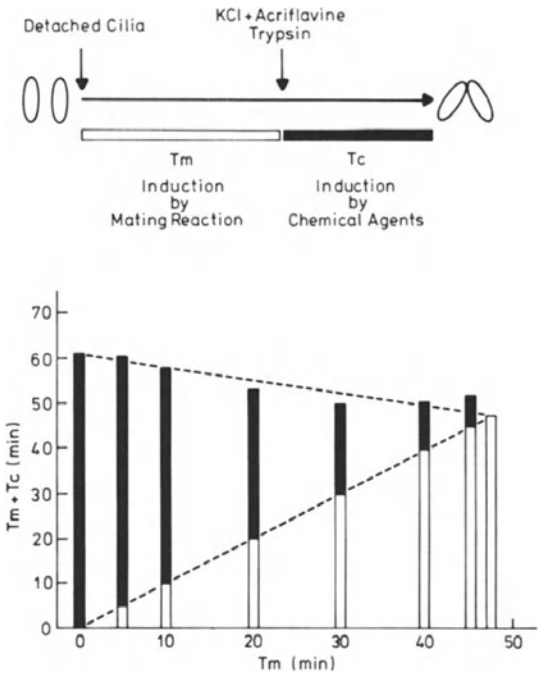


Fig. 10. Induction of conjugation by successive treatments by detached cilia and by chemical agents, change-the-booster experiment, in *Paramecium multimicronucleatum*. (Modified from MIYAKE, 1969)

if trypsin was added in the last quarter of the time, holdfast pairs were formed later, although the number of pairs were much less than in the control without the trypsin treatment. The result indicates that a part of the machinery for the holdfast-union induction continues to some extent after the mating reaction is blocked.

To study how the induction of conjugation by the mating reaction progresses, the mating reaction with detached cilia was stopped at various times and then the induction of conjugation was resumed once again ("change-the-booster experiment"). For the second induction or the second booster, the mating reaction could hardly be used because trypsin-treated cells lose the ability to undergo the mating reaction and remain non-reactive for some time. Therefore the chemical induction, which is not affected by trypsin, was used for the second booster. Cells were mixed with detached cilia of the opposite mating type and after T_m minutes, the mating reaction was blocked by trypsin. At this moment, the chemical induction was initiated and the time needed to induce the holdfast union by this induction, Tc, was measured. As Tm increased, Tc decreased and the values of $T_m + Tc$ were roughly between the times required for inducing holdfast union by each of the two methods used individually (Fig. 10). As expected, $(T_m + Tc)$ was roughly constant when the two methods took the same time to induce holdfast union. The result indicates: (1) The induction by the mating reaction and that by chemical

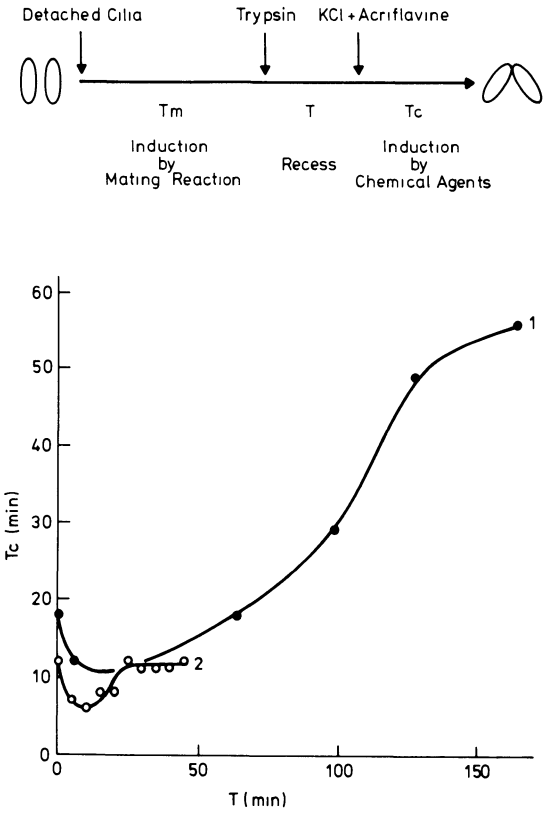


Fig. 11. Effect of the recess time introduced between the two inductions of conjugation in the change-the-booster experiment (see Fig. 10) in *Paramecium multimicronucleatum*. (Modified from MIYAKE, 1969)

agents are additive, and (2) the holdfast-union-inducing effect of the mating reaction is continuously and proportionally accumulative.

In order to examine the stability of this accumulated effect, recess times of various periods were introduced between the two inductions of the change-the-booster experiment. The induction was initiated by detached cilia and after Tm minutes it was blocked by trypsin. After T minutes of recess, the chemical induction was initiated and the time needed to induce the holdfast union by this second induction, Tc, was measured. Two of such experiments are shown in Fig. 11. Tm was 35 minutes in experiment 1 (curve 1) and 30 minutes in experiment 2 (curve 2). Detached cilia and chemicals, if used singly, induced the holdfast union in 50 and 55 minutes respectively in experiment 1, and 42 and 46 minutes respectively in experiment 2. As the recess time T increased, Tc decreased first, remained unchanged for some time, and then continuously increased until it reached the time required for chemical induction to induce the holdfast union without any previous treatment. The first decrease of Tc agrees with the previously described result, namely that the effect of the mating reaction continues for some time after

it is blocked. The later increase of Tc should be due to the breakdown of the accumulated effect of the mating reaction. The result of experiment 1 indicates that the effect of 35 minutes of mating reaction completely disappears 2.5 hours after the reaction is blocked.

The process of the induction of holdfast union which emerges from these experiments is as follows: during the mating reaction a hypothetical factor continually increases and at a certain threshold the holdfast union is formed. When the mating reaction is blocked, the increase in the level of this factor continues for some time and then stops. Thereafter the level remains constant for some time but gradually decreases and drops to zero within a few hours. This factor may be regarded as the holdfast substance which was postulated by METZ (1948, 1954) as a material basis for the holdfast union.

Induction of holdfast union in *P. multimicronucleatum* is inhibited by actinomycin S_3 and puromycin if cells are treated by these agents for some time before the formation of the union. Ficin and lipase also inhibit the holdfast union in the same species but these enzymes inhibit it even if they are added immediately before the formation of the union. These results suggest that the factor or the holdfast substance is a lipoprotein transcribed and translated prior to the holdfast union (MIYAKE, 1969). The finding in the same species that actinomycin S_3 and puromycin also inhibit the local degeneration of cilia which precedes the holdfast union (MIYAKE, unpublished) may support this hypothesis. This interesting but highly speculative hypothesis must be tested by further investigations.

In *P. aurelia* the mating reaction induces not only conjugant pair formation but also the nuclear changes characteristic of conjugation. This was demonstrated by treating isolated single cells by killed cells of the complementary mating type (METZ, 1947). A much higher percentage of these individually treated cells underwent the nuclear changes than in the untreated control cells some of which underwent "autogamy", another reorganization process in *P. aurelia*.

In autogamy, all the nuclear reorganization processes in conjugation except the exchange of gametic nuclei take place in a single cell (DILLER, 1936; SONNEBORN, 1939, 1947). The sister haploid nuclei of the post meiotic division fuse in the same cell yielding homozygosity for all genes. Autogamy is known to occur in some ciliates including *P. aurelia* and some stocks of *E. minuta* (NOBILI, 1966; SIEGEL and HECKMANN, 1966) but not in the other ciliates mentioned in this paper.

The result of the METZ' experiment described above indicates that the mating reaction can induce autogamy. Since natural autogamy occurs in single cells without any cell interaction, the first step of the induction of natural autogamy must differ from that of the mating-reaction-induced autogamy. However, it would be reasonable to postulate that the mechanism of the nuclear reorganisation in conjugation and autogamy merge somewhere in their induction processes (METZ, 1948). This problem will be discussed again in section V.

In ciliates other than *Paramecium*, the cell agglutination during preconjugant interaction is much less conspicuous. In these ciliates cells were

reported to swim freely during the waiting period before any visible reaction was detected even if cells are mixed under appropriate conditions for mating. In some ciliates like *B. intermedium* and *E. patella* in which the preconjugant interaction is mediated by gamones in the medium, the waiting period is explained as the time needed for the gamones to make cells adhere. However, in many other ciliates gamones were not found in the medium making this explanation untenable. Thus a hypothesis was presented by HECKMANN and SIEGEL (1964) to explain the waiting period of *E. crassus* by the positive feedback of the mating reaction. They assume that cells have a small amount of mating-type substances on their surface before they meet the complementary mating type. Although their amount is too small for the occurrence of a visible agglutination, these substances react at the initial contact between cells of complementary mating types and serve to raise cellular concentrations of mating-type substances to a high enough level to allow cells to undergo a visible agglutination and eventually induce them to form conjugant pairs. This hypothesis is strongly supported by a recent discovery that the immediate mating reaction actually occurs between cells of complementary mating types of *E. crassus* if they are mixed under appropriate conditions (MIYAKE and NOBILI, 1974). Although the initial mating reaction of this species is never so strong as in *Paramecium* it starts in a few seconds after the mixing and therefore it proves one of the basic assumptions of the positive-feedback hypothesis that cells have mating-type substances before they meet the other mating type. On the other hand such a feedback system is not unrealistic since a positive-feedback system has already been an established fact in the preconjugant interaction of *B. intermedium* (MIYAKE and BEYER, 1973). The positive-feedback hypothesis of the mating reaction appears to be a useful guide in future investigations of the preconjugant interaction of ciliates.

IV. Conjugant Cell Interaction

The formation of a conjugant pair is followed by a series of changes, as briefly described in sections I and II. A well established interaction at this stage is the exchange of genetic information between the two conjugants of a pair. Although less well established, conjugants also exchange information to regulate the process of conjugation. These two kinds of interaction will be treated as "genetic" and "developmental" interactions, respectively.

A. Genetic Interaction

The behaviour of nuclear genes in conjugation was first demonstrated in *P. aurelia* to follow the Mendelian rules of segregation and independent assortment by SONNEBORN (1939). Since then, the transfer of nuclear and cytoplasmic genetic factors from cell to cell in a conjugant pair and the interaction between nuclear genes and cytoplasmic factors have been intensively investigated by SONNEBORN and others. As a result of these investigations, which have been occasionally reviewed (SONNEBORN, 1947; BEALE, 1954;

KIMBALL, 1964; PREER, 1969), it has been shown, not only that the haploid micronucleus is regularly transferred from the conjugant to the other partner of a pair but also that some of the microscopically visible structures may also be transferred. These structures include kappa and related particles of *P. aurelia* (SONNEBORN, 1959), symbiotic *Chlorella* in *P. bursaria* (SIEGEL, 1960), cortical structures in *P. aurelia* (BEISSON and SONNEBORN, 1965), and possibly mitochondria in *P. aurelia* (ADOUTTE and BEISSON, 1970). The fact that intracellular structures as large as micronuclei and *Chlorella* are transferred from cell to cell suggests that conjugants are connected by cytoplasmic bridges. Indeed, electronmicroscopical investigations have demonstrated that they exist in *T. pyriformis* (ELLIOT and TREMOR, 1958), *P. aurelia* (JURAND and SELMAN, 1969), *P. caudatum* (VIVIER and ANDRÉ, 1961); VIVIER, 1962), *P. multimicronucleatum* (INABA et al., 1966), *E. vanus* (NOBILI, 1967). Many of these cytoplasmic bridges are less than 1 μ in width but a bridge as wide as 10 microns was also observed in *P. aurelia* (SCHNEIDER, 1963) and *B. intermedium* (OTOTAKE, 1969). The exchange at the molecular level was autoradiographically investigated in *T. pyriformis* (MCDONALD, 1966). When ^{3}H-histidine labelled and unlabelled cells were induced to conjugate, the radioactivity soon entered unlabelled cells and after about 7 hours, when micronuclei were still undergoing the first meiotic division, about 30 percent of the whole radioactivity of a pair was detected in the originally unlabelled conjugant. In a similar experiment in which cells were labelled by ^{3}H-uridine, about 40 percent of the radioactivity was found in the originally unlabelled partner 7 hours after the pair formation. These results indicate that the exchange of genetic and non-genetic information can occur to a large extent between the two cells of a conjugant pair.

For the study of cytoplasmic genetic factors, gamone-induced homotypic pairs of *B. intermedium* may be successfully used, since no nuclear changes in conjugation occurs in this pair and hence the exchange of nuclear genetic factors are not expected to occur. The cytoplasmic transfer in this pair was demonstrated in homotypic pairs induced by gamone II between the red (wild type) and the white (mutant) cells, both of mating type I. In some of such pairs, the white cell gradually turned pink, although after separation it became white again while the red cell remained red (MIYAKE and BEYER, 1973).

B. Developmental Interaction

In *B. intermedium* the nuclear changes characteristic of conjugation occur only in heterotypic pairs and not in homotypic pairs (MIYAKE and BEYER, 1973), indicating clearly that the interaction between conjugants of complementary mating types is needed for the further progress of conjugation. However, in *P. aurelia* (METZ, 1947), *P. caudatum* (HIWATASHI, 1949a) and in *P. multimicronucleatum* (MIYAKE, 1964) homotypic pairs induced by killed cells or detached cilia of the opposite mating type undergo the nuclear changes. In the last two species the preconjugant interaction alone appears to be

unable to induce the nuclear changes because killed cells and detached cilia failed to induce these changes in isolated single cells of *P. caudatum* (HIWATASHI, 1955) and *P. multimicronucleatum* respectively, while in *P. aurelia* (METZ, 1947) the preconjugant interaction alone can induce the nuclear changes as described in section III B.

In other words these differences between ciliates are due to two pairs of factors: (1) Whether the preconjugant interaction is enough to induce the nuclear changes or the participation of the conjugant interaction is additionally needed, and (2) when the latter is the case, whether the homotypic conjugant interaction induces the nuclear changes or the heterotypic interaction is needed. Two questions may now be raised: (1) Is the preconjugant interaction effective in inducing the nuclear changes *only* in *P. aurelia* or is it more or less effective also in other species although it may not be enough by itself to induce the nuclear changes? (2) When the conjugant interaction is needed for the nuclear changes, are the participating substances the same as those in the preconjugant interaction?

For the first question, inductions of the nuclear changes by conjugation-inducing chemicals and by certain killers may provide some hints. In *P. aurelia*, conjugation-inducing chemicals induce the nuclear changes in isolated cells. In *P. multimicronucleatum* (MIYAKE, 1968b, 1968c, 1969, 1970a), conjugation-inducing chemicals+ficin induce in non-united cells the nuclear changes including the formation of new macronuclear anlagen and their development into functional macronuclei. Here ficin completely inhibits the cell union while the nuclear changes progress in single cells. This method was also successfully applied to *P. aurelia* (MIYAKE, 1970a). In *P. caudatum*, ITO (1969) found that conjugation-inducing chemicals induce nuclear changes similar to those in conjugation in isolated cells. Thus it appears to be a general fact, at least in *Paramecium*, that conjugation-inducing chemicals are effective in inducing the nuclear changes without the participation of the conjugant union. Similarly, certain killers of *P. aurelia* (PREER, 1948; SONNEBORN, personal communication) and *P. bursaria* (CHEN, 1945) induce in some stocks of sensitive cells nuclear changes similar to those in conjugation and this induction appears to occur with and without the cell union. These results indicate that those agents which can induce a conjugant union can also induce the nuclear changes without the participation of the cell union. Considering that (1) the pathway of the induction of conjugation by the mating reaction and that by the chemical agents join into one as shown by the change-the-booster experiment (Fig. 10), and that (2) the mating reaction induces the nuclear changes in one of the *Paramecium* species, it may not be very unreasonable to postulate that the mating reaction is more or less effective in inducing the nuclear changes also in other species of *Paramecium*. This possibility may be experimentally tested by the change-the-booster experiment since by using this method even a small effect of the mating reaction can be detected.

