

THE CORRELATION EFFECT FOR A HISTIDINE LOCUS OF NEUROSPORA CRASSA¹

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FOR different organisms rare wild type segregants have been observed in crosses involving independently isolated mutants which all show the same phenotypic effect. In all cases in which markers on one or both sides of these mutational sites were present the segregants which are wild type with respect to the isolocal³ markers are associated with outside marker combinations which in the crossing over theory would be assigned to 1, 2, and even 3 crossovers between and outside the isolocal markers. The correlation between these "recombinations" is so high that the occurrence of wild type segregants cannot be explained by statistically independent crossovers. The first report of this kind was given by DEMEREC (1928) who described the phenomenon as reverse mutation associated with crossing over in heterozygotes of *Drosophila virilis*. That neither statistically independent crossovers nor clusters of (reciprocal) crossovers can explain the results has most clearly been shown for *Neurospora crassa* by M. B. MITCHELL (1955 a,b), who found that of four asci containing a wild type segregant for the isolocal markers none contained the reciprocal double mutant. Further results have been published for *Neurospora* by GILES (1955), ST. LAWRENCE (1956), and DE SERRES (1956), for *Aspergillus nidulans* by PRITCHARD (1955) and for phages by STREISINGER and FRANKLIN (1956). Irregular asci have also been found in yeast by LINDEGREN (1955) and ROMAN (1956).

In this situation several points of view can be or have been suggested. Agreement among all workers in the field seems now to exist for the statement that there occur regions of "intimate" (or "effective") pairing statistically distributed along the chromosome. In these regions events take place which result in the irregular segregation for the isolocal markers and the correlated appearance of the described 1, 2 and 3 fold "recombinants".

Concerning the events within such a region of intimate pairing two different attitudes to the problem can be stated.

The conversion theories

On the one hand one may use the (unproven) assumption of exactly reciprocal material exchanges (after duplication) as cause for the recombinations between more

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³ We shall call different mutations *isocal* if they cause the same phenotypic effect with respect to a given (for instance biochemical) test and if they are confined to the same small genetic region. This definition merely comprises the preliminary biochemical and genetic results which ordinarily are easy to obtain without referring to a particular model. It leaves open to further experimental specification whether the different mutations are identical or different, whether they give wild type segregants in two factor crosses or not, and whether they belong to the same cistron (BENZER 1956) or to different ones.

distant loci and suppose that the correlation effect is due to an additional phenomenon being observable only for isolocal or very close markers. This additional effect shall be called "*conversion*". A conversion, as here defined, could either be due to a "*directed mutation*" (LINDEGREN 1955), that is, a mutation of one strand under the influence of the homologous strand during pairing, or due to a *transfer*, that is, the exact duplication of a chromosomal piece with insertion into the homologous strand (MITCHELL 1955 a,b). In order to explain the frequent appearance of "3 fold recombinants" one has to assume, then, a high correlation between the occurrence of both events, the conversion and the reciprocal exchange. This correlation is supposed to come about because the two events have a high chance to occur simultaneously within the same region of intimate pairing.

Two alternatives may be considered concerning the chromosomal places at which the reciprocal material exchanges between any two of the four chromatids occur: In the *classical picture* exchanges can only occur *between* the biochemically functional units (genes). Therefore different occurrences (alleles) of the same gene can never recombine by such exchanges and wild type segregants can only be obtained by a directed back mutation. In contrast, a modern viewpoint supposes the gene to be a linear structure of a certain length within which exchanges can occur, these exchanges being more or less equally probable for each place of this chromosome region. This concept was strengthened by the results of BENZER (1955) who showed for *phages* that a large number of isolocal mutations belonging to the same cistron³ are separable by recombination, and can, in fact, be arranged in a linear map. For other organisms a linear structure of the gene has not been proven, although the complexity of adenine loci in *Neurospora crassa* (DE SERRES 1956) as well as in yeast (ROMAN 1956) are indications in this direction.

