LINKAGE AND FUNCTION IN NEUROSPORA[1]

S. R. GROSS[2] AND ADELE FEIN

Rockefeller Institute, New York, N.Y.

Received February 1, 1960

TIGHT linkage of genes controlling the synthesis of enzymes involved in related physiological reactions is prevalent in Salmonella and *Escherichia coli* (DEMEREC and HARTMAN 1959). Extensive linkage of this sort has not been reported in Aspergillus and Neurospora. Instead, most of the genes concerned with the reaction sequences studied in the enteric bacteria have been found to be distributed throughout the genome of the fungi. Departures from a completely random distribution of functionally related genes have been reported frequently (see PONTECORVO 1958, for review). However, if one excludes those cases which probably involve complex cistronic relationships relative to the synthesis of a single protein, few linked loci have been observed that are functionally related but distinct. Moreover, tight linkage of more than two distinct functionally related genes has, up to now, not been established in the fungi.

The question can then be asked whether clustering of functionally related genes is a result of a selective mechanism peculiar to the physiological activity of the enteric bacteria or whether the selective mechanism is universally operative but more subtly expressed in other organisms. In this report evidence is presented indicating that the genetic determinants of four enzymes involved in the synthesis of the precursors of the aromatic amino acids and *p*-aminobenzoic acid (PAB) in Neurospora are closely linked. Furthermore, some specific qualitative and quantitative changes in the enzymatic complement of the mutant strains will be discussed.

Specific interest in the linkage relationship of the genes controlling the synthesis of the enzymes involved in the synthesis of the aromatic ring arose as a consequence of an apparent paradox displayed by a mutant strain of *Neurospora crassa* (C161), obtained by METZENBERG and MITCHELL (1958), which did not utilize shikimic acid for growth as a sole replacement of the requirement for the aromatic amino acids and PAB. The genetic and biochemical evidence suggested that this mutant was unable to carry out a reaction prior to the synthesis of the hydroaromatic compounds that have been established as intermediates in the synthesis of the aromatic amino acids of *E. coli* (DAVIS 1955) and Neurospora (TATUM, GROSS, EHRENSVÄRD, and GARNJOBST 1954). Failure of this mutant strain to respond to shikimic acid for growth as distinct from another, Y7655, (TATUM *et al.* 1954) which does, suggested that either an absolute permeability

[1] This investigation was supported in part by Research Grant C1267 from The National Cancer Institute, Public Health Service, Bethesda, Md.

[2] Present address: Microbiology Dept., Duke University Medical Center, Durham, N.C.

barrier to shikimic acid was present in C161 or that the mutant strain was not capable of converting shikimic acid to the aromatic moiety of the amino acids as well as being blocked in a reaction prior to the synthesis of 5-dehydroquinic acid (DHQ), the first of the hydroaromatic precursors of shikimic acid (WEISS, DAVIS, and MINGIOLI 1953). The data presented below indicate that C161 is missing the entire enzymatic apparatus necessary to convert 2-keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate (KDHP) to at least one product of shikimic acid.

MATERIALS AND METHODS

Biological: Table 1 contains all pertinent information regarding the origin of mutant strains used in this investigation. The symbols *arg*, *arom*, *inos* and *pan* refer to gene loci that are involved in the synthesis of arginine, the aromatic amino acids and PAB, inositol, and pantothenic acid, respectively. Locus numbers were assigned to *arom* mutants in the order in which sufficient genetic and/or functional dissimilarities were observed. Since subsequent investigation has indicated that C161 probably is an aberration of some sort, *Ab* has been added to the locus designation (*arom-2*) assigned and the locus number reserved for the point mutation involving the first missing reaction in the metabolic sequence in strains bearing *arom-2 Ab*. The mutant strains will be referred to by their locus designation throughout.

The standard types used were 38-2a and 38-8A. These were obtained from SY7A and Y7655a (*arom-1*) after five successive sib crosses. Both *arom-2 Ab* and *arom-3* were backcrossed three times to the standard strains in order to obtain reasonable homogeneity and the proper heterocaryon compatibility background.