For the second question, available evidence is too limited to discuss in detail the specific substances which participate in the induction of nuclear

changes in conjugants, but circumstantial evidence suggests that the gamones in the preconjugant interaction can also participate in this induction. The observation in *P. aurelia* that a cell, which has first agglutinated with a cell of the opposite mating type, can temporarily adhere to a cell of the same mating type suggests that cell-bound gamones are transferred by the mating reaction (SONNEBORN, 1937). Therefore, it is quite possible that in homotypic pairs induced by killed cells or detached cilia of the complementary mating type, the transferred gamone or even whole detached cilia are sandwiched between contacted cell surfaces and, as they partially fuse, gamones or gamone-receptor complexes are eventually incorporated into the cell. Naturally, such an incorporation should be carried out more efficiently in heterotypic pairs. In *B. intermedium* gamones are excreted, but massive transfer of gamones between conjugants is expected to occur since the gamone is found inside the cell (MIYAKE and BEYER, 1973) and since the cytoplasmic fusion occurs to a large extent at conjugation (OTOTAKE, 1969). These incorporated gamones or gamone-receptor complexes might play an important role in the induction of the nuclear changes.

These discussions lead to a hypothesis that only one kind of reaction, the gamone-receptor reaction, is needed as the trigger for the induction of the nuclear changes as well as for the induction of cell union. The validity of this hypothesis may be tested, for example, by the microinjection technique (KOIZUMI, 1970). The fact that the induction of the nuclear changes in *P. aurelia* does not need conjugant cell union could be due to a relatively low threshold value of the trigger mechanism. Whether it is related to the characteristic of this species capability to carry out the mechanism of natural autogamy is to be studied in the future.

One of the striking cytoplasmic phenomena in conjugation is the reversible fusion of cell membranes. It may begin with the degeneration of cilia, followed by the close contact, local disappearance, reformation and separation of the cell membrane, and ends up with the regeneration of cilia. Such a reversible partial fusion of cells appears to be an excellent device for cell communication, although its general importance in biological systems is still to be demonstrated. In the investigation of this reversible cell union, the induction of homotypic cell union by gamone II in *B. intermedium* described in section III-A will provide a reasonably simple system, amenable to experimental analysis, because here a single kind of molecule with a known structure induces and maintains the cell union without leading to other processes in conjugation.

V. General Discussion

As described above, ciliate cells can form a conjugant union only after the preconjugant cell interaction which often continues for as long as one hour. Therefore, for the occurrence of conjugation, cells of complementary mating types should: (1) Come together close enough to undergo the preconjugant interaction, (2) stay together close enough to continue the interaction, and

(3) make direct contact to form a conjugant union when they gain the capacity to unite. Although no chemotactic mechanism to guide the specific approach of complementary mating types has been found in free living ciliates, to which belong all the ciliates described in this paper except *Tokophrya*, they have developed mechanisms to effectively use their first accidental encounters for the realization of conjugation. This will be discussed in three ciliates, *B. intermedium*, *Paramecium*, and *E. crassus*, which have been dealt with in detail in the previous sections. In *B. intermedium*, cells communicate by soluble gamones. When they feel the presence of potential mates in the vicinity by a minute amount of the gamone excreted by the latter, they start producing more gamone of their own. Cells of the complementary mating type will respond to this by the increase in the production of the first gamone. Meanwhile gamones make the interacting cells adhesive and upon contact such cells may loosely agglutinate with each other. Once it occurs, the interaction by gamones is more efficient and they become more and more likely to accomplish conjugation. In *Paramecium*, potential mates cannot recognize each other until they accidentally collide, since they carry all of their gamones on their surface. However, the amount of gamone they carry is so high that they may agglutinate to each other at the first contact and may remain so until they form conjugant pairs. In *E. crassus* the mechanism is similar to that in *Paramecium*, but their reaction is relatively lukewarm at the beginning. The amount of gamone on their surface is seldom enough to assure a permanent engagement. However, their contacts stimulate gamone production and later contacts will be more like those in *Paramecium*.

To summarize, they have two mechanisms to increase the chance of conjugation: (1) Agglutination of cells, and (2) the positive feedback of the preconjugant interaction. The first mechanism is seen more or less in all the three examples, but the second mechanism has not been reported in *Paramecium*. Does this really not occur in *Paramecium*? The mating reaction in this ciliate can be very intense at the beginning of the preconjugant interaction and in such cases the detection of its further increase can hardly be expected. However, it might be detected in such cases when the inital mating reaction is not strong but later many conjugant pairs are formed as suggested by MIYAKE and NOBILI (1974).

Irrespective of whether this feedback mechanism is found in *Paramecium* or not, the gamone induction that has already been demonstrated to occur in *B. intermedium* and *E. crassus* is worthy of intensive investigation, because it appears to be one of the simplest models of the transmission, modulation and amplification of information in biological systems. Probably most accessible for molecular study is the gamone II induction by gamone I in *B. intermedium*. Here a low concentration of a single kind of molecule induces the production of another kind of informational molecule, gamone II, within about two hours in a single type of cell. This problem may be studied by stepwise investigation of, (1) the biosynthetic pathway of gamone II, (2) enzymes participating in this pathway, (3) the receptor for gamone I, (4) the nature of information

produced by the interaction between the gamone I and its receptor, and (5) the mechanism by which this information causes the functioning of the enzymes described in (2). It should be noted that gamone I induces not only the production of gamone II but also the homotypic cell union between type II cells. However, such a homotypic union is not followed by further changes in conjugation and therefore in this system the two processes, namely the induction of gamone synthesis and that of cell union, may be investigated under conditions that are effectively separated from other complicated processes in conjugation. Similarly, the enhancement of the gamone I production by gamone II may be studied with the same advantages. The investigation of the gamone-receptor reaction may be easier here because gamone II is a stable substance of known molecular structure.

The relationship between the two types of systems of the preconjugant interaction represented by *Blepharisma* and *Paramecium* respectively will now be discussed. These two systems differ not only by, (1) the excretion versus non-excretion of gamones, but they also differ by, (2) the absence versus presence of the immediate cell agglutination when complementary mating types meet. However, the second difference can be simply a consequence of the first, because, if gamones of *Blepharisma* were cell bound, the gamone-receptor reactions might result in a cell agglutination. Another difference between the two systems is (3) the postulated number of the specific substances involved. In *Blepharisma* a receptor was postulated for each of the two gamones making the total number of specific substances at least four. These receptors are postulated because gamones of complementary mating types do not appear to react with each other and because a mild treatment by trypsin deprives the cells of the ability to respond to the gamone. On the other hand, in *Paramecium* only a pair of mating-type substances were postulated, since there was no evidence for the presence of more specific substances (METZ, 1948, 1954); according to this view, the mating-type substance is a gamone and a gamone receptor at the same time. However, this difference might also be a consequence of (1), because, if gamones are cell bound, their isolation is more difficult, and if they are not isolated, the evidence for the presence of their receptors will be harder to obtain. Although the view of METZ appears to be still generally valid at present, failures to observe the agglutination between detached cilia of complementary mating types in *P. bursaria* (COHEN and SIEGEL, 1963), *P. caudatum* (HIWATASHI, personal communication) and *P. multimicronucleatum* might be taken as evidence that two gamone-receptor pairs participate also in the mating reaction of *Paramecium*; since these detached cilia can stick to living cells of the complementary mating type, their failure to agglutinate with one another might indicate that only the gamone but not the receptor is functional in detached cilia. The failure to demonstrate any effect of detached cilia of complementary mating type on the reactivity of detached cilia in *P. aurelia* (BYRNE, 1972) may also be similarly considered. To summarize, until further evidence is obtained, the system of *Blepharisma* and that of *Paramecium* should not be regarded as

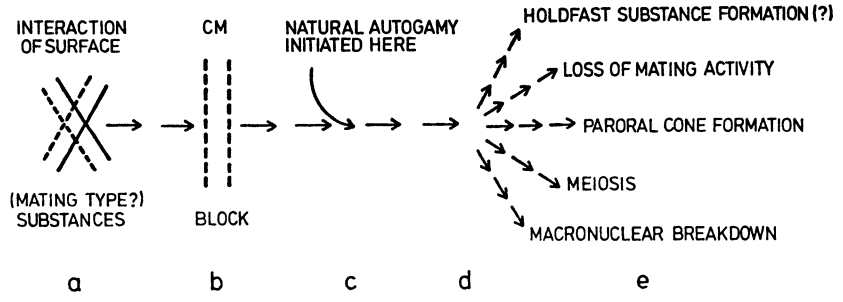


Fig. 12. Scheme for activation in conjugation and autogamy in *Paramecium*. (Redrawn from METZ, 1948)

greatly different except with regard to the point that gamones are excreted in the medium in the former but not in the latter. Incidentally, the system of the preconjugant cell interaction in *E. patella* described in section III A appears to be more reasonably explained by assuming that a cell possesses the receptor for the gamone which is not excreted by this cell, rather than by assuming that only gamones react with each other.

Recently a new type of cell interaction was reported in *T. pyriformis* (PHILLIPS, 1971). Like other ciliates, well fed *Tetrahymena* cells must be moderately starved to gain the capacity to conjugate. It was demonstrated that during this process of gaining the mating capacity, a small number of cells excrete into the medium a heat stable factor which makes other cells competent to undergo the preconjugant interaction. If a population does not contain such a cell, the whole population remains incompetent. The effective factor appears to be not mating-type-specific although the conclusive evidence for this is still lacking. Another recent report by TAKAHASHI and HIWATASHI (1970) might be considered in relation to this type of factor. They demonstrated that repeated washing of *P. caudatum* cells by some salt solutions deprived of them the ability to undergo the mating reaction. Since a fresh culture medium is effective to restore the lost reactivity, the involved factor does not appear to be very specific substance. The exact nature of the cell interaction in these experiments should be revealed by further studies.

In his pioneering work on the mechanism of conjugation, METZ (1948, 1954) postulated that processes in conjugation are related to the initial trigger event through a predetermined chain of reactions because these processes follow in an orderly sequence from the initial reaction. Based mainly on works on *P. aurelia*, he presented a hypothetical scheme for activation in *Paramecium* which is a series of chain reactions beginning with the mating reaction and eventually branching into various processes in conjugation (Fig. 12). Since CM stocks can undergo the mating reaction and induce normal cells to conjugate while they themselves cannot undergo conjugation, some block, namely the CM block, was supposed to prevent the progress of the chain reaction in CM cells. Since CM cells can undergo natural autogamy, METZ placed the CM block between the initial mating reaction and the position c of Fig. 12

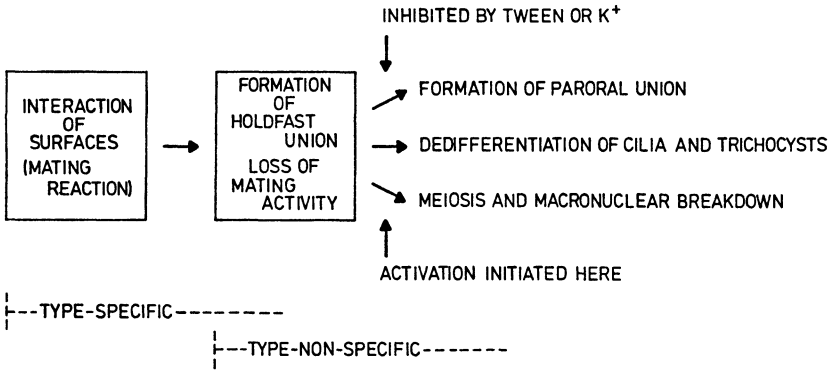


Fig. 13. Scheme for activation in conjugation in *Paramecium caudatum*. (Redrawn from HIWATASHI, 1955)

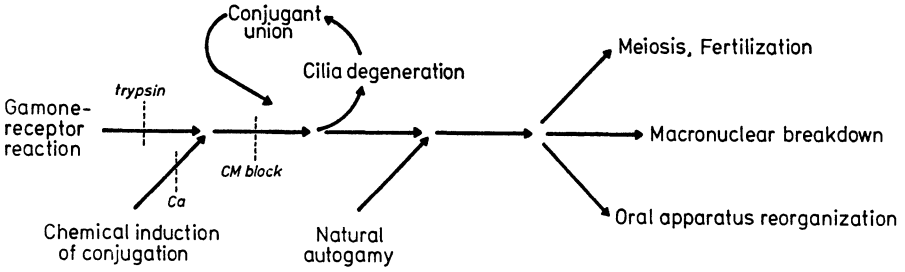


Fig. 14. Scheme for activation in conjugation and autogamy in ciliates mainly based on studies on *Paramecium* and *Blepharisma*. Those shown in italics are inhibitors specific to a step indicated by a dotted line. These inhibitors, chemical induction of conjugation, natural autogamy and cilia degeneration have been studied in *Paramecium*

where a mechanism of natural autogamy initiates the reaction chain. He suggested that holdfast-substance formation may branch from the main activation chain at a place between b and c of Fig. 12, because the holdfast union is not formed in natural autogamy. Demonstrating that the mating reaction is not sufficient to induce the nuclear changes in *P. caudatum*, HIWATASHI (1955) placed the holdfast-union formation in the main activation chain in his scheme for this species (Fig. 13).

Based on these previous works, new experimental results, and discussions described in this paper, an attempt was made to construct a new scheme in which the formation of conjugant union branches from the main activation cain but it feedbacks positively to the main activation chain (Fig. 14). The relative importance of this feedback process may differ from ciliate to ciliate: It appears to be essential in *P. caudatum*, *P. multimicronucleatum* and *B. intermedium*, while it seems to play only an accessory role in *P. aurelia*. The mechanism of this feedback has not yet been clarified. It could be the continuation of the interaction by gamones of the preconjugant interaction as suggested in section IV B, but it could also be a yet unknown mechanism. The exact place where the feedback process rejoins the main activation chain is also to be determined by further investigations.

Although this scheme is still far from perfect, a project to describe its individual steps in molecular terms and to construct a whole picture of the molecular mechanism of conjugation of ciliates is now under way. Such a project would have seemed more like a dream a few decades ago, but now the outstanding achievements in prokaryote biology and the recent success in determining the molecular structure of one of the ciliate gamones make it realistic. It is hoped that in the process of carrying out this project, much will be learned about the regulatory mechanisms of eukaryotic cells.