There are thus two ways within the conversion model in which wild type segregants can arise in crosses involving two different isolocal mutants. Either a conversion can give rise to the unreciprocal appearance of a wild type allele, or an exchange between the two isolocal markers (if the mutations occurred at different places of a linear map) causes the combined appearance of a wild type and a double mutant recombinant.—In the classical picture the results of crosses involving two isolocal mutants should usually show a different behavior according to whether the isolocal markers belong to the same or to two different genes. When they belong to the same gene only directed backmutations could give rise to wild type segregants and without further exchanges in the marked region the parental type of outside markers would be obtained. The frequent appearance of the recombinant type of outside markers can be understood only when both a directed backmutation and an exchange on the right or the left of the isolocal markers often occur together within the same region of intimate pairing. See figure 1. Because we might expect the exchanges to occur with about equal frequency on the right or the left side of the isolocal markers, the two recombinant types of outside markers should be about equally frequent. An order of the isolocal markers could then not be established. When the two isolocal markers belong to two different genes, reciprocal exchanges could also occur *between* the two markers. One recombinant type would therefore be more frequent than the other, thus allowing one to arrange the markers in linear order.

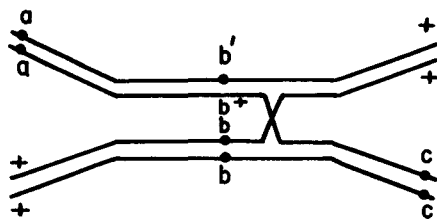


FIGURE 1.—Production of the type ab^+c in the classical picture of the directed mutation hypothesis by a “directed backmutation” of the b' allele and an additional reciprocal exchange.

If, in contrast to the classical picture, the “modern” standpoint is applied, in addition to conversions of one mutant, reciprocal exchanges would be expected to occur in crosses with nonidentical (or overlapping) isolocal markers, not only to the right or left of the markers but also between them, irrespective of whether they belong to the same gene or not. For marker distances for which the two mechanisms occurred with comparable frequency they could be detected in the same cross. However often one of the two limiting cases would be approached: For distant markers the classical effect of reciprocal exchanges would be predominant while on the contrary for very close markers the frequency of conversions (or directed mutation) could outweigh the occurrence of exchanges between the markers, resulting in an equal frequency of both recombinant types of outside markers.

The experimental evidence does not yet allow a decision. The 4-factor crosses of MITCHELL (1955 a,b) and GILES (1955) show in combination with wild type for the isolocal markers very unequal frequencies for the two recombinant types of outside markers. These results suggest in the conversion model that, at least for these markers, reciprocal exchanges can occur not only outside but also between the two isolocal markers. Yet these results cannot be taken as proof for the modern viewpoint because it has not been proven that the two markers in MITCHELL'S or GILES' case belong to the same gene. Another set of crosses has been reported by ST. LAWRENCE (1956) in some of which the two recombinant types of outside markers occur nearly equally frequent (excepting the crosses with the seemingly aberrant q^4 marker). Such results cannot be regarded as a proof for the classical picture either, because the two isolocal markers might be so close that exchanges between them are much rarer than a conversion of one of them.

We conclude that the correlation effect can be explained by assuming that within regions of intimate pairing the mechanisms of conversion occur *in addition to* reciprocal material exchanges between or within genes.

A generalized crossover (switch) theory

On the other hand, one may prefer an interpretation of the correlation effect in which the events taking place within a region of intimate pairing do not involve two distinct but only one mechanism. This attitude is influenced by recent observations with phages revealing a strong correlation between the appearance of 1, 2 and 3 fold “recombinants” within short regions of the genetic map (STREISINGER and FRANKLIN 1956; DOERMANN, CHASE and EDGAR, personal communication). On the one hand, it seems impossible to explain these results by statistically independent exchanges

alone or by conversions alone. On the other hand, it appears rather improbable that two different mechanisms, such as exchanges *and* conversion, should both be involved in the production of three-fold "recombinants" for such a simple DNA structure. In attempting the construction of a unified theory, we can also use the results in phages (BENZER 1955) which suggest that a recombination between two markers, belonging to the same cistron, can occur in a large number of places between the markers. In order to explain the correlated occurrence of 1, 2 and 3 fold "recombinants" and the irregular segregation by just one mechanism we are led to an hypothesis which we will call the *switch hypothesis* and formulate as follows:

Within a region of intimate pairing two new chromosomal strands duplicate partially along one parental strand, partially along the other, switching forth and back from one information source to the other. The points at which switching can occur are continuously distributed along the genome. In other words, the probability that a switch occurs within a region of intimate pairing is approximately the same for each point within the region.