The growth requirements of the *arom* mutants were satisfied by the addition of 40 mg each of phenylalanine, tyrosine, tryptophan and 0.25 mg PAB per liter of VOGEL's synthetic medium (VOGEL 1956). Shikimic acid, 400 mg per liter was

TABLE 1

Mutant strains

Locus designation	Isolation number	Mutagen	Reference
arg-5	27947	X-ray	B*
arom-1	Y7655	N-Mustard	B*
arom-2 Ab	C161†	UV	METZENBERG and MITCHELL (1958)
arom-3	C163†	UV	METZENBERG and MITCHELL (1958)
bal: balloon	B56	UV	PERKINS (1960)
fl: fluffy	Lindegren	Spontaneous	B*
inos	89601†	N-Mustard	B*
pan-1	5531	X-ray	B*
pe: peach *pe*^{m}	Y8743m	Spontaneous?	B*

* B, BARRATT *et al.* (1954).
† Derivatives of the original isolates that would form heterocaryons with Stanford-Yale stocks were used.

added in place of the aromatic amino acids and PAB. The requirements for arginine and inositol were satisfied by the addition of 100 mg L-arginine and 10 mg of inositol per liter. Crosses of the *arom* mutants were found to be most fertile when the crossing medium was supplemented with 0.1 to 0.25 of the aromatic requirement. Random ascospore isolates were obtained by the sorbose plating method (NEWMEYER 1954).

Chemical: The strains used in the enzymatic analysis were grown in standing culture for three to four days in synthetic medium supplemented with the aromatic amino acids plus any other specific compound required for growth. The grinding and extraction procedure has been described (GROSS 1958). Generally, only a two- to four-hours extraction in 0.1 M Tris-HCl buffer *p*H 8.0 was employed.

In order to avoid error introduced by the conversion of DHS to PCA by 5-dehydroshikimic acid dehydrase (DHS dehydrase) and the endogenous TPN reduction exhibited by fresh extracts of Neurospora, measurements of the specific activity of 5-dehydroshikimic acid reductase (DHS reductase and 5-dehydroquinase) were done on 0 to 0.75 saturated $(NH_4)_2SO_4$ fractions after freezing and thawing (which inactivates more than 80 percent of the DHS dehydrase). 2-Keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate synthetase (KDHP synthetase) was measured in 0.4 to 0.55 $(NH_4)_2SO_4$ fractions dialyzed for 12 hours against M/30 *p*H 7.4 KPO_4 buffer with 10^{-4} M thioethanol. 5-Dehydroquinic acid synthetase (DHQ synthetase) was measured in similarly dialyzed 0.35 to 0.75 saturated $(NH_4)_2SO_4$ fractions. PCA oxidase and DHS dehydrase were measured directly in crude extracts.

In all cases where no enzyme activity was found, the entire crude extract as well as precipitates obtained by stepwise $(NH_4)_2SO_4$ fractionation (0.2 saturated $(NH_4)_2SO_4$ per step) were examined for activity.

DHS reductase was measured in a reaction mixture containing 1 μ mole shikimic acid, 10 μ moles triphosphopyridine nucleotide (TPN), 0.1 ml enzyme preparation in 2 cc 0.1 M Tris-HCl buffer *p*H 8.0. The initial reaction velocity was determined by the increase in optical density at 340 mμ (Σ_{340} TPNH $= 6.22 \times 10^3$) during the first five minutes of the reaction.

5-Dehydroquinase activity was measured by following the production of 5-dehydroshikimic acid (DHS) from DHQ (MITSUHASHI and DAVIS 1954). The reaction mixture contained 0.1 ml enzyme preparation plus 1 μ mole DHQ in 3 ml 0.1 M Tris-HCl *p*H 7.4. Because of end absorption of the crude $(NH_4)_2SO_4$ fraction, the reaction velocity was determined by measuring the increase in absorption at 240 mμ (DHS *p*H 7.4 $\Sigma_{240} = 9.7 \times 10^4$) instead of 234 m$\mu$.

The activity of PCA oxidase and DHS dehydrase were measured in 2 ml 0.1 M Tris-HCl *p*H 7.4 by the spectrophotometric and colorimetric procedures described previously (GROSS, GAFFORD, and TATUM 1956; GROSS 1958).

KDHP synthetase activity was measured by the procedure of SRINIVASAN and SPRINSON (1959). The synthesis of DHQ from KDHP was followed by measuring the disappearance of KDHP colorimetrically and the appearance of DHQ as determined by bioassay using an *arom* auxotroph of *Aerobacter aerogenes* A170-

143S1 (SRINIVASAN, KATAGIRI, and SPRINSON 1959). All bacterial strains mentioned in this paper were the generous gift of DR. B. D. DAVIS.

A unit of enzyme activity is defined as the amount of enzyme that converts 1 mμ mole substrate per minute under the assay conditions described. Protein concentrations were determined by the procedure of LOWRY, ROSEBROUGH, FARR, and RANDALL (1951).

DHS and DHQ were isolated from culture filtrates of mutant strains of *E. coli* by the procedures of SALAMON and DAVIS (1953) and WEISS, DAVIS, and MINGIOLI (1953). Cyclohexylammonium erythrose 4-phosphate dimethylacetal and the barium salt of 2-keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate (KDHP) were the generous gifts of DR. D. B. SPRINSON. Sodium erythrose-4-phosphate was prepared by the procedure of BALLOU, FISHER, and MCDONALD (