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The Pathogenesis of Autoimmunity in New Zealand Black Mice

NORMAN TALAL[1] and ALFRED D. STEINBERG[2]

Table of Contents

1. Introduction

This paper will review the immune disease of New Zealand Black (NZB) mice and their F_1 hybrids produced by mating with New Zealand White mice (NZB/NZW). It will attempt to highlight important recent observations made in many laboratories around the world without exhaustively reviewing all papers written on the subject. Previous reviews have emphasized pathological changes and disease descriptions in New Zealand mice (HOWIE and HELYER, 1968; HELYER and HOWIE, 1963a). The present effort will stress the immunological abnormalities of these mice. An attempt will be made to relate these immunological abnormalities with genetic and viral factors known to be important in their disease processes. The authors and their co-workers have studied approximately 20000 NZB and NZB/NZW mice with regard to natural history, immunology, pathogenesis of disease and therapy. They will draw upon this experience when possible to fill in details not available in published reports.

The inbred New Zealand mouse strains were bred for coat color for use in cancer research by Dr. MARIANNE BIELCHOWSKY. NZB mice were found to

1 Chief, Clinical Immunology and Arthritis Section, Veterans Administration Hospital, 4150 Clement Street, San Francisco, California 94121 and Professor of Medicine and Microbiology, University of California, San Francisco, San Francisco, California.

2 Senior Investigator, Arthritis and Rheumatism Branch, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health Bethesda, Maryland.

die prematurely with severe anemia and hepatosplenomegaly. In 1959, BIELCHOWSKY and co-workers described the autoimmune hemolytic anemia of NZB mice, the first report of a spontaneously occurring autoimmune disease (BIELCHOWSKY et al., 1959). HELYER and HOWIE observed the spontaneous appearance of a different autoimmune process in NZB/NZW F_1 mice: the formation of antinuclear antibodies, lupus erythematosus (L.E.) cells and renal disease resembling human systemic lupus erythematosus (HELYER and HOWIE, 1963b). NZB mice also develop significant renal disease; however it does not occur as reliably or in as severe a form as in the female NZB/NZW F_1 hybrid.

The spontaneous diseases of NZB and NZB/NZW mice are so similar to human autoimmune hemolytic anemia and the nephritis of systemic lupus erythematosus that they provide unique models for these human autoimmune diseases. The advantages of these animals in therapeutic investigations are obvious. Study of these mice may also lead to a better understanding of the pathogenesis of the human diseases. However, the diseases of New Zealand mice offer the possibility of understanding the pathogenesis of a variety of autoimmune phenomena as well as associations between autoimmunity and neoplasia. The interrelationships between genetic and viral factors may be better understood. The role of both upon the immune system should provide valuable information for the field of clinical immunology. Finally, a knowledge of the derangements of living organisms often leads to a better understanding of the normal. It shall be seen that much progress has been made toward the attainment of several of these goals.

2. Natural History of NZB and NZB/NZW Mice

NZB mice consistently develop an autoimmune hemolytic anemia early in life (Table 1). The causative anti-erythrocyte auto-antibody first appears about 3 months of age, is of the incomplete warm antibody type, and is detected by the direct antiglobulin test. Within a month or two, free unbound antibody may be detected by the indirect antiglobulin test. This antibody, either bound or free, has been characterized as IgG (NORINS and HOLMES, 1964) in contrast to a complete IgM anti-erythrocyte antibody which may occur late in life (MELLORS, 1965). Antibody titers generally increase with age, although individual animals may differ markedly with regard to both age of onset and subsequent antibody titers. Reticulocytosis, which is often marked and anemia develop in a majority of animals by fifteen months. Splenomegaly with extramedullary hematopoiesis occurs in most animals by eighteen months, but is variable between 9 and 18 months. A histologically severe membranous glomerulonephritis is frequently found in the absence of clinically significant renal functional deterioration. Later in life severe proliferative glomerulonephritis may be found (MELLORS, 1965; MELLORS, 1966c). An arteritis resembling human polyarteritis nodosa has been reported to occur in 10% of NZB mice (HICKS, 1966). Lupus erythematosus cells and positive

Table 1. Natural history

NZB	NZB/NZW
Anti-Erythrocyte Antibodies	Anti-Nuclear Antibodies
Hemolytic Anemia	Immune-Complex Nephritis
Renal Disease	Lymphoid Infiltration of Organs
Lymphoid Infiltration of Organs	Proteinuria
Death from	
1. Hemolytic Anemia 2. Renal Disease 3. Malignancy	Death from Uremia

antinuclear antibody test in high titer are found in a minority of NZB mice, usually late in life. An antibody to murine thymocytes was described by SHIRAI and MELLORS (1971) to occur very early in the life of NZB mice. The possible role of this autoantibody in the pathogenesis of a variety of immunological abnormalities will be discussed in a later section.

Histological lesions of the thymus have been described (BURNET and HOLMES, 1965a; DE VRIES and HIJMANS, 1966; DE VRIES and HIJMANS, 1967), but their relationship to autoimmune phenomena remains uncertain. Hyperplasia of lymphoid follicles in the spleen and lymph nodes develops throughout the first year of life terminating in extreme proliferation of lymphoid cells throughout the body, including the thymus, lungs and salivary glands. This may result in frank lymphoid malignancy in as many as 20% of the animals (DE VRIES and HIJMANS, 1967; MELLORS, 1966b); however, distinguishing between neoplasia and non-malignant lymphoid proliferation may be extremely difficult. NZB mice have been found to be particularly susceptible to bacterial infection (KAYE and HOOK, 1964), and such infection may be the cause of death in some animals. The majority die of their autoimmune disease and a few die of lymphoid malignancy. NZB mice survive an average of 15 to 18 months with males living about a month longer than females. MELLORS and co-workers have studied in depth a C-type murine leukemia virus present in NZB mice throughout life (MELLORS and HUANG, 1967; MELLORS et al., 1971). This virus may have a role in various autoimmune processes.

NZB/NZW mice differ quantitatively from NZB mice with regard to manifestations of autoimmunity. Hemolytic anemia is much less significant and rarely occurs prominantly before the onset of severe renal disease. Anti-erythrocyte antibodies are overshadowed in the F_1 hybrid by antinuclear antibodies. These antinuclear antibodies may be detected as early as two months of age. They are heterogeneous consisting of antibodies to single and double-stranced DNA and RNA as well as antibodies to nucleic acid-protein complexes and nuclear proteins. Anti-DNA antibodies (STEINBERG et al., 1969a) and death from nephritis (BURNET and HOLMES, 1965a) occur earlier in females. Typical LE cells are detectable after 4 months of age and are found at some time during the life of most animals. Between 3 and 6 months

of age immunoglobulin and complement are first detectable by immunofluorescence in the glomeruli of NZB/NZW mice (AARONS, 1964; LAMBERT and DIXON, 1968a). Later fibrin, alpha and beta globulins and albumin are also found (NAIRN et al., 1966). All these proteins are also present in the urine (SHARARD, 1967).

Electron microscopic studies of kidneys from New Zealand mice of various ages have generally disclosed electron dense deposits in the mesangium by about 4 months of age, followed by sub-epithelial deposits and later sub-endothelial deposits. The electron microscopic and immunofluorescent observations are consistent with renal histological changes found by light microcopy. The earliest lesions are usually evident in the mesangium. These abnormalities are seen at about 5–6 months of age and consist of deposition of fibrinoid material and localized proliferation. Later, proliferative changes may involve much of the glomerulus. Focal areas of necrosis may be present, particularly at the periphery of a tuft. Basement membrane thickening, focal areas of glomerular sclerosis and proliferation of Bowman's capsule typical of diffuse membrano-proliferative glomerulonephritis are found in the advanced renal lesion. Different glomeruli may be found in various states of disease activity, sclerosis being a common finding in some.

The incidence of proteinuria rapidly increases in female NZB/NZW mice after 6–7 months of age and death from renal failure generally follows the onset of massive proteinuria by 3–4 weeks (LAMBERT and DIXON, 1968b). Average survival for virgin females is about 300 days with a 1–2 month variation in different laboratories. Males have been found to have early proteinuria not related to significant renal disease (LAMBERT and DIXON, 1968a). Later in life they also develop proteinuria and renal histological changes similar to those of their female litter-mates. However, the changes in the males are less constant, develop more slowly and are less predictable. On the average, males live 100–150 days longer than the females. It appears that lymphoid neoplasia occurs with somewhat lower frequency in the NZB/NZW mice than in the NZB, perhaps because many die of renal failure before neoplasms develop. However malignancy is observed in these mice especially thymomas, with an incidence between 1 and 5 percent.

3. Genetic Studies

Large numbers of mating, back-cross and other genetic studies have been performed with NZB mice. The first was the study of HELYER and HOWIE (1961) in which the hemolytic anemia observed in homozygous NZB mice was modified to glomerulonephritis in (NZB × NZY)[3] F_1 hybrids (Table 2). Subsequently these workers described the (NZB × NZW) F_1 hybrid (HELYER and HOWIE, 1963b) which has been studied extensively in many laboratories because of the severe glomerulonephritis, antinuclear antibodies and immunologic dysfunction that they develop. Markers of disease and survival are

3 In all hybrids the mother will be listed first and the father second.

Table 2. Genetic features

1. NZB crossed to NZY or NZW increases severity of renal disease
2. NZB × NZW F_2 to F_5 decreases severity of autoimmune disease
3. NZB × NZW similar to NZW × NZB therefore disease is not sex linked and not dependent upon material passed by mother *in utero* or via colostrum
4. NZB crossed to non-autoimmune strains of mice lead to F_1 mice with mild Coombs positive late in life
5. Backcross experiments suggest that more than one gene is involved

indistinguishable in NZB × NZW and NZW × NZB mice (GHAFFAR and PLAYFAIR, 1971; BURNET and HOLMES, 1965a). The disorder is therefore, neither sex-linked nor determined by passage of material either *in utero* or via colostrum. This latter point has been further substantiated in a series of experiments by BARNES and co-workers (1972). They transplanted ova from NZB mothers into pregnant normal CFW mothers. The direct Coomb's test, anemia and reticulocytosis were comparable in all mice (normal NZB, ovum transplantation derived NZB and milk fostered NZB). They concluded that the stimulus to develop the disease must be present prior to the stage of uterine transplantation. The same investigators have also studied a small number of allophenic (tetraparental) NZB: CFW chimeras prepared by fusing ova from hymozygous NZB and CFW matings. Autoimmune disease was suppressed in these allophenic mice just as it often is in F_1 animals derived from mating between NZB and non-New Zealand strain mice.

Autoimmune phenomena are not linked to coat color despite the original breeding for coat color (HOWIE and HELYER, 1965). BIELSCHOWSKY and BIELSCHOWSKY (1964) found that F_2 mice derived from NZB × NZC matings had 74% incidence of hemolytic anemia and F_1 mice a 100% incidence. They postulated that a single dominant gene was operative. Other F_2 mice were later also found to have autoimmune disease (HOWIE and HELYER, 1965; BRAVERMAN, 1968).

All generations through F_5 were found to have autoimmune disease with decreasing and variable severity (BRAVERMAN, 1968). BRAVERMAN described a 50% incidence of Coombs positivity, antinuclear factor and LE cell tests in F_2 NZB × NZW mice (BRAVERMAN, 1968). From the analysis of renal disease, antinuclear antibody and Coombs positivity in F_1, F_2 and back-cross mice he concluded that there is a dominant gene in NZB mice which determines Coombs positivity and an additional modifying gene which allows the expression of antinuclear antibody. Renal disease did not correlate with the autoantibodies and appeared to be determined by several genes. In the authors' experience (unpublished) renal disease and antibodies to DNA and RNA are much less prominent in F_2 as compared to F_1 mice derived from NZB × NZW crosses.

A variety of other crosses of NZB mice with non-autoimmune strains have produced F_1 hybrids with relatively mild autoimmune disease generally

occurring later in life (BURNET and HOLMES, 1965b). Backcrosses of the F_1 mice to the parent strains resulted in no segregation of autoimmune disease, backcross mice expressing disease intermediate in severity between the parent and F_1. These studies suggest that although genetic factors are important in the expression of autoimmune phenomena in New Zealand mice, several genes are probably involved. One very interesting cross, NZB×AKR, was studied by HOLMES and BURNET (1966). They observed an inverse relationship between the early development of lymphoid neoplasia (characteristic of the AKR strain) and of autoimmunity (characteristic of the NZB strain).

A study by GHAFFAR and PLAYFAIR (1971) has examined the question of the genetic basis of autoimmune phenomena in NZB mice. They studied crosses of NZB mice with both NZW and Balb/c mice and the F_1, F_2 and backcross offspring were tested for antinuclear antibodies, Coombs positivity and glomerulonephritis. Unlike most investigators, they found no sex differences and so pooled data for all measures. Tests were carried out at 9 months of age, a time when most NZB mice are positive and most NZB×Balb/c F_1 hybrids are negative. There was no good genetic explanation for antinuclear antibodies. This may be explained in part by the heterogeneous group of antibody specificities measured in the antinuclear antibody test. Looking at specific antinuclear antibodies (e.g. anti-single or double-stranded DNA) might have been more informative. Coombs test results were explainable on the basis of recessive genes or gene. It was suggested that a single recessive gene might give a positive test late in life, whereas a double genetic dose might give a stronger test early in life. Renal immunofluorescent staining studies in NZB×NZW crosses suggested inheritance on the basis of a single dominant gene. Positive results were lower in the NZB×Balb mating suggesting the presence of another recessive gene in both New Zealand strains, but absent in Balb/c mice, which may give a positive result when the first gene is also present, and perhaps an earlier one if two of the first genes are present. Perhaps the most important observation was that the data could not be explained solely by a virus, but that at least some genetic factors were operative in the autoimmune phenomena observed in New Zealand mice. It appears that the final word regarding the exact genetic inheritance of various autoimmune markers is not yet available. It is possible that all of the markers or most of them may be secondary to other abnormalities which may show rather simple inheritance. These primary abnormalities may be influenced by a variety of environmental and hormonal and perhaps other genetic factors so that the secondary autoimmune markers appear to have rather complex inheritance. We anxiously await a definition of this problem.

4. Immunologic Studies

An effective immune response requires the cooperative interaction of several different types of cells. Two of these are lymphocytes and a third is the macrophage. Lymphocytes are classified into two main groupings according

to their function. B-lymphocytes arise as bone marrow stem cells and migrate directly to peripheral lymphoid organs (i.e. lymph nodes and spleen) where they participate in the formation of germinal centers and synthesize and secrete antibody of defined specificity. Early antibody is IgM while later antibody belongs primarily to the IgG immunoglobulin class. The distinguishing characteristic of B-cells is the presence of readily detectable immunoglobulin on their surface membranes. This immunoglobulin is a receptor for antigen and can easily be demonstrated by immunofluorescent methods using anti-immunoglobulin reagents.

The other lymphocyte called the T-cell, also has its origin in the bone marrow but it migrates from there to the thymus where it differentiates in an entirely different way. Mature T-cells leave the thymus to populate the thymic-dependent areas in lymph nodes and spleen. T-cells do not produce antibody but, rather, mediate a host of immunologic functions that are classified as cell-mediated immunity. These include delayed hypersensitivity, allograft rejection, and immune surveillance against malignancy. They appear to perform these activities by releasing a variety of soluble mediating factors after specific interaction with antigen. Certain non-specific stimulators of T-cells, such as the plant mitogen phytohemagglutinin (PHA) can also cause the release of these soluble mediators. T-cells, although they appear to have receptors for antigen in their membranes, do not fluoresce with anti-immunoglobulin sera.