Because of the observed unreciprocal production of "recombinants" (MITCHELL 1955 a,b) we suppose that switching is often unreciprocal within small dimensions, although markers outside the switch area segregate reciprocally. Because of the observed correlation effect we suppose that several switches (at least 3) may occur within a region of intimate pairing; the average number of them is yet undetermined.

To illustrate these notions for a specific case, consider the cross in figure 2. In such a cross an ascus may arise containing two P1, one P2 and one irregular segregant like P2 except that it is wild type for *b* and *b'*. (For example, ascus 2 and 4 in MITCHELL'S (1955a) case.) With the switch hypothesis this could be interpreted as seen in figure 3: One chromatid duplicates along one parent and because of the close pairing switches over to the other parent duplicating along there for a while (or incorporating an already duplicated piece) and returning then to its parent chromatid. The other "new" chromatid duplicates along the other parent and when it comes to the place that served as template for the first one, this has already separated off its piece so that nothing prevents the later arriving strand from duplicating along the same template.

This line of speculation then leads one to attempt to correlate the true crossovers with the abnormal segregation in a more direct manner than by saying merely that both are statistically favored by close pairing. On the contrary, one is led to the point of view that the irregular segregation displays the finer details of crossing over.

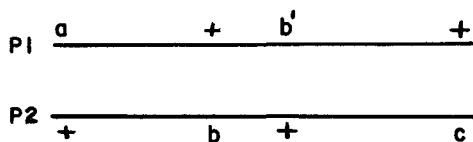


FIGURE 2.—A four factor cross involving two isoclonal markers *b* and *b'* and two outside markers *a* and *c*, as plotted in the modern picture.—Such a cross is for instance given in the paper of M. B. MITCHELL (1955a) where here stands

a for *pyr-1*
c for *co*

b for *pdxp*
b' for *pdx*

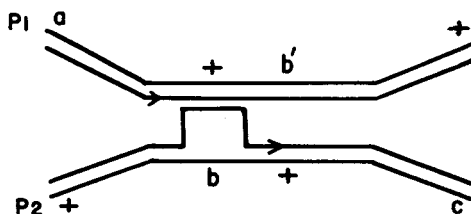


FIGURE 3.—A four factor cross resulting in a wild type segregant with respect to the isocal markers for one strand and in three parental strands. Explanation by the switch hypothesis: One chromatid duplicates along the P2 parent, switches near the b^+ marker by chance over to the P1 parent duplicating along there for a while (or incorporating a preformed piece) and then switches back to the P2 parent. The other chromatid duplicating a little later along P1 finds the part around b^+ vacant again and thus proceeds duplicating along P1. No recombination is observable for the outside markers in this tetrad.

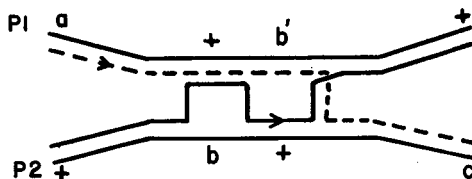


FIGURE 4.—Production of a three fold recombinant $++++$ according to the switch theory. One chromatid replicates along the P2 parent, switches three times and then proceeds replicating along the P1 parent. The other chromatid, coming somewhat later along P1, finds the P1 parent vacant also in the region of the marker b^+ , because the first chromatid has here already separated off. At a later point this chromatid finds the P1 parent occupied thus having only the P2 parent open for further replication. In this way the reciprocal appearance of the two recombinant types of more distant markers in a cross is obtained.

The general picture is this: In a region of close pairing (= crossover region) multiple switches take place, involving in any one region only two of the four strands (but the strands involved in different regions are not correlated). These switches may be and in general will be, nonreciprocal in detail, but they must be reciprocal at the ends of the region of intimate pairing, as illustrated in figure 4.

The switch hypothesis can explain the phenomena in small dimensions by just one mechanism, and it yields for larger marker distances the classical results of reciprocal crossovers: A region of intimate pairing containing an odd number of switches for each of two chromatids results in an observable "crossover", while an even number of switches in a chromatid is not detectable for classical marker distances. It is not yet clear whether a second mechanism, such as sister strand exchanges, is necessary for large dimensions to explain 4-strand crossing over, or whether the special nature of the duplicating molecule (DNA) makes it possible to understand by the same mechanism why randomly any two of the four strands are involved in any one region of intimate pairing.

It must be noted that the difference between the conversion hypothesis (once the notion of an order of isocal markers within a gene has been accepted) and the switch hypothesis is only slight because in both hypotheses extra duplicated pieces are transferred. A decision could be made by a statistically significant number of

ascus dissections in a cross involving two isocal and two close outside markers, because these theories differ in the *maximum* number of strands which can be involved in any one switch region. In the conversion hypothesis the conversion of a new strand should occur *during* the duplication and a "correlated" material exchange between any two of the four strands *after* the duplication. Therefore, *three* strands may be involved and those asci containing two strands recombinant for the outside markers should show a wild type segregant for the isocal markers as often in a strand which is recombinant for the outside markers as in one which is parental. In contrast, the switch hypothesis assumes that only *two* strands are involved in recombination within any one region of intimate pairing. Therefore in those asci containing two strands recombinant for the outside markers the wild type segregant with respect to the isocal markers should practically always be found in a strand having the recombinant type of outside markers. Some indication in this direction is given by the reported ascus dissections of M. B. MITCHELL (1956): in none of the four asci containing a wild type segregant for the isocal markers are more than two strands involved in the production of "recombinants".

In all considerations the possibility that isocal markers can be arranged linearly with respect to each other and with respect to outside markers plays an important role. This concept of order has not been verified experimentally except partially for phages (BENZER 1955; DOERMANN, personal communication) which may, but need not, involve the same recombination mechanism as other organisms. Therefore a study of three isocal histidine mutants was undertaken, using crosses between pairs of these mutants in all three combinations and employing three outside markers. It was hoped to obtain in this way also some more information as to whether the correlation effect is produced by a mutation like phenomenon or by the insertion of larger chromosomal pieces.

The marker order (GILES 1955), the recombination frequencies of the distant markers and the kind of crosses performed are clear from the scheme given in figure 5. Not only two, but three outside markers were used because this allows one to distinguish histidine independent recombinants from several other possible types: wild type contaminations, pseudowild types (still carrying the *hist* markers) and heterocaryons (for instance, also between a histidine independent recombinant and a *hist* marker containing parent type). If such types would appear very frequently, one should very frequently obtain a mycelium that is neither dependent on inositol (*inos*) nor on p-aminobenzoic acid (*pab*), while for the real histidine independent recombinants less than 1 in 100 spores should be of this independence (since the map distance between *inos* and *pab* is one unit).

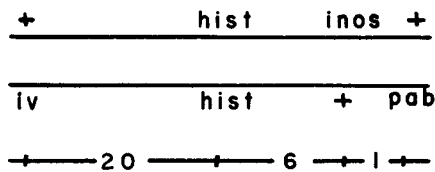


FIGURE 5.—Scheme of the crosses performed between pairs of *hist* markers and three outside markers. The three histidine markers are called *hist-1/1*, *hist-1/2*, *hist-1/3* or shortly 1, 2, 3. The properties and origins of all markers are given in table 1.

MATERIALS AND METHODS

The mutant strains were obtained from M. B. MITCHELL. The three histidine mutants (HAAS *et al.* 1952) should be isogenic, at least in the environment of the *hist* locus, because they were all produced from the same wild type. The origin and growth requirements of the different mutants can be seen from table 1. All mutants belong to linkage group V (BARRATT *et al.* 1954).

In order to obtain the different mating types or to eliminate an undesired growth behavior (e.g., microconidial fluffy) a mutant was crossed to wild p3178-2 a or wild p3177-4 A. The necessary double and triple mutants were obtained by crossing, plating the spores on very slightly supplemented medium, placing the smallest growing plants into growth tubes and later testing for biochemical deficiencies.

The heterocaryon tests between pairs of *hist* mutants were performed by putting very concentrated suspensions of conidia in water at one end of a "race" tube which was half filled with Fries minimal medium + agar (BEADLE and TATUM 1945) and containing either none or a limiting histidine supplement.