T-cells cooperate with B-cells for maximal antibody production. Antibody synthesis by B-cells to most antigens is markedly diminished if T-cells are absent. B-cells recognize the haptenic determinants on complex antigens while T-cells recognize the carrier portion. Not all antigens elicit T-cell cooperation, and others can circumvent the T-cell requirement if presented in a highly polymerized form. The exact mechanism of B-T cell cooperation is not known, but a recent hypothesis by FELDMANN (1972) is most intriguing. He proposes that T-cells contain monomeric IgM on their surface membranes. This receptor IgM, after interacting with antigen, is shed from the T-cell surface and binds to specific sites on macrophages. The antigen, still attached to IgM molecules on the macrophage surface, is held in a configuration particularly favorable for stimulation of B-cells, perhaps in a phasic orientation resembling polymeric antigen. B-cells are thus stimulated for maximum production of antibody.

Both B and T cells develop immunologic tolerance, but the kinetics and duration of tolerance is different in each population. B-cell tolerance comes on more slowly and is short-lived.

Certain lines of evidence point to a negative cooperative interaction between B and T cells. For example, three recent studies indicate that a population of T-cells capable of suppressing *in vitro* B-cell antibody responses are generated by treatment of spleen cells with the plant mitogen concanavalin A (DUTTON, 1972; RICH and PIERCE, 1972; SJOBERG et al., in press). Furthermore, several laboratories have reported that *in vivo* immune responses to

Table 3. Immunologic abnormalities

1. Early immunologic maturation
2. Selective humoral hyperresponsiveness early in life
3. Loss of tolerance to foreign and self antigens early in life
4. Impaired cellular immunity later in life
5. Loss of recirculating thymus derived cells
6. Spontaneous production of anti-thymocyte antibody

thymus-independent antigens can be suppressed by T-cells. Baker found that anti-thymocyte serum (which suppresses T-cell functions) paradoxically increased the antibody response to Type III pneumococcal polysaccharide (BAKER et al., 1970). This increase was largely prevented by addition of thymocytes (BAKER, STASHAK et al., 1970). Others have reported enhanced antibody responses to polyvinylpyrrolidone after adult thymectomy (KERBEL and EIDINGER, 1972) and T-cell suppression of antibody responses to E. coli endotoxin (MOLLER and MICHAEL, 1971). Likewise, the suppression of genetically-determined allotypes in mice is relieved by anti-lymphocyte serum (JACOBSON et al., 1972). These and similar experiments have been interpreted as indicating the presence of a population of regulating T-cells which can inhibit B-cell synthesis of antibody. When these T-cells are themselves suppressed or eliminated by thymectomy or anti-thymocyte serum, then B-cell synthesis becomes more active.

Much information is available on the immune response of New Zealand mice (Table 3). Before reviewing these results, it is necessary to emphasize that the immunologic properties of these mice varies greatly depending upon age and stage of disease. In general, their immune status can be best characterized as an imbalance in which B-cell activity and antibody responses are excessive and T-cell activity or cell-mediated immunity is depressed (TALAL, 1971). As discussed subsequently, this disordered state of immune regulation would leave them particularly susceptable to unusual forms of virus infection, auto-antibody formation and malignancy.

New Zealand mice appear clinically normal for the first 3–5 months of life. Some laboratories have especially studied the immune responses of young mice lacking clinical disease in an attempt to gain information of potential relevence to the subsequent development of autoimmunity. New Zealand mice achieve full immunologic maturity when still very young. Within the first week of life, they have antibody responses to sheep erythrocytes equal to that seen in adult animals (EVANS et al., 1968). Other strains require several weeks to achieve comparable levels of immunologic maturity. This premature development of immune competence is also seen with cell-mediated responses, since very young New Zealand mice can cause regression of viral-induced sarcomas more rapidly than age-matched control strain animals (GAZDAR et al., 1971).

New Zealand mice make excessive antibody response to a variety of experimental antigens, including foreign proteins, sheep erythrocytes and

synthetic nucleic acids (STAPLES and TALAL, 1969; PLAYFAIR, 1968; CEROTTINI et al., 1969; STEINBERG et al., 1969b). However, their responses to pneumococcal polysaccharide, hemocyanin, and erythrocytes from pigs and chickens are within normal range indicating some selectivity in their hyperresponsiveness.

Adult New Zealand mice show a resistance to the induction and maintenance of immunologic tolerance to soluble foreign proteins and sheep erythrocytes (WEIR et al., 1968; STAPLES and TALAL, 1969; STAPLES et al., 1970). Using soluble ultracentrifuged bovine gamma globulin to induce tolerance, STAPLES and TALAL (1970) found that New Zealand mice over 5–6 weeks of age were neither made tolerant nor immunized by this treatment. New Zealand mice 3 weeks or younger developed a temporary state of tolerance that was lost in 2–4 weeks (STAPLES et al., 1970). Several control strains developed a long-lasting tolerance. Thymectomized and lethally irradiated NZB mice repopulated with young (i.e. under 3 weeks of age) or older spleen, bone marrow and thymus cells and then given tolerogenic BGG developed the type of tolerance characteristic of the transplanted cell population (STAPLES et al., 1970). The tolerance experiments were extended by studying another antigen, sheep erythrocytes and the induction of tolerance by use of a cytotoxic drug, cyclophosphamide (JACOBS et al., 1971). Transfer of tolerant New Zealand or C57Bl/6 bone marrow and thymus cells into lethally irradiated syngeneic mice brought about tolerance in the recipients. When tolerant bone marrow or thymus cells were mixed in such a way that one population came from normal non-tolerant donors, it was found that the New Zealand thymus failed to transfer tolerance. Similarly, PLAYFAIR (1971) reported that the NZB thymus has a raised threshold for tolerance induction to sheep erythrocytes. He found that 2×10^8 sheep erythrocytes produce specific tolerance in Balb/c thymus but not in NZB or NZB $\times$ Balb/c F_1 thymus. Thus, T-cells which are responsible for a more sustained tolerance than are B-cells, seem unusually resistant to tolerance in New Zealand mice. Left with primarily B-cell tolerance, which is short lived, New Zealand mice maintain tolerance poorly to a variety of antigens. As a consequence perhaps, they are unable to remain tolerant to the leukemia virus which they harbor from birth (MELLORS and HUANG, 1966; PROSSER, 1968).

Older New Zealand mice have marked deficiencies of T-cell functions demonstrated by decreased responsiveness to mitogens (LEVENTHAL and TALAL, 1970), impaired ability to induce graft-vs-host disease (CANTOR et al., 1970) and to reject tumors (MELLORS and HUANG, 1966) or skin grafts (GELFAND and STEINBERG, 1973a). They have marked alterations in their lymphoid population with deficiency of long-lived recirculating lymphocytes in lymph nodes and spleen (DENMAN and DENMAN, 1970; ZATZ et al., 1971). The cause of impairment in cellular immunity in aging New Zealand mice was studied in the model of skin allograft rejection (GELFAND and STEINBERG, 1973a). Old (eight month old) female NZB/NZW mice, but not mice of control strains, rejected skin grafts significantly more slowly than did young mice of the

same strain. Splenectomy did not lead to further prolongation of graft survival in old NZB/NZW mice in contrast to all other groups, suggesting a deficiency in a spleen cell subpopulation in old NZB/NZW mice. Prompt graft rejection could be restored to old NZB/NZW mice by small numbers of young NZB/NZW spleen or lymph node cells. The cells present in young animals and lacking in old were found to be recirculating thymus-derived cells that were both corticosteroid and ATS sensitive. These cells were found to synergize with relatively corticosteroid resistant subpopulations of splenic T-cells present in old mice.

An attempt was made to transfer the defect in cellular immunity from old NZB/NZW mice to young syngeneic recipients. Old spleen cells were ineffective in transferring prolonged graft survival to young mice; however, serum from old NZB/NZW mice was found to transfer the defect to young NZB/NZW mice (GELFAND and STEINBERG, 1973b). The serum factor could be absorbed with young spleen cells, but not old spleen cells, consistent with the functional studies. Furthermore, the serum factor was absorbed with thymocytes from young animals suggesting that the factor was similar to the thymocytotoxic antibody discussed in the next section.

Three recent studies have investigated the number and distribution of T and B cells in lymphoid organs using specific antisera to theta, B-cell antigens (MBLA) or immunoglobulin (STOBO et al., 1972; WAKSMAN et al., 1972; STUTMAN, 1972). The development and distribution of theta antigen is essentially normal except in older mice (over 200 days of age) who show a decline in theta (STOBO et al., 1972a; STUTMAN, 1972). The normal development of theta-bearing T-cells in thymus and peripheral lymphoid organs merely indicates that any functional abnormality in a subpopulation of T-cells (e.g. deficiency of suppressor T-cells) is not reflected in a change in this surface membrane antigenic marker. Interestingly, NZB thymocytes showed an accelerated development of reactivity to the mitogen concanavalin A, another indication of premature immunologic maturation in these animals (STOBO et al., 1972a).

The decline in cell-mediated immunity and in theta-positive lymphocytes first appeared at about 6 months of age, when antibody responses to sheep erythrocytes were still normal or increased. This decrease in theta-positive cells in thoracic duct appeared before overt autoimmunity was detected, but it became more marked as autoantibodies developed. In two studies, the number of B-cells bearing immunoglobulin receptors was diminished although there was no abnormality in MBLA antigen or in complement-receptor lymphocytes.

STOBO et al. (1972b) used antiserum to mouse-$\varkappa$ chain to detect the number of lymphocytes with this light chain marker on their membranes. By three different techniques (cytotoxicity, immunofluorescence and autoradiography), the lymphoid population of NZB mice at all ages starting from birth had a decreased number of such immunoglobulin-bearing lymphocytes. Antiserum to λ chain and to specific heavy chains confirmed this result. Thus, the NZB

lymphoid organs contained a large percentage of lymphocytes bearing neither theta nor immunoglobulin markers on their surface. This percentage was 23–27% in the spleen and 11–21% in the lymph nodes, compared to 6–12% and 0–5% respectively in four normal control strains. These "null cells" appear not to be macrophage or hematopoietic stem cells and may represent a minor lymphoid population present in normal mice but greatly expanded in NZB mice.

BHOOPALAM et al. (1971), employing the technique of immunocytoadherence, have observed a decrease in IgG and IgM receptor lymphocytes in NZB mice over 6 months of age, a time when there are marked elevations of serum immunoglobulins (particularly IgM). Using an antiserum that recognizes an idiotypic determinant on IgM, they find an increased number of lymphocytes with this idiotypic determinant on their membrane receptors in aging Coomb's positive NZB mice.

An additional thymic deficiency has been observed in New Zealand mice. Thymosin, a thymic hormone which can convert bone marrow cells into T-cells, has its counterpart in a "thymosin-like" material present in normal mouse serum and in serum from New Zealand mice under 3–6 weeks of age (BACH et al., personal communication). By two months, an age at which tolerance to foreign proteins can no longer be induced, thymosin-like activity has disappeared from the serum of NZB and NZB/NZW mice. Somewhat later, there is a change in splenic and lymph node rosette-forming cells corresponding to that seen in neonatally thymectomized normal mice.

The cellular immunological abnormalities of New Zealand mice are explainable by a progressive loss of thymic function with age. Recent studies of thymic suppressor function may help to explain some of the humoral immune abnormalities as well. Autoimmune disease in New Zealand mice is accelerated by neonatal thymectomy. This acceleration is prevented by thymic grafts from two week old NZB/NZW mice suggesting the presence of suppressor cells in the young thymus (STEINBERG et al., 1970). Thymic grafts from ten week old NZB/NZW mice were completely ineffective. This suggests that the suppressor function is lost by 10 weeks of life. Recent studies confirm that with age there is a loss of suppressor T-cells in both NZB/NZW (CHUSED et al., 1973) and NZB mice (BARTHOLD et al., 1973). In addition to an age dependent loss of T-cell suppression of B-cell response, there appears also to be a similar loss of T-cell suppression of T-cell responses (HARDIN et al., 1973). The loss of suppressor T-cells may help to explain the humoral hyperresponsiveness of New Zealand mice, their impaired tolerance induction and maintenance, and the loss of self tolerance leading to autoimmunity.

5. Natural Thymocytotoxic Autoantibody (NTA)

New Zealand mice have a variety of autoantibodies reactive with nonorgan specific antigens such as nucleic acids (described in the next section) and organ specific antigens such as erythrocytes and liver. A recently described autoantibody to T-cells may have particular importance in view of the central

role of T-cells in immunological reactions of all types. This natural thymocytotoxic autoantibody (NTA), similar to the anti-theta antibodies obtained by immunization (Raff, 1969) was discovered by Shirai and Mellors (1971). They found that many NZB mice developed this NTA in the first month of life, and that at three months of life all NZB mice were positive. NZB/NZW mice were not found to be positive until five to seven months of age at which time half of these mice were found to have NTA (Shirai and Mellors, 1971). At that age a portion of C57 Bl/6, AKR, and 129/J mice were also positive. The NTA could be absorbed with cells from thymus, lymph node, spleen or brain from adult mice. In newborn mice the reactive antigen was present only in the thymus. Gel filtration suggested that the NTA was an antibody of the IgM class.

Follow-up studies by the same workers confirmed and expanded the original observations (Shirai and Mellors, 1972; Shirai et al., 1972; Shirai et al., 1973). Some NZB/NZW mice were found to be positive at two months of age, and one-half of the NZB/NZW mice three to six months of age were positive. However, NZB$\times$C57 Bl/6 F_1 mice with much milder autoimmune disease than NZB/NZW F_1 mice had an equal prevalence of NTA. Sera were generally more reactive at 4°C than at 37°C. The antibody killed 98% of thymocytes, 62% of thoracic duct cells, 64% of peripheral blood leukocytes, 58% of lymph node cells, 33% of spleen cells, 3% of bone marrow cells and less than 1% of fetal liver cells. These findings suggested that the antigenic specificity of NTA was very similar to that of theta alloantigen. Lymphoid cells from congenitally athymic nude mice were not sensitive to NTA as would be predicted. It appears that NTA is very similar to anti-theta antibody, but containing specificity of both C3H and AKR anti-theta alloantibodies. Such an autoantibody could be responsible for a variety of immunological abnormalities of New Zealand mice. Early in life it could reduce the effect of suppressor T-cells leading to antibody hyperresponse. Later in life it might be responsible for progressive loss of all T-cell function and might be responsible, in part, for the development of malignancy.

6. Immune Complex Nephritis and Antibodies to Nucleic Acids

The nephritis in NZB/NZW mice (and in human lupus patients) is related to the glomerular deposition of immune complexes and complement (Lambert and Dixon, 1968). Not all auto-antibodies have the same tendency to deposit in the kidney. Immunofluorescent and elution studies indicate that DNA and its antibody is concentrated in the renal deposits, implicating this particular immune complex in the pathogenesis of the nephritis.

There is a second immune complex system which deposits in the glomeruli of New Zealand mice. This system involves antibodies directed against antigens of the mouse leukemia virus (Mellors et al., 1969). However, quantitatively this seems to be less important than nuclear antigen-antibody complexes (Dixon et al., 1971).