For crosses, supplemented WESTERGAARD-MITCHELL (1947) minimal medium was used in large tubes with filterpaper strips. Because all mutants containing the *iv* marker produced only a small number or no protoperithecia, always the *hist inos* double mutants were taken as protoperithecial parents in the final crosses. The cultures used for inoculation or fertilization were each time tested for their biochemical deficiency. For each cross another slant was taken and in this way a large number of independently prepared crosses obtained.

To select in the final crosses for the histidine independent recombinants, Fries medium supplemented with *iv*, *inos* and *pab* was used on plates. For plating the spores a soft agar layer technique was used in order to obtain an equal distribution of the spores over the plate and all spores in one plane, so that counting under the microscope was easy. The soft agar was the same supplemented Fries medium but with 0.5 percent agar. The spores were added to a bottle with liquid soft agar medium which was kept in a 45°C bath. The bottle was shaken strongly to separate all spores from each other and then onto each of as many plates as possible 2 ml of the liquid were pipetted and spread immediately by movements of the plate. The heavy spores

TABLE 1
The mutant strains and their properties

Marker	Isolation number	Growth on minimal medium	Mutagen used	Crosses grew best with the supplement, per 100 ml. of
<i>hist-1/1</i>	C91-R4	none	UV	20 mg histidine or more
<i>hist-1/2</i>	C84-R4	none	UV	same
<i>hist-1/3</i>	C85-R4	none	UV	10 mg histidine or more
<i>inos-t</i>	83201-s-5	grows in 25° none in 35°	X-ray	0.4 mg i-inositol
<i>iv</i>	39709	none	UV	30 mg L-isoleucine plus 40 mg L-valine
<i>pab</i>	1633	leaky	X-ray	4 γ p-aminobenzoic acid

The *iv* mutant grows slowly on supplemented medium; all other mutants grow like wild with the given amounts of supplement.

sink to the bottom of the soft agar layer before this can harden. Germination is induced by heating either the bottle before or the plates after the distribution of the soft agar for 40 minutes at 60°C. Spores containing the *hist* marker can just germinate in this medium, producing one or two very short hyphae. Only the histidine independent spores (and pseudowilds) can grow out further. At first we plated at most 10,000 spores per plate, scanned each plate after *ca* 20 hours growth at 25°C and picked the rare number of large growing plants which were all clearly originating from one spore. Biochemical tests showed that none of these plants was a wild or pseudowild type. Because neither with the heterocaryon test nor here had we observed heterocaryons, we had good reason to believe that the number of heterocaryons produced would at least be small when we altered the procedure by using somewhat more spores on a plate and the colony producing sorbose medium (supplemented Fries medium containing one percent sucrose and one percent sorbose) (see NEWMAYER 1954). In this sorbose medium the growth of the *hist* spores is as tiny as without sorbose while the larger growing plants produce colonies which can be seen with the bare eye after three to four days. Scanning some of these plates after two days in the microscope we observed that each little colony clearly arose from a single spore. Only then did we take more spores per plate. (The highest number was in one case 80,000!) This still gave usually none or one colony per plate, rarely more.

The hyphae of these colonies grow through the upper surface of the soft agar layer while the little hyphae of the *hist* deficient spores stay beneath. For the *hist* deficient spores no feeding by the colonies could be observed with the microscope. It can therefore be expected that the protruding hyphae of a colony are mostly free from *hist* deficient hyphae. The experimental results confirm this (see table 2). Thus these hyphae were picked and transferred into slants with Fries minimal medium supplemented with *iv*, *inos* and *pab*. After one or two days the plates were inspected once more. Occasionally one more colony was found on a formerly unopened plate. It was picked too and usually contained the *inos* or the *iv* marker.

Because of the large number of spores on the plates a special counting device was constructed which made it possible to count (with 36 fold magnification and a semi-dark field illumination) the number of germinated and ungerminated spores in four areas of given size, and to scan systematically the whole plate when this was necessary. Multiplying the counted number, which mostly was larger than 500, by a given factor gave a good estimate of the number of spores on one plate. The number of spores on all the other plates pipetted from the same bottle was then assumed to be equal. In a check the counting variation from plate to plate was less than 20 percent; but the concentration of the spores was slightly larger at the rim of the plate.

EXPERIMENTAL RESULTS

We first applied the heterocaryon test in order to see whether any of the three *hist* markers belong to different cistrons. In these tests no histidine independent heterocaryons have been found. This negative result shows that either no heterocaryons have been built at all or that the different histidine dependent nuclei within a heterocaryon cannot crossfeed each other.

For the final crosses the necessary double and triple mutants were produced and crossed in the described manner: *hist inos* × *iv hist pab*. In the crosses with the *iv hist-1/1* parent the *pab* marker was not present because lack of time had prevented its inclusion.

The spores of the final crosses were plated in the described manner on selective medium containing only *iv*, *inos* and *pab* supplements. Hyphae of the large growing plants were picked and tested for their biochemical deficiencies, and the numbers of germinated and not germinated spores were counted. The germination was generally larger than 70 percent.

The results of these crosses are summarized in table 2. The self-crosses show that spontaneous reversions of the *hist* markers are negligibly small. In all crosses with two different *hist* alleles histidine independent colonies occurred with a frequency of one to five in 100,000. The mycelia of the histidine independent colonies, when tested for the other biochemical deficiencies, proved to contain about equal frequencies of all four types of outside marker combinations (not considering now the *pab* marker). This shows that recombination between the outside markers is correlated with the appearance of *hist*⁺ segregants, similar to the results already published by the aforementioned authors. In our case, similar to that of ST. LAWRENCE, but in contrast to those of MITCHELL and of GILES, the inequality between the two recombinant types of outside markers is very small, the difference in our numbers not being significant. This large correlation effect can certainly not be explained by the ordinary crossover theory, because from two factor crosses the expected probability of obtaining in

TABLE 2
Result of the crosses

Cross	Total living in 10 ⁶	Hist. independent segregants	<i>iv inos</i> recombinant	++ recombinant	<i>inos</i> parent	<i>iv</i> parent	Wild type (contaminants?)
1 × 1	0.66	0	—	—	—	—	1
2 × 2	1.41	0	—	—	—	—	0
3 × 3	2.80	0	—	—	—	—	0
1 × 2	4.82	35	7	5	11	10	2
2 × 1	3.21	49	8	13	11	17	some of the plants counted as ++?
1 × 3	2.62	73	15	11	24	22	1
3 × 1	2.18	62	10	14	21	17	some of the plants counted as ++?
2 × 3	1.83	101	30	25	24	20	2
3 × 2	2.66	120	20	27	44	29	1

The first parent in the cross is always the *hist inos* parent, the second the *iv hist pab* parent. In the cross *hist-1/2 inos* × *iv hist-1/1* = 2 × 1 and the cross 3 × 1 the *pab* marker was not present. In the crosses containing the *pab* marker no type containing either the *pab* or the *inos* marker was found besides those counted as contaminants. Only in the cross *hist-1/3 inos* × *iv hist-1/2 pab* = 3 × 2 two types have been found containing both the *inos* and the *pab* marker. One of them (*inos pab*) was counted as *inos* parent, the other (*iv inos pab*) as *iv inos* recombinant. In the crosses containing the *pab* marker all the “++ recombinants” and the “*iv* parents” carry also the *pab* marker which for convenience was not written in the table.

addition to a crossover between two *hist* markers a crossover in the *iv. . hist* region would be 0.2 and in the *hist. . inos* region 0.06. Thus a triple recombinant should occur only in about 0.012 of all cases.

The biochemical tests showed that for crosses involving the *pab* marker none of the colonies contained hyphae which, carrying the *iv* marker, were independent of both inositol and p-aminobenzoic acid. Therefore the number of colonies containing a heterocaryon between histidine independent nuclei and nuclei containing one of the parent type of marker combinations seems in our case negligibly small.