Immune complex nephritis can be induced in normal mice by neonatal infection with viruses (OLDSTONE and DIXON, 1971) such as lymphocytic choriomeningitis (LCM). In this disease, viral immune complexes are present in the kidney but antibodies to nuclear antigens are not. Thus, anti-DNA antibodies appear to be a unique feature of disease in human lupus and in New Zealand mice.

Antibodies to DNA develop progressively in New Zealand mice and can be easily measured by radioactive binding assays which employ either precipitation of immune complexes by ammonium sulfate (STEINBERG et al., 1969) or entrapment of radioactive DNA-antibody complexes on nitrocellulose filters (TALAL and GALLO, 1972).

The same methods can be used to detect the presence of antibodies to RNA which also occur with greatest frequency in NZB/NZW mice and human lupus patients (TALAL et al., 1970; STEINBERG et al., 1969; SCHUR et al., 1971). Antibodies to double-stranded RNA can be measured using radioactive reovirus RNA or synthetic polyinosinic-polycytidylic acid (poly I·poly C) as antigen. Since double-stranded RNA is relatively rare in mammalian tissues unless they are infected with viruses, these antibodies to RNA may arise because of immunization to viral nucleic acids. This possibility is supported by the finding that these antibodies have greatest specificity for viral double-stranded RNA, next for synthetic double-stranded RNA and least for mammalian ribosomal or transfer RNA (TALAL et al., 1970).

7. Nucleic Acids as Antigens

Since anti-nucleic acid antibodies are a hallmark of both murine and human lupus, a short summary of the antigenicity of nucleic acids seems appropriate.

Antibodies to nucleic acids have been induced in experimental animals by a variety of immunization procedures. The most reliable methods have been the immunization of rabbits with nucleic acid bound either covalently or electrostatically to an immunogenic protein carrier. Such methods led to the production of large quantities of antibody to single and multiple stranded nucleic acids as well as to oligonucleotides, nucleotides, nucleosides and bases (PLESCIA and BRAUN, 1969). Antibodies to natural and synthetic nucleic acids were produced in this way. Although antibodies to double-stranded RNA and single-stranded DNA were easily elicited, antibodies to native DNA could not be induced. STOLLAR (1970) has found that antibodies to synthetic double-stranded DNA can be induced in rabbits but only if the DNA does not cross react with native DNA. Thus, deoxyguanylic·deoxycydidylic acid (dG·dC) complexed to a protein will induce antibody; however, dAT which cross-reacts with native DNA is ineffective.

Early studies in rabbits suggested that nucleic acids were haptens (PLESCIA and BRAUN, 1968; YACHNIN, 1962). That is, an immune response could be induced to them only if they were attached to an immunogenic protein carrier.

Antibodies were produced in New Zealand and other mice by immunization with single-stranded DNA coupled to an immunogenic protein (LAMBERT and DIXON, 1970). The first suggestion that nucleic acids might be immunogenic in their own right (i.e. without being complexed to a protein carrier) came from studies originally conducted in New Zealand mice with poly I·poly C (STEINBERG et al., 1969). In these studies New Zealand mice given multiple injections of poly I·poly C had accelerated production of antibodies to RNA and DNA. Subsequent studies have suggested that anti-RNA production was accelerated at least in part by antigenic stimulation whereas the anti-DNA production was largely attributable to an adjuvant action of poly I·poly C (POWELL and STEINBERG, 1972). Studies in non-autoimmune strains of mice confirmed that poly I·poly C is recognized immunologically without a protein carrier and even without adjuvant (STEINBERG et al., 1970; STEINBERG et al., 1971). Other investigators have also induced antibody to synthetic double-stranded RNA in rodents (THOBURN and KOFFLER, 1971; FIELD et al., 1972). Guinea pigs were found to respond with antibody production but not delayed hypersensitivity to poly I·poly C or poly A·poly U (VAN BOXEL and STEINBERG, 1972).

A recent study of the antibody response to poly I·poly C in mice and rabbits detail the time course of the response, immunoglobulin class of antibody, and role of adjuvant and carrier (PARKER and STEINBERG, 1973). It appears that with the exception of a mutant strain of CBZ mice (SCHER et al., 1973) all mice respond to poly I·poly C aqueous solution with a short lived IgM antibody response that peaks at 4 days. This is not altered by complexing the RNA to an immunogenic protein carrier. However, if the RNA is injected in complete or incomplete FREUND'S adjuvant, the IgM peak is followed by a later prolonged IgG peak. This second peak is heightened by complexing the RNA with a protein carrier prior to emulsification in adjuvant. The response to poly I·poly C without a carrier is relatively thymic independent. Neonatal thymectomy does not diminish the immune response to poly I·poly C in complete adjuvant in New Zealand mice (STEINBERG et al., 1970) or in incomplete adjuvant in Balb/c×DBA/2 F_1 mice (CHUSED et al., 1973). Thymus deprived animals are capable of responding to poly I·poly C in aqueous solution; and anti-thymocyte serum plus poly I·poly C leads to acceleration rather than depression of antibody formation CHUSED et al., 1973). Taken together, these studies indicate that the immune response to nucleic acids may require rather little helper T-cell function and that T-cell function may be inhibiting. The loss of such inhibiting T-cells with age in NZB/NZW mice is associated with the spontaneous production of antibodies to nucleic acids (CHUSED et al., 1973).

8. Modifiers of Disease

In an earlier section, the ability of genetic factors to modify disease in New Zealand mice was discussed. In this section (Table 4) examples will be given of modification (either acceleration or retardation) of autoimmune

Table 4. Alteration of natural history of NZB/NZW mice

Agent	Effect on renal disease
1. Nuclear Antigens	Worse
2. Viral Infection	
a) LCM, Polyoma	Worse
b) LDV	Improved
3. Inteferon Inducer	
a) Statalon	Improved
b) Poly I·Poly C	Worse
4. Malarial Infection	Improved[a]
5. Anti-Lymphocyte Serum	
a) Without Tolerance	Worse
b) With Tolerance	Improved[a]
6. Steroids	Improved
7. Cyclophosphamide	Improved
8. Azathioprine	Improved[b]

[a] No positive effect on renal disease of NZB mice.
[b] Not effective in hemolytic anemia of NZB mice.

phenomena by chemical substances, interferon inducers, viruses and other infections. Therapeutic agents such as corticosteroids, anti-lymphocyte sera and immunosuppressive drugs are covered in a later section.

The first reported modification of autoimmune disease was the acceleration of renal disease in NZB/NZW mice following injection of heat-denatured DNA into anti-DNA positive mice, and the accelerated appearance in young NZB/NZW mice of anti-nuclear antibodies and proteinuria by injections of DNA complexed to methylated albumin (LAMBERT and DIXON, 1968). It is of interest that similar injection of native DNA was without effect (LAMBERT and DIXON, 1968). Likewise, acceleration of anti-nuclear antibody production and death from immune complex glomerulonephritis occurred following infection of NZB/NZW, NZB and NZW mice with both LCM and polyoma virus (OLDSTONE and DIXON, 1969; TONETTI and OLDSTONE, 1970). The former contains RNA and the latter DNA. This acceleration occurred without affecting the severity of the autoimmune hemolytic anemia of NZB, NZW or NZB/NZW mice.

Multiple injections of the interferon inducer, poly I·poly C, were given to parent NZB and NZW mice and to their NZB/NZW offspring starting from birth so that the latter would receive continuous exposure to relatively high levels of interferon (STEINBERG et al., 1969). Rather than suppressing the effects of endogenous or exogenous virus, such poly I·poly C treatment led to accelerated production of antibodies to both double-stranded RNA and DNA (STEINBERG et al., 1969). In addition there was an acceleration of glomerulonephritis in the parent strains as well as in the NZB/NZW offspring (CARPENTER and STEINBERG, 1970). It was found that this treatment induced RNA anti-RNA immune complex glomerulonephritis and in NZB/NZW mice

this preceded and complicated the naturally occurring immune complex disease (Carpenter and Steinberg, 1970). Because the synthetic nucleic acid and experimental virus infections all accelerate disease, it was concluded that a unique genetic predisposition was present. It is of interest that New Zealand mice respond to multiple injections of poly I·poly C with anti-nuclear antibody production much more easily than most other mice (Steinberg et al., 1971) and similarly respond with antinuclear antibody following LCM and polyoma infection in contrast to other strains (Tonetti and Oldstone, 1970). New Zealand mice showed progressively rising titers of LCM between 6 weeks and 6 months of age whereas other strains of mice showed no increase during that period (Tonetti and Oldstone, 1970). This ability to handle viral infection may be genetically conditioned or related to immunologic factors. A germ-free environment (which would not eliminate viral infections) had no effect on disease (East et al., 1967).

Not all viral infections and interferon inducers accelerate autoimmune disease in New Zealand mice. Statalon injected subcutaneously into NZB/NZW mice every two weeks starting at birth (20 μg/g) led to a reduction in antinuclear antibodies (Lambert and Dixon, 1970b). Furthermore, infection of New Zealand mice with lactate dehydrogenouse virus (LDV) led to a marked reduction in mortality from renal disease associated with a reduction in anti-DNA antibodies (Oldstone and Dixon, 1972). The protective effect of LDV was greater when administered at 4 weeks of age than at 10 weeks of age. The authors point out that LDV has profound effects upon the immune system; however, the mechanism of this favorable effect remains unknown. A similar phenomenon has been observed following infection of New Zealand mice with the malarial parasite *Plasmodium berghei*. Such parasitic infection led to marked reduction in proteinuria and protection of NZB/NZW mice from fatal glomerulonephritis (Greenwood and Voller, 1970a). The same workers found that although *Plasmodium berghei* infection of NZB mice delayed the onset and reduced the severity of autoimmune hemolytic anemia, the occurrence of antinuclear factor was the same as in control NZB mice (Greenwood and Voller, 1970b). In contrast to the NZB/NZW mice, malaria infected NZB mice had more proteinuria and more severe renal disease by histology and immunofluorescence (Greenwood and Voller, 1970b). The immunofluorescence was positive for mouse IgG and complement, but not malarial antigens. It is unclear why malarial infection led to rather different outcomes in NZB and NZB/NZW mice. It may be suggested that an understanding of the mechanisms of disease modification, especially that observed with LCM and LDV infection would lead to a better understanding of the autoimmune process and provide some insight for a more rational approach to therapy.

9. Pathogenesis

Although much is known about genetic, viral and immunologic factors in New Zealand mouse disease, the way in which these influences inter-relate

Table 5. Pathogenetic factors in autoimmunity

Factor	New Zealand mice	Human Lupus Erythematosis
Genetic	Multiple autosomal genes	Family and twin studies; HL-A types
Viral	C-type leukemia particles Antibodies to viral agent	Virus-like cytoplasmic inclusions Antibodies to viral antigens
Immunologic	(Proven) Defect in T and B cells	(Possible)

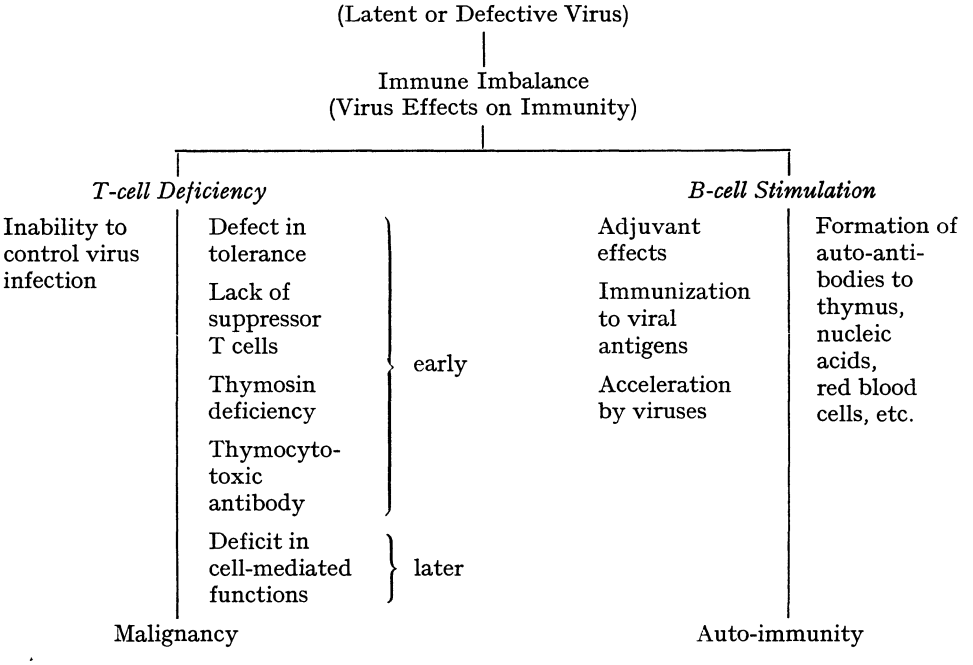
is still to be discovered. The problem is extremely complex because there are so many opportunities for mutual influences that primary processes are difficult to determine. Table 5 lists the major evidence that these three factors are involved both in New Zealand mouse disease and in human lupus erythematosus. The genetic studies in the mice have already been reviewed. In man, the role of genetic factors is less clear cut. Lupus has been reported in multiple members of a single family and in about twelve pairs of monozygotic twins (LEONHARDT, 1967). Leukocyte typing studies indicate that there is a statistically greater likelihood of developing lupus if certain major histocompatibility antigens (HL-A) are present (GRUMET et al., 1971). The explanation for these observations is uncertain but they would seem to implicate genetic factors in pathogenesis.

New Zealand mice harbor a C-type virus particle easily demonstrated by electron microscopy. In addition, they make antibodies to Gross viral antigens and deposit some of these as immune complexes in the glomeruli (MELLORS et al., 1969). Whether this virus is the common Gross leukemia agent found in all mouse strains and wild mice is a very crucial issue. Two studies suggest that the NZB virus is different from Gross virus in certain respects, particularly in its growth properties in tissue culture (LERNER et al., 1972; LEVY and PINCUS, 1970). If it is different from the Gross agent, as seems likely, how did these differences arise and are they in some primary way related to the unique form of autoimmune disease that develops in New Zealand mice?

A similar problem of virus complicity arises in human lupus. However, the problem there is even more difficult because there is no agreement that the particle is a virus.

A cytoplasmic inclusion consisting of tubular structures resembling myxoviruses is present in the endothelial cells of kidney and skin as well as in lymphocytes. The incidence in lupus renal biopsies is 80–90% in most series while the incidence in other chronic nephritides is generally under 25 % Similar "virus-like" inclusions have also been observed in human autoimmune diseases related to lupus but also in a wide variety of other unrelated disorders, albeit in much lower incidence. Some believe that the structures are not viruses but rather non-specific cellular responses to injury or to virus infection. The resolution of this problem must await definitive chemical or virologic characterization of the structures in question. Curiously, lupus patients also have high titers of serum antibody to many different viruses

Table 6



including myxoviruses and EB virus. These high titers have been ascribed to a general state of immunologic hyperactivity, since the levels correlate with absolute γ-globulin concentration.

The immunologic factors can be generalized as an imbalance between excessive B-cell activity and diminished T-cell activity. However, New Zealand mice are not the equivalent of neonatally thymectomized or congenitally athymic "nude" mice. Certain T-cell functions, like cell-mediated immunity, are generally intact until late in life. Moreover, the distribution of theta antigen in the peripheral lymphoid organs is likewise essentially normal in young mice. Never the less, the possibility that autoantibody-producing B-cell clones appear as a consequence of T-cell failure remains highly attractive. As already discussed, it depends upon the existence of suppressor T-cells which may be lacking in New Zealand mice early in life. At this same age, T-cell tolerance is difficult to induce and serum thymosin-like activity is declining prematurely. Other T-cell functions remain intact until later. Future investigation should attempt to characterize and directly measure this suppressor function in New Zealand mice.

This concept of pathogenesis is presented schematically in Table 6. As a consequence of genetic or viral factors, a state of immune imbalance arises with its consequent changes in T and B cell functions. The ultimate pathological consequences are lymphoid malignancy and auto-immunity. The genetic component could be due as well to a latent or defective virus integrated into the mouse genome and indistinguishable from it. The immune

imbalance could arise as a consequence of virus influence on lymphocytes. Indeed natural or experimental virus infections can stimulate or inhibit T and B cell functions and produce many of the immunologic abnormalities exhibited by New Zealand mice, including the resistance to immune tolerance (NOTKINS et al., 1970). Viruses can act as immunologic adjuvants or as antigens to stimulate or induce B cell responses. Many viruses (such as LCM or polyoma) accelerate autoimmunity in New Zealand mice. Furthermore, T-cells are important in host defense against viral infection and T-cell deficiency may lead to defective control of virus.

10. Treatment

A variety of therapeutic approaches are theoretically available for treating the autoimmune disease of New Zealand mice. Which therapeutic modality one chooses first depends to some extent on ones ideas regarding pathogenesis and etiology. If the disease is a genetic one, genetic "counciling" would be the first approach. This appears to have favorable results for F_1 hybrids of NZB mice with C_3H mice, and for almost all backcrosses of F_1 mice to non-autoimmune strains of mice. Other efforts would be directed at the individual animal who already has the genetic predisposition to the disease and has or has not yet manifested autoimmune phenomena. If the defect be an immunological deficiency, replacement therapy might be in order. Immunological hyperresponsiveness might be treated with a variety of immunosuppressive regimes. On the other hand, if the disease is thought to be due to a viral agent, therapeutic efforts would be directed primarily at the virus or the secondary effects of the virus. Finally, if it is thought that both genetic and environmental factors are important, some combination therapy might be tried.

Another approach to therapy is the use of New Zealand mice as animal models of human autoimmune diseases. To that way of thinking, therapy of autoimmune phenomena in the mice might merely be used as a testing ground for a variety of therapeutic modalities which might ultimately be applied to humans. Such studies might stress therapy which might easily be acceptable for human patients, such as immunosuppressive drugs; and minimize efforts at relatively impractical (at the present) modalities, such as cellular reconstitution and viral superinfection.

Several immunosuppressive and lymphocytic drugs have favorably altered the natural history of autoimmune disease in New Zealand mice. NZB mice have been treated with anti-lymphocyte globulin with suppression of the autoimmune hemolytic anemia; however, there was no favorable effect upon hyperglobulinemia, lymphoid infiltrates, and renal disease (DENMAN and RUSSELL, 1971). Corticosteroid therapy was also found to be effective for the autoimmune hemolytic anemia of NZB mice (CASEY and HOWIE, 1965). There was a reduction of spleen size and lymphoid tissue in the treated mice; however relapses occurred after drug therapy was stopped. Azathioprine and 6-mercaptopurine have not led to amelioration of autoimmune disease when

given in high dose; however they have been associated with the development of lymphoid malignancy, especially when given to young animals (CASEY, 1968a; CASEY, 1968b).

Both anti-lymphocyte serum (ALS) and anti-thymocyte serum (ATS) have been given to NZB/NZW F_1 mice. In the first studies (DENMAN and DENMAN, 1966; STROM et al., 1968) the ATS was found to accelerate disease. In one study there was increased mortality without increased severity of the renal histological changes (STROM et al., 1968). This result was attributed to acceleration of the renal disease (DENMAN and DENMAN, 1966). When more carefully investigated (DENMAN et al., 1970), it was found that the response to ATS depended upon the method of administration. Without prior induction of tolerance, acceleration of renal disease occurred, presumably due to the formation of antibodies to the heterologous serum proteins and the resultant additional immune complexes. The IgG fraction of the ALS did not reduce antinuclear antibodies nor the elevated IgM levels in these NZB/NZW mice.

The long term cyclophosphamide therapy was found by RUSSELL and HICKS (1968) to markedly reduce the incidence of lethal kidney disease in NZB/NZW mice. Cessation of therapy at the age of one year did not reduce the efficacy of therapy. Furthermore, even relatively short courses of therapy led to improvement in the renal disease of the hybrid mice (RUSSELL and HICKS, 1968). Corticosteroids and azathioprine have also been found to be effective in the treatment of the renal disease of the NZB/NZW mice (GELFAND and STEINBERG, 1972); corticosteroids and azathioprine (HAHN, 1972) have also been reported to be more effective than single drug therapy (HAHN, 1972). The combination of all three drugs—cyclophosphamide, azathioprine, and methylprednisolone was superior to all single and double drug regimes with regard to proteinuria, anti-DNA antibodies, renal histological changes, and survival (GELFAND et al., 1972). An attempt was made to improve upon drug therapy by the technique of drug-induced tolerance. Treatment with the synthetic double-stranded RNA, polyinosinic·polycytidylic acid plus cyclophosphamide led to reduced antibodies to nucleic acids (STEINBERG and TALAL, 1971) and a three week increase in survival (unpublished) as compared with cyclophosphamide treated controls.

High dose intermittent or single dose therapy is so effective that it is difficult to improve upon it. Tolerance induction has not been possible with other less effective immunosuppressive drugs nor have attempts at tolerance to DNA been successful. However, high dose intermittent cyclophosphamide therapy has been found to be superior to daily low dose therapy (STEINBERG et al., 1972).

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Nucleic Acid Reassociation as a Guide to Genetic Relatedness among Bacteria

RICHARD L. MOORE[1]

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I. Introduction

A substantial amount of effort has been devoted to the classification of bacteria by microbiologists of the past and the present and, no doubt, will be by those of the future. Studies on the morphology, structure and biochemical properties of bacteria have provided us with much useful information, but even with the advent of computer-aided taxonomy, there is no satisfactory natural classification for bacteria. Although the number of characteristics used, and the labor to obtain them is steadily increasing, it is still uncertain whether all are of equal value or whether some should be weighted more heavily than others. Fossil records of bacteria have been reported (DELEY, 1968; SWAIN, 1969) although their existence has been disputed by others (MYERS and MCCREADY, 1966). Whether or not fossil records exist, the relatively simple structure of bacteria would still limit the amount of information available for investigating the genealogy of bacteria. It has been recognized for some time that genetics might some day make a contribution toward the solution of this problem (ROPER, 1962). JONES and SNEATH (1970) have discussed the role of genetic transfer studies in bacterial taxonomy. Changes in polynucleotide and protein composition which accompany the evolution of organisms have been reviewed by MCCARTHY (1965; 1967) and others (Evolving Genes and Proteins, 1965; BRYSON and VOGEL, eds.).

If the genotype of a bacterium could be read directly from its DNA, the position of that bacterium relative to other living organisms could be readily

1 Division of Pathology, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada. T2N 1N4.

established. Although this cannot yet be accomplished, the overall similarity between the genotypes of different organisms can now be estimated from the degree of nucleotide sequence complementarity between their respective DNA. By this approach phenotypic similarities may also be estimated with the use of messenger RNA, since these molecules represent copies of the genes being expressed. However, examination of the actual characteristics of an organism by the more classic procedures will continue to be important in providing a wealth of useful information.

Present techniques for measuring nucleotide sequence similarities between nucleic acids originate from the work of Doty et al., (1960) and Marmur and Lane (1960). They demonstrated the separation of the two complementary strands of a DNA molecule and the specific reassociation of the two strands with the bases again in register. This work also stimulated the development of current techniques for determining bacterial genome sizes and the base compositions of their DNA.

Nucleic acid reassociation studies have been extremely useful in determining the phylogenetic relationships between various organisms. The tremendous impact which this approach and other advances in genetics and biochemistry have had on bacterial taxonomy during the past ten years is best demonstrated in recent reviews on the *Enterobacteriaceae* (Sanderson, 1971; Middleton and Mojica-a, 1971; and Brenner and Falkow, 1971) and the *Bdellovibrios* (Starr and Seidler, 1971). The scope of this article will be limited to nucleic acid reassociation studies, and the reader is referred to the above references and to previous reviews of modern approaches to bacterial taxonomy by Marmur et al., (1963a) and Mandel (1969).

II. DNA/DNA and DNA/RNA Reassociation

A. Methods

The first experiments using strand separation and reannealing to examine the similarity of base sequences between DNA of different bacteria were carried out by Schildkraut et al., (1961). Single-stranded DNA labeled with heavy isotopes was allowed to reassociate in solution with unlabeled DNA from a different source. Hybrid molecules were detected by their difference in buoyant density in CsCl gradients. This method was also used for the detection of RNA/DNA hybrids (Marmur et al., 1963b). An assay for measuring the rate and extent of complex formation between radiolabeled RNA and single-stranded DNA was later introduced by Nygaard and Hall (1963). This assay was based on the discovery that RNA/DNA complexes were retained by nitrocellulose membrane filters while free RNA was not bound. Thus, it was only necessary to allow the nucleic acids to react in solution, pass the solution through a filter and determine the amount of radiolabeled RNA/DNA complex bound by the filter. Although both these methods are useful in examination of the formation of heteroduplexes by nucleic acids, they have been largely replaced in studies of bacterial relatedness by a similar method

of reassociation in solution in which hydroxyapatite chromatography is used to separate the heteroduplexes that are formed from the unreacted single-stranded nucleic acids (BERNARDI, 1965; MIYAZAWA and THOMAS, 1965; BRENNER et al., 1969).

The first quantitative reassociation studies for determining bacterial DNA base sequence homologies were performed by MCCARTHY and BOLTON (1963) using the DNA-agar method (BOLTON and MCCARTHY, 1962). In this method single-stranded DNA is trapped in an agar gel and incubated with the radiolabeled, single-stranded DNA to be examined for base sequence similarity. After incubation, the DNA agar is washed to remove the unreacted DNA and then washed under conditions designed to remove the radiolabeled DNA fragments which were bound to the trapped DNA. Base sequence similarity between DNA and RNA can also be examined by means of this method. MCCARTHY and BOLTON (1963) showed that the degree of relatedness observed among bacteria from the family *Enterobacteriaceae* was essentially the same whether determined by DNA/DNA reassociation or from reactions between DNA and pulse-labeled RNA. This has also been observed in reassociation studies with other groups of bacteria (DELEY and PARK, 1966; MOORE and MCCARTHY, 1969; GIBBONS and GREGORY, 1972).

One of the most common techniques presently in use for assessing the degree of homology between nucleic acids for taxonomic purposes is the DNA-filter method of GILLESPIE and SPIEGELMAN (1965). This is similar to the DNA-agar method except that the disassociated strands of DNA are immobilized on nitrocellulose membrane filters instead of being trapped in an agar gel. The DNA filter is incubated in the reaction mixture containing radiolabeled RNA or DNA, washed, dried, and placed directly into scintillation fluid for measurement of the quantity of bound radiolabeled nucleic acid.

The reassociation of the complementary strands of a bacterial DNA follows second-order kinetics. The rate of reassociation is determined by the concentration of the DNA, genome size, the size of the fragments used for reassociation, base composition, and the temperature and salt concentration of the solution (MARMUR et al., 1963b; BRITTEN and KOHNE, 1968; WETMUR and DAVIDSON, 1968). Conditions which give the optimal rate of reaction are also necessary for maximum base pairing within the reassociated product, so these factors must be carefully considered when determining the similarity of base sequences among nucleic acids.

Information on the MW of a DNA can be obtained from the rate of reassociation of the complementary strands. The rate is commonly expressed as the $C_o t_{0.5}$, the concentration of DNA in moles of nucleotide per liter times the time in seconds required for 50% reassociation. BRITTEN and KOHNE (1968) showed that the rate of reassociation of bacterial or viral DNAs is directly proportional to the size of the genome. This relationship exists because the vast majority of the cistrons of a viral or bacterial DNA are composed of unique base sequences. This observation has made it possible to conveniently measure the genome size of a bacterium by comparing the rate of

reassociation of its DNA to the rate obtained with DNA from a bacterium with a known genome size such as *Escherichia coli*. This is readily accomplished with a recording spectrophotometer. The formation of double stranded complexes between complementary strands of the DNA is observed by the hypochromic shift. The rate of reassociation is determined by the rate of change in absorbance. The genome size for a number of bacteria has now been obtained by this method (MOORE and MCCARTHY, 1969; BAK et al., 1969, 1970; KINGSBURY, 1969; GILLIS et al., 1970; SEIDLER and MANDEL, 1971; BRENNER et al., 1972a; SEIDLER et al., 1972; MOORE and HIRSCH, 1973). The molecular weights of bacterial DNA determined by this method, range from 2.3×10^8 daltons for *Chlamydia trachomatis* (KINGSBURY, 1969) and 4.4×10^8 daltons for certain *Mycoplasma* species (BAK et al., 1969) to 3.7×10^9 daltons for *Serratia marcescens* (GILLIS et al., 1970). The results with this approach appear to be comparable to the results with chemical or electron microscopic methods. Findings in the author's laboratory agree with those of WETMUR and DAVIDSON (1968) and indicate the necessity for a correction of the $C_o t_{0.5}$ by 1.8% for every mole percent GC above 51% and −1.8% for every mole percent below this value (see SEIDLER and MANDEL, 1971 for discussion).

GILLIS et al., (1970) found that bacteria with limited enzymatic capabilities had relatively smaller genomes than other bacteria. This is in general agreement with the observation that the amount of DNA per haploid genome has increased during the evolution of simple organisms to the more complex higher organisms (MIRSKY and RIS, 1951; MCCARTHY, 1965, 1967; DELEY, 1968). Measurements of the genome sizes of *Mycoplasma* species by BAK et al., (1969) have provided convincing evidence that the sterol-requiring and human T strains examined by them cannot be closely related to the non-sterol requiring *Mycoplasma laidlawii* related strains since they have genomes of only half the size. The lack of base sequence similarity between the DNA of *Mycoplasma* species and that of bacterial L-forms (MCGEE et al., 1965, 1967; ROGUL et al., 1965), and the small size of the mycoplasma genome suggest these organisms belong to a separate bacterial group.

Renaturation rates may also be used to determine the degree of base sequence homology between DNAs. If two identical disassociated DNAs are allowed to reassociate under optimal conditions in the same incubation mixture, the reassociation rate of the mixture is expected to be the same as if they were incubated separately. If two DNAs with no base sequences in common are allowed to reassociate, the reassociation rate is expected to be equal to the sum of their rates when incubated separately. Thus, a mixture of two DNAs with partial base sequence similarity will exhibit a rate of reassociation somewhere in the range between their rate alone and the sum of their rates. Although this method is somewhat less sensitive than those described above, it is convenient. With the use of a spectrophotometer one may readily determine the DNA base composition, genome size and relatedness of other DNAs with a reasonably sized sample, by a similar technique and

without preparing radiolabeled DNA. In one study of this type, measurements of the reassociation rates for DNAs from extremely halophilic bacteria also helped to establish the non-episomal nature of their satellite DNA component (MOORE and MCCARTHY, 1969).