The crosses containing the *pab* marker gave in a total of 329 histidine independent colonies altogether six colonies whose conidia behaved biochemically as wild type (two of them growing slightly better with than without inositol). Thus the number of wild type contaminants or heterocaryons was small and it can be expected that this number was also small in crosses with the *iv hist-1/1* parent (without *pab*). These six types were backcrossed to wild, the spores plated on minimal medium and incubated for 12 hours at 35°C. For four types all observed germinating spores gave wild type growth, which was also assured by testing some of them biochemically. The two types growing slightly better on inositol came from crosses between *hist-1/2* and *hist-1/3*. They evidently contained mostly inositol nuclei (besides wild types) because the ratio of inositol dependent and independent spores was 5:3 in one and 5:2 in the other case. It might be that originally *inos* colonies were contaminated with wild type after picking. In any case neither in the heterocaryon test nor in the crosses have wild type heterocaryons (or pseudowilds) between any two different *hist* markers been detected. Thus the three *hist* markers belong to the same cistron.

DISCUSSION

Since in crosses with different *hist* markers the frequency of histidine independent segregants is rather different, one is tempted to arrange linearly the isosomal markers with respect to each other (fig. 6). A monotonical increase of recombination frequency with marker distance, allowing an ordering of the markers, is expected from the modern version of the conversion model as well as from the switch model. In the classical picture, however, which assumes that chromatid exchanges can occur only between the genes, an order of three isosomal markers could be obtained only if each mutation belonged to a different gene. This seems improbable in our case because no heterocaryons have been found between any two *hist* markers and there is no biochemical evidence that this *hist* locus should consist of three biochemically functional units (genes). All three mutants are blocked in the production of the same enzyme. See Ames (1957).

In classical genetics it is postulated that the recombination frequencies are additive for marker distances that are not too long. This certainly cannot be expected to be true for markers whose distances are comparable to the average length of a region of intimate pairing since a wild type segregant can appear not only when this region lies between the markers, but also when it is overlapping one of them. Thus the recombination frequency is always expected to be a little larger than the "map distance" or, in other words, the sum of the two smaller recombination frequencies between three markers is expected to be larger than the largest recombination fre-

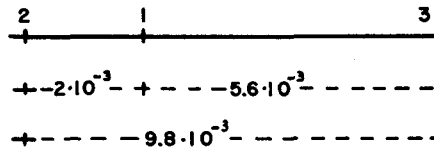


FIGURE 6.—Recombination frequencies between pairs of *hist* markers in percent. The values in reciprocal crosses differ only slightly in crosses 1×3 and 3×1 or in 2×3 and 3×2 , while in the cross 2×1 the recombination frequency is twice the one of cross 1×2 . These variations may be considered as a measure for the heterogeneity of the genetic background.

quency (except when one of the mutations involves an appreciable deletion). For our crosses the experimental error is too large to allow a significant statement about additivity.

The proposed possibility of arranging our three *hist* markers in a linear order can only be regarded as a further indication against the classical view but not as a proof. For it is well known from classical genetics that variations in the genetic background may give rise to recombination frequencies from which a wrong marker sequence is deduced. Because the used double and triple mutants are fairly isogenic such a wrong inference seems not probable in our case but an independent proof would be convincing. The ordinary kind of accurate test, three factor crosses, seems at present not feasible for *Neurospora*, using the isolocal markers alone, because it is very difficult to isolate a double mutant without a selective technique. However, it should be possible to determine the order of the isolocal markers from crosses involving one or two outside markers, although the correlation effect blots out somewhat the frequency differences. When only one outside marker is used, the correlation effect is mostly so pronounced that it is hard to obtain a statistically significant difference between the two types which are wild type segregants for the isolocal markers. Thus the ordinary three factor test should for isolocal markers be replaced by a four factor test involving two outside markers. This can be seen in the cases studied by MITCHELL (1955 a,b) and by GILES (1955) where the two recombinant types of outside markers occur with very different frequencies. For these cases it seems reasonable, considering that the mentioned possible models explain the correlation effect, to propose the following rule for determining the order of two isolocal markers with respect to the outside markers: The more complicated events within a region of intimate pairing give rise to the less frequent recombinant type of outside markers. This definition leads to the same marker sequence whichever of the proposed models is used. For MITCHELL'S (1955a) case one obtains, for instance, the order *pyr-1 pdx**p pdx co*. The same order should result from the "reciprocal" cross (in which just the two isolocal markers are interchanged). Indeed, this is the case in the crosses of MITCHELL and of GILES. The proof for the proposed rule should either come from its consistency in several crosses with different isolocal markers or by comparison with a three factor cross involving three isolocal markers.