The spectrophotometric method has been discussed by DELEY et al., (1970) and SEIDLER and MANDEL (1971). Because of the effect of the size of the DNA fragments on the rate of reassociation, it is important in this method that the size of the fragments of the DNAs to be compared are the same. In addition, this method and the procedures mentioned above require DNA prepared from cells in the stationary growth phase in order to avoid an unequal distribution of certain regions of the DNA due to partial replication of the DNA molecules. SEIDLER and MANDEL (1971) find the DNA from exponential phase *E. coli* reassociates 6% faster than DNA from stationary phase cells. When the cells are grown in rich medium, an additional replication fork is formed on the DNA molecule and the difference increases to 15%.

Analysis of the reassociated product by examination of the thermal denaturation profile is very useful in providing additional information on the nature of the complex (MCCARTHY and BOLTON, 1964; BOLTON and MCCARTHY, 1964; MCCARTHY, 1967; JOHNSON and ORDAL, 1968). The reassociation experiments discussed above measure the quantity of DNA fragments or RNA molecules which undergo sufficient base pairing with the complementary sites of a DNA to form a stable complex under the conditions used for reassociation. The resistance to thermal denaturation of such a complex reflects the number and kinds of paired bases which exist in the complex. The heterogeneity of the fragments with respect to base pairing and base composition are reflected by the width of the thermal denaturation profile. In duplexes between nucleic acids with only partial homology of their base sequences, the $T_{m(e)}$ (the temperature at which 50% of the bound complex is eluted) of the thermal denaturation profile is always lower than that of the homologous complex. The difference between the $T_{m(e)}$ of the homologous complex and the heteroduplex ($\Delta T_{m(e)}$) is approximately 1.5°C per 1% of the unpaired bases which exist in the heteroduplex (BAUTZ and BAUTZ, 1964; LAIRD et al., 1969; ULLMAN and MCCARTHY, 1973). This relationship does not apply, however, if the chain length of the complex is less than that required for specificity, or if the salt and temperature conditions do not assure maximum registry of the base pairs.

B. Problems

One of the problems which influenced the results of some of the earlier studies was the failure to carry out reassociation experiments under the proper conditions of temperature and salt concentration. Meaningful results can be obtained at incubation temperatures 20°–30°C below the T_m (the temperature at the midpoint of the hyperchromic shift observed at a wavelength of 260 nm during thermal denaturation) of the native DNA in solutions containing 0.15 M to 0.33 M Na^+. The maximum rate of duplex formation and specificity

of base pairing occurs at this temperature. This is easily determined from the relationship between the overall base composition of a DNA and the T_m, and between salt concentration and T_m established by Marmur and Doty (1962). Because of the acceptance of DNA base composition as a taxonomic guide for microorganisms, the base compositions of a large number of strains have been reported in the literature of the past decade. This information is to be given for organisms listed in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (in press). Where the base composition of a DNA is unknown or the strain of bacteria uncertain, the first step of a nucleic acid reassociation study should be the determination of the base composition. Either the method of Marmur and Doty (1962) or Schildkraut et al., (1962) may be used, although the latter may be preferable because of its accuracy and the small sample size required. The best way to determine whether or not the conditions used for reassociation are correct for proper base pairing is to compare the $T_{m(e)}$ of reassociated DNA from the reference strain to be used with the T_m of the native DNA (Johnson and Ordal, 1968). These values usually differ by 0 to 2°C. Homologous RNA/DNA complex $T_{m(e)}$ are about 4°C less than the T_m of native DNA because of their lower stability.

A common problem which is encountered with the filter method is the background noise caused by non-specific binding. Much of this is avoided by using highly purified nucleic acid preparations and sufficiently stringent conditions for reassociation. Methods have been proposed for lowering the level of non-specific binding by pretreatment of the filters with a solution of albumin, polyvinylpyrrolidone and Ficoll (Denhardt, 1966), by carrying out reassociation in solutions containing dimethylsulfoxide (Legault-Démare et al., 1967) or formamide (Gillespie and Gillespie, 1971), by washing the filter-bound complexes after incubation with a solution of high pH (Warnaar and Cohen, 1966) or by treatment with enzymes after incubation (Yankofsky and Spiegelman, 1962a; Sutton, 1971). However, if enzyme treatment is employed, the bound DNA or RNA may be altered and no longer suitable for analysis by thermal denaturation experiments (McCarthy and Church, 1970).

The reacting DNA is normally sheared to single stranded fragments between 100,000 to 300,000 daltons by sonication or passage through a French pressure cell and RNA prepared by the usual procedures is also generally of this size. This increases the sensitivity of the direct binding experiment since the base sequence homology with shorter stretches of DNA is being examined. However, since about 15 base pairs are required for the specificity of the reaction at the optimal temperature for reassociation (Niyogi and Thomas, 1967; McConaughy and McCarthy, 1967; McCarthy, 1967), the presence of fragments smaller than this in the nucleic acid preparations will decrease the sensitivity and may add to the problem of background noise.

Elution of immobilized DNA from agar or filter preparations is also a common problem, particularly at higher temperatures of incubation. Formamide can be used to reduce the temperature required for optimal conditions of reassociation (Bonner et al., 1967; McConaughy et al., 1969; Gil-

LESPIE, 1971). The added advantage of this is in greatly reducing hydrolysis of RNA when very long periods of incubation are required, although this is rarely necessary in studies involving microorganisms. The use of high molecular weight DNA in making agar or filter preparations also helps to reduce elution during incubation. The immobilization of DNA on filters requires particular care in ensuring complete denaturation of the DNA, the purity of the DNA preparation and the use of filters previously tested for their ability to retain the immobilized DNA. The greater tendency of bacterial DNA to bind to nitrocellulose filters compared with the DNA from higher organisms may be due to the difficulty in obtaining highly purified high MW preparations of DNA from higher organisms and the greater tendency for certain redundant base sequences of these DNAs to undergo rapid reassociation.

In our laboratory, DNA-filter preparations are incubated for 2 hours in the solution of DENHARDT (1966) at the salt concentration and temperature to be used for reassociation, allowed to drain on filter paper and placed in the reaction mixture. This short incubation probably removes most of the DNA which would be eluted during incubation as well as serving to block sites on the filter which undergo non-specific binding. We found that the amount of DNA eluted from the filter during 16 hours of incubation in 0.30 M NaCl and 0.03 M Na citrate at 66°C is rarely more than 1–10%. Increases in temperature, however, greatly increase this amount (MOORE and MCCARTHY, 1967). In any case, the amount of DNA bound to the filter before and after incubation may be determined by the use of radiolabeled DNA or by eluting the DNA from the filter with 0.5 N $HC1O_4$ at 75°C for 15 minutes and reading the A_{260nm} of the solution against a blank from a control filter (CHURCH, 1972).

Experiments of the competition type where non-labeled RNA or DNA is used to compete with the binding of radiolabeled RNA or DNA to homologous DNA are less affected by loss of DNA from the filters than direct binding measurements. Since only one radiolabeled DNA or RNA is required, this is a very convenient way to compare the degree of base sequence homology of a large number of different bacterial DNAs to a single reference species.

Radiolabeled nucleic acid preparations with an adequate specific activity for reassociation experiments are difficult to prepare from a number of bacteria because of the dilution of the radioisotope by components of the complex media required for their growth. However, this problem can be circumvented with procedures currently available for *in situ* radiolabeling of nucleic acids (SMITH et al., 1971; COMMERFORD, 1971; GETZ et al., 1972; SCHERBERG and REFETOFF, 1973).

Extrachromosomal elements, such as R-factors, F-factors and bacteriophages are an important component of the bacterial gene pool and are potential sources of difficulty in reassociation experiments. These elements are related to one another and to the host chromosome to various extents (FALKOW et al., 1969; BRENNER and FALKOW, 1971). If DNA for base sequence similarity studies is prepared from cells containing a significant quantity of extrachromosomal DNA, the results will not be reliable. BRENNER and FALKOW (1971) emphasize

the importance of examining the various bacterial strains to be used in such studies for the presence of extrachromosomal elements.

Further details on the methods and problems in studies on the reassociation of nucleic acids may be found in the investigations of the various bacterial groups listed in Table 1 and in reviews by Mandel, 1969; McCarthy and Church, 1970; Kennell, 1971; Brenner and Falkow, 1971 and Church, 1972.

III. Evolution of the Ribosomal Cistrons

The discussion of the previous sections is concerned with the use of nucleic acid reassociation studies in making generalized comparisons between the genetic complements of different bacteria. The ideal situation, of course, is to know the degree of similarity between individual genes of various organisms. Some genes are lacking entirely between different strains and others differ to various extents. Such permutations establish the taxonomic level to which the information on a given gene is most usefully applied. However, comparisons have been made among only a few genes and even on these the information is still extremely limited.

The most intensively investigated region of the bacterial chromosome is that portion responsible for the production of ribosomal RNA (r-RNA). Yankofsky and Spiegelman (1962a, 1962b) demonstrated the ability of r-RNA to undergo specific base pairing with complementary sites on the DNA molecule. The sites for the 16S and 23S classes of r-RNA were found to be distinct from one another as a result of the inability of one size class to compete for binding sites of the other and by the requirement for both size classes to be present in order to completely saturate the available sites of the DNA (Yankofsky and Spiegelman, 1963). A difference in the overall base composition of these two species was also noted (Attardi and Amaldi, 1970). An analogous situation exists with the 28S and 18S classes of r-RNA found in eukaryotic organisms. There is a loose clustering of the 23S and 16S r-RNA cistrons together with the small 5S class of r-RNA and transfer RNA (t-RNA) (Yankofsky and Spiegelman, 1962b; Oishi and Sueoka, 1965; Oishi et al., 1966; Cutler and Evans, 1967; Dubnau et al., 1965; Smith et al., 1968; Colli and Oishi, 1969; Colli et al., 1971; Doolittle and Pace, 1971). In general, the number of ribosomal cistrons is in proportion to the total amount of genetic information possessed by an organism. The number varies from zero in the viruses to several million in amphibians. Most bacterial DNAs examined so far contain 5–10 cistrons for each of the large r-RNAs. However, in *Mycoplasma* species (KID) there is only one cistron for each (Ryan and Morowitz, 1969). The base composition of r-RNA from bacteria is in the range of 54% GC and does not differ greatly from that of other organisms. There is no apparent connection between the base composition of r-RNA and the DNA of an organism. Together, these findings suggest that the base sequences of the ribosomal cistrons have a greater similarity among different organisms than the base sequences which make up the bulk

Table 1. A listing of various bacterial groups for which phylogenetic relationships between selected members have been determined by nucleic acid reassociation studies. (See text for explanation of methods.)

Bacterial group	Method	Reference
"Acetic acid bacteria"	in solution — buoyant density	De Ley and Friedman (1964)
"Actinomycetes"	filter	Farina and Bradley (1970)
"Actinomycetes"	agar	Yamaguchi (1967)
Aerococcus	filter	Shultes and Evans (1971)
Agrobacterium	filter	De Ley and Tijtgat (1970)
Bacillus	in solution — filtered	Doi and Igarashi (1965)
Bacillus	filter	Dubnau et al. (1965a)
Bacillus	agar	Takahashi et al. (1966)
Bacillus	agar	Takahashi and Saito (1968)
Bacillus	filter	Herndon and Bott (1969)
Bacillus	filter	Van der Plaat et al. (1969)
Bdellovibrio	filter, spectrophotometric	Seidler et al. (1972)
Bedsoniae	agar	Gerloff et al. (1966)
Bifidobacterium	filter	Scardovi et al. (1970)
Bifidobacterium	filter	Scardovi et al. (1971)
Brucella	agar	Hoyer and McCullough (1968a)
Brucella	filter	Hoyer and McCulllough (1968b)
Brucella	agar	McCullough (1968)
"Budding and prosthecate bacteria"	filter	Moore and Hirsch (1972)
Chlamydia	agar, filter	Kingsbury and Weiss (1968)
Chlamydia	filter	Weiss et al. (1970)
Clostridium	filter	Lee and Riemann (1970a)
Clostridium	filter	Lee and Riemann (1970b)
Clostridium	filter	Cummins and Johnson (1971)
Clostridium-Propionibacterium	filter	Johnson (1970)
Corynebacterium-Propionibacterium	filter	Johnson and Cummins (1972)
Enterobacteriaceae	in solution — bouyant density	Falkow et al. (1962)
Enterobacteriaceae	agar	McCarthy and Bolton (1963)
Enterobacteriaceae	agar	Brenner et al. (1967)
Enterobacteriaceae	agar, hydroxyapatite	Brenner and Cowie (1968)
Enterobacteriaceae	agar, filter, hydroxyapatite	Brenner et al. (1969)

Table 1 (continued)

Bacterial group	Method	Reference
Enterobacteriaceae	hydroxyapatite	BRENNER and FALKOW (1971)
Enterobacteriaceae	hydroxyapatite	BRENNER et al. (1972a)
Enterobacteriaceae	hydroxyapatite	BRENNER et al. (1972b)
Enterobacteriaceae	hydroxyapatite	BRENNER et al. (1973)
Enterobacteriaceae	hydroxyapatite	CROSA et al. (1973)
Enterobacteriaceae-"Myxobacteria"	filter	MOORE and MCCARTHY (1967)
Enterobacteriaceae-Bdellovibrio	spectrophotometric	SEIDLER and MANDEL (1971)
"Halophilic bacteria"	filter	MOORE and MCCARTHY (1969)
Haemophilus	filter	BOLING (1972)
Lactobacillus	in solution — filtered	MILLER et al. (1971)
Lactobacillus	hydroxyapatite	SIMONDS et al. (1971)
Leptospira	agar	HAAPALA et al. (1969)
Listeria	filter	STUART and WELSHIMER (1973)
Moraxella	filter	JOHNSON et al. (1970)
Mycobacterium	agar	GROSS and WAYNE (1970)
Mycobacterium-Nocardia	filter	BRADLEY (1973)
Mycoplasma	in solution — filtered	REICH et al. (1966a)
Mycoplasma	in solution — filtered	REICH et al. (1966b)
Mycoplasma	in solution — filtered	SOMERSON et al. (1966)
Mycoplasma	in solution — bouyant density	NEIMARK (1967)
Mycoplasma	agar	WALKER (1967)
Mycoplasma	filter	PETERSON and POLLOCK (1969)
Mycoplasma-"L-forms"	agar	MCGEE et al. (1965)
Mycoplasma-"L-forms"	agar	ROGUL et al. (1965)
Mycoplasma-"L-forms"	agar	MCGEE et al. (1967)
"Myxobacteria"	filter	JOHNSON and ORDAL (1968)
"Myxobacteria"	filter	JOHNSON and ORDAL (1969)
Neisseria	filter	KINGSBURY (1967)
Neisseria	filter, hydroxyapatite	KINGSBURY et al. (1969)
Neisseria	filter	WEISS et al. (1971)
Nocardia	filter	CLARK and BROWNELL (1972)
Nocardia-Streptomyces	agar	TEWFIK and BRADLEY (1967)
Pasteurella	agar	RITTER and GERLOFF (1966)