In our experimental results, as in the crosses reported by ST. LAWRENCE (1956), the frequency difference of the recombinant types of outside markers is small, probably because the isolocal markers are very close to each other. Therefore, the difference in our numbers is not significant; but these numbers are in reciprocal crosses at

least compatible with the assumption that the markers 1 and 2 are closer to the *iv* locus than is marker 3. A convincing proof for the modern view point has in the meantime been given by the author, using the experimental results with a *pab* locus. (Freese 1957.)

In the introduction we raised the question of whether the frequent appearance of the parental type of outside markers in combination with wild type for the isolocal markers is produced by a "directed backmutation" or by an insertion of a larger chromosomal piece into the homologous chromatid (as in the transfer or the switch model). From our experiments the directed backmutation seems improbable: In this model the probability for the appearance of a directed backmutation should be a specific property of the mutated place alone (because our strains are isogenic at least in the neighborhood of this chromosomal part) and should at least not depend in a regular fashion on the distance of other markers. The frequency for the occurrence of a parent type of outside markers should then be approximately equal to the probability f_i that the marker *hist-1/i* has been back mutated to wild. Grouping together always reciprocal crosses with their proper statistical weight, we get for the different crosses values for the f_i which can be seen in table 3. There are striking differences in the estimate of the same f_i in two different crosses (especially for f_2). It therefore seems improbable that directed backmutations are the cause for the frequent appearance of parental types of outside markers, unless the intimate pairing is appreciably decreased in the neighborhood of a mutation. Such a decrease of pairing seems improbable as long as a mutation involves only a change of a small number of DNA nucleotides. In our case for instance the correlation effect extends over the whole distance between our most distant *hist* markers and therefore the pairing area must be at least as large as this distance. This involves a large number of DNA nucleotides and thus pairing should not be influenced appreciably by the change of just some of them. Therefore it seems very probable that larger chromosomal pieces, duplicated along one parent, can be inserted into the new chromatid duplicating along the other parent. This qualitative argument is not affected by the possibility of independent crossovers outside the *hist* region (the correction being small).

TABLE 3
Values of f_i in units 10^{-6}

Cross between:	1 and 2	1 and 3	2 and 3
f_1	3.5	8.7	—
f_2	2.6	—	11.8
f_3	—	9.1	14.3

f_i is the frequency of those histidine independent segregants which have the same combination of outside markers as the parent containing the *hist-1/i* (marker).

e.g. In the cross *hist-1/1 inos* × *iv hist-1/2 pab* f_1 is the probability to obtain *inos* and f_2 the probability to obtain *iv pab* among the total number of germinating spores. From table 2 one obtains the following weighted average of f_1 from the crosses 1 × 2 and 2 × 1:

$$f_1 = \frac{11 + 17}{4.8 + 3.2} \cdot 10^{-6} = 3.5 \cdot 10^{-6}$$

The foregoing considerations make it probable that either the transfer model (accepting the possibility of an order of isocal markers) or the switch model or something similar are the right explanations for the observed phenomena in small dimensions.

If the statement that chromosomal pieces are sometimes duplicated along one strand twice while not at all on the homologous strand should prove correct, a severe limitation would be placed on models trying to explain the duplication of chromosomes.

SUMMARY

A genetic study involving three closely linked histidine requiring mutants and three other linked markers has been undertaken. Histidine independent heterocaryons (or pseudowilds) between any two histidine mutants have not been found. Crosses between any two different histidine mutants yield rare histidine independent progeny while self-crosses do not. The recombination frequencies suggest an order for the three histidine markers with respect to each other. The histidine independent progeny contains parental and both recombinant types of outside markers with about equal frequency. These results cannot be explained by classical genetics. A reason has been given why the events occurring in small regions of intimate pairing and resulting in the observed correlation effect seem not to involve mutation-like phenomena but rather the insertion of chromosomal pieces, replicated along one parent, into the homologous chromatid. How the incorporation of this piece comes about is a question of more specific models, some of which have been mentioned in the introduction. A model-independent rule for ordering pairs of isocal markers with respect to the outside markers has been given which is similar to the rule of classical genetics.

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