Table 1 (continued)

Bacterial group	Method	Reference
Pseudomonas	agar	De Ley and Park (1966a)
Pseudomonas	agar	De Ley et al. (1966a)
Pseudomonas	filter	Ballard et al. (1970)
Pseudomonas	filter	Palleroni et al. (1970)
Pseudomonas	agar, filter	Rogul et al. (1970)
Pseudomonas	filter	Palleroni and Doudoroff (1971)
Pseudomonas	filter	Palleroni et al. (1972)
Pseudomonas	filter	Ralston et al. (1972)
Pseudomonas	filter	Pecknold and Grogan (1973)
Pseudomonas	filter	Ralston et al. (1973)
Rhizobium	agar	Kern (1968)
Rhizobium-Agrobacterium	filter, spectrophotometric	Gibbins and Gregory (1972)
Rhizobium-Agrobacterium-Chromobacterium	agar	Heberlein et al. (1967)
Streptococcus	in solution — filtered	Weissman et al. (1966)
Streptococcus	filter	Mehta and Hutchinson (1970)
Streptococcus	filter	Coykendall (1971)
Streptomyces	filter	Monson et al. (1969)
Streptomyces	filter	Okanishi and Gregory (1970)
Vibrio	in solution — filtered	Basden et al. (1968)
Vibrio	agar	Hanaoka et al. (1969)
Vibrio	agar	Kiehn and Pacha (1969)
Vibrio	hydroxyapatite	Citarella and Colwell (1970)
Vibrio	filter	Anderson and Ordal (1972)
Vibrio	hydroxyapatite	Staley and Colwell (1973)
Xanthomonas	in solution — bouyant density, agar	Friedman and De Ley (1965)
Xanthomonas-Pseudomonas	agar	De Ley and Friedman (1965)
Xanthomonas-Pseudomonas	agar	De Ley et al. (1966b)
Xanthomonas-Pseudomonas	agar	Park and De Ley (1967)
Xanthomonas-Pseudomonas	filter	Mürata and Starr (1970)
Xanthomonas-Pseudomonas-Enterobacteriaceae	spectrophotometric, filter	De Ley et al. (1970)

of the DNA. Direct support for this was provided by the work of Doi and Igarashi (1965) and Dubnau et al., (1965a). They observed that the r-RNA of *Bacillus subtilis* was able to reassociate with DNA from other members

of the genus *Bacillus* to a greater extent than pulse-labeled *B. subtilis* RNA. A considerable, although louer amount of hybrid formation was even observed between *B. subtilis* r-RNA and DNA from bacteria too distantly related to undergo hybrid formation with pulse-labeled RNA. Investigations on the genus *Bacillus* have been extended by others (Takahasi et al., 1967; Takahashi and Saito, 1968; Pace and Campbell, 1971b). Among bacteria belonging to the family *Enterobacteriaceae*, the amount of hybrid formation between *Escherichia coli* r-RNA and the DNA of closely related species is almost as great as with *E. coli* DNA and a substantial amount of hybrid formation occurs with distantly related species of different genera. On the other hand, complex formation between *E. coli* DNA or pulse-labeled RNA and the DNA even of closely related species is significantly less than in the homologous reaction (Attardi et al., 1965; Moore and McCarthy, 1967; Kohne, 1968; Brenner et al., 1969). Similar results are observed among strains of myxobacteria (Moore and McCarthy, 1967), *Desulfovibrio* (Pace and Campbell, 1971a), *Clostridium* (J. Johnson, personal communication), *Bdellovibrio* (Seidler et al., 1972), *Moraxella* (Johnson et al., 1970) and extremely halophilic bacteria (Moore and McCarthy, 1969). This has also been demonstrated in eucaryotic microorganisms as distantly related as bacteria, protoza, fungi, plants and animals (Bendich and McCarthy, 1970). However, the degree of similarity between the ribosomal cistrons of procaryotic and eucaryotic organisms is much less than among organisms of the same cell type. The evolutionary discontinuity between procaryotes and eucaryotes which this suggests is consistent with the observed differences in the base composition and size of the r-RNA, the size of the ribosomal particles, the differential sensitivity of their ribosomes to various antibiotics and the specific enzymes required for protein synthesis (Attardi and Amaldi, 1970; Pestka, 1971; Ciferri and Parisi, 1970). Similar information has been used to support the theory that the chloroplasts of plants originate from blue-green algae. Further support was provided by reassociation experiments between the chloroplast DNA of *Euglena gracilis* and r-RNA from blue-green algae and photosynthetic bacteria (Pigott and Carr, 1972). A considerable degree of hybrid formation was obtained with blue-green alga r-RNA and detectable amounts with r-RNA from photosynthetic bacteria.

Because of this evolutionary conservatism, reassociation studies with r-RNA are potentially very useful in determining taxonomic relationships among distantly related bacteria. However, when the amount of hybrid formation with r-RNA from a reference strain is measured by direct binding, some bacterial DNAs of a group often give much higher values than expected. For example, the DNA base compositions of *Proteus vulgaris* and *E. coli* differ by 13% GC, and there is no apparent base sequence homology between them. Yet the amount of *E. coli* 23S r-RNA which forms complexes with *P. vulgaris* DNA is greater than with the DNA of the more closely related species, *Enterobacter aerogenes*, and even slightly larger than the homologous reaction with *E. coli* DNA (Moore and McCarthy, 1967). The reasons for

this are not yet entirely clear. A difference in the number of ribosomal cistrons present in various DNAs could help to explain observations of this kind. This has been demonstrated in at least one case involving ribosomal cistron comparisons among plants (MATSUDA and SIEGEL, 1967). Almost 12 times the amount of hybrid was formed between tobacco r-RNA and pumpkin DNA than between tobacco r-RNA and tobacco DNA because of the larger number of ribosomal cistrons in pumpkin DNA. It would be very useful in bacterial r-RNA/DNA reassociation studies to have more complete information on the number of ribosomal cistrons present in various bacteria. Base sequence differences between individual ribosomal cistrons of the same genome could also affect the results of direct binding measurements. The existence of heterogeneity among the ribosomal cistrons is still uncertain, however. This possibility has been discussed by DOI and IGARASHI (1966), MOORE and MCCARTHY (1967, 1968), NOMURA (1970), ATTARDI and AMALDI (1970) and BIRNSTIEL et al., (1971).

Fortunately, the r-RNA/DNA hybrids obtained by direct binding experiments can be further analyzed by thermal disassociation to gain an approximate measure of the degree of base-pairing which exists within them. It was previously shown that the thermal stability of pulse-labeled RNA/DNA hybrids decreased sharply with the divergence of the bacterial strains and rougly in parallel to the decrease in the amount of hybrid formed. The thermal stability of r-RNA/DNA hybrids also decreased with strain divergence, but much more slowly and not necessarily in proportion to the amount of hybrid formed (MOORE and MCCARTHY, 1967). BENDICH and MCCARTHY (1970) examined the thermal stability of hybrids between pea 25S or 16S r-RNA and DNAs from a wide variety of very distantly related organisms. They observed that the thermal stabilities of the 25S hybrids were increasingly less stable than 16S hybrids as the degree of an organism's relatedness to pea became less. Similar results were obtained with the 23S species of *E. coli* r-RNA.

The usefulness of this approach was also demonstrated in experiments concerned with the taxonomic relationships between distantly related bacteria with different salt requirements for their growth (MOORE and MCCARTHY, 1969). A direct correlation was found between the salt concentration required for the growth of a particular strain and the thermal stability of the hybrid between the DNA of this strain and 23S r-RNA from the rod-shaped, extremely halophilic reference bacterium. The extent of ribosomal cistron similarity correlated well with their relatedness obtained by a taxonomic scheme based on their salt requirements (LARSEN, 1962).

The use of the competition type of experiment for ribosomal cistron comparisons is often preferred to direct binding measurements. JOHNSON et al., (1970) used this method to demonstrate the distant relationship between the oxidase-negative *(Acinetobacter)* and oxidase-positive moraxellas. Little or no nucleotide sequence similarity was detected between these two groups by DNA/DNA reassociation, while 66–69% homology was observed between the ribosomal cistrons. SEIDLER et al., (1972) used r-RNA competition measure-

ments together with other techniques to demonstrate the difference between *Bdellovibrio bacteriovorus* strains and the new species which they proposed, *B. starrii*. Their work also suggested a distant relationship between the bdellovibrios and vibrios. Pace and Campbell (1971 b) examined the ribosomal cistron base sequence similarities between distantly related bacteria which had different temperature requirements for growth. Previous studies had shown a correlation existed between the maximum temperature for the growth of a particular bacterium, the thermal stability of the ribosomes themselves and the overall base composition of its r-RNA (Pace and Campbell, 1967). This suggested a possible relationship between the base sequence of a r-RNA and the maximum temperature for growth of the cell. The r-RNA from various bacteria was examined for the ability to interfere with the reassociation between r-RNA and DNA of the thermophilic *Bacillus stearothermophilus* or the mesophilic *E. coli*. In contrast to the correspondence observed between the relatedness of the ribosomal cistrons and similarities between the salt requirements of various bacteria, no correlation was observed between similarities in their temperature requirements and the degree of base sequence homology of their ribosomal cistrons. This is the expected result if the heat stability of ribosomes is due to their overall structure and not the r-RNA alone. This is supported by the work of Altenberg and Saunders (1971) who demonstrated that the ribosomal subunit from a thermophilic bacterium can influence the structure of the subunit from a mesophilic bacterium and increase the heat stability of the functional unit.

In both *E. coli* and human KB cells, 5S r-RNA has a length of 120 nucleotides and there are regions of nucleotide sequence homology between them (Brownlee et al., 1968; Forget and Weissman, 1967). A comparative study among bacteria of this molecule has not yet been done. This could be particularly fruitful because its comparatively small size makes direct chemical analysis of the nucleotide sequence more feasible.

All of the above studies indicate that the base sequences of the ribosomal cistrons have been more stable to change during evolution than the majority of other genes. This should allow alterations in the base sequences of some of the ribosomal cistrons without severely affecting the cell. The evolutionary conservatism of the ribosomal cistrons implies that there is a severe restriction placed on such change. Presumably this restriction arises from the complex structure-function relationships required for the ribosome to carry out its key role in protein synthesis. Better knowledge of the reason for the evolutionary stability of the ribosomal cistrons would increase our present understanding of the mechanisms of protein synthesis as well as providing a more solid basis for its use in taxonomy.

IV. Evolution of Other Regions of the Chromosome

Goodman and Rich (1962) examined the nucleotide sequence similarities of transfer RNA (t-RNA) among species of *Enterobacteriaceae*. These studies

were continued on this group of bacteria by BRENNER et al., (1970) and extended earlier to species of the genus *Bacillus* by DUBNAU et al., (1965 a). The findings of these investigations indicate that the nucleotide sequences of the t-RNA cistrons show at least as much conservatism as the r-RNA cistrons. This is not surprising, since, like r-RNA, the size and base compositions of t-RNAs from a wide variety of organisms show a high degree of similarity (MCCARTHY, 1965). Further investigations on this limited region of the chromosome can be expected to provide useful taxonomic information in the future.

During their studies on t-RNA cistron similarity among *Bacillus* species, DUBNAU et al., (1965 a) also examined the inter-specific transformability of various genetic markers. They found that the number of transformants for the nutritional markers, tryptophan, leucine, adenine and methionine rapidly declined with the divergence of a species from the reference strain. Transformation frequencies corresponded well to the amount of cross reaction with pulse-labeled RNA from the reference strain, suggesting that these genes have evolved at approximately the same rate as the majority of other genes. In contrast, the transformability of genetic markers for resistance to erythromycin, streptomycin and micrococcin was much greater among the various strains and more closely correlated to the results obtained by t-RNA/DNA and r-RNA/DNA reassociation. Similar results were obtained by CHILTON and MCCARTHY (1969) during an investigation to determine the effect of map position on the rate of evolution of DNA base sequences in species of *Bacillus*. Results from transformation and DNA homology experiments revealed areas of genetic conservatism located near the loci for r-RNA. The degree of conservatism decreased with increasing distance from these loci.

Genetic tests using transformation and conjugation are of great interest and should be one of the most important guides in taxonomy. However, idiosyncrasies in the application of such methods, suggest that their greatest current usefulness in taxonomy may be in the calibration of methods of more general application (MARMUR et al., 1963 a; MANDEL, 1969).

The extent of reassociation between partially purified *E. coli* lactose operon messenger RNA and the DNA of selected strains of *Enterobacteriaceae* was investigated by BRENNER et al., (1969). The relative amount of cross-reaction was generally the same as that with bulk *E. coli* messenger RNA. However, the greater cross-reaction of lactose messenger RNA shown by two of the *Salmonella* species is of considerable interest. No hybrid formation was observed with *Proteus mirabilis*, *Proteus morganii* or *Serratia marcescens* DNA. Similar experiments were reported by DENNY and YANKOFSKY (1972) with partially purified *E. coli* tryptophan messenger RNA. A considerable amount of hybrid formation was observed with DNA of other *Enterobacteriaceae*. Thermal stability measurements of the hybrids suggested that these genes have a high degree of base sequence complementarity in this group of bacteria.

An alternative approach which can be used for examining individual genes or small groups of linked genes depends on their transfer by genetic means

into a bacterium with little or no similarity in nucleotide sequence to the DNA to be used for comparison. Preliminary studies of this kind with *Salmonella-Escherichia* genetic hybrids and *P. mirabilis* carrying the *E. coli* F-lac$^+$ plasmid have been reported (Brenner et al., 1969).

V. Conclusions

Abundant use has been made of DNA/DNA and DNA/RNA reassociation in bacterial taxonomy since the development of these techniques a few years ago. Determination of the genetic relatedness of new bacterial isolates to other bacterial strains by this approach is becoming more and more routine. The groups of bacteria in which nucleotide sequence homologies have been investigated are listed in Table 1 together with the methods used for reassociation. In comparing the results obtained by different investigators, it must be remembered that the amount of complex formation between nucleic acids is dependent on the conditions of reassociation. The temperature and salt concentration must therefore be specified for proper interpretation of relative binding values. At the present time, there unfortunately are no established criteria for directly converting nucleotide sequence information to speciation.

If proper attention is paid to the incubation criteria, small differences in nucleotide sequences can be detected between the DNA of very closely related bacteria, such as different strains of *E. coli* (Brenner et al., 1972a). However, other approaches are most useful for determining relationships between distantly related bacteria. Comparison of the overall base composition of DNAs is now a well accepted procedure for determining whether or not two bacteria may be related. A method for predicting the maximum nucleotide sequence homology between two DNAs from their respective base compositions has been described by de Ley (1969). The predicted values can be adjusted to include the effects of differences in genome size. The greater ease with which genome size determinations can now be made should encourage their more frequent use in assessing relatedness between bacteria. Relationships between distantly related groups of bacteria may also be determined from reassociation studies employing limited regions of the chromosome or their RNA products, such as t-RNA or r-RNA. Further studies on the nucleotide sequence similarities of other restricted regions of the chromosome would also be valuable in understanding the evolution of the bacterial chromosome.

The various kinds of information which can be obtained from the reassociation of bacterial nucleic acids should be of tremendous interest to future bacterial taxonomists, both by posing new questions and providing answers to old ones.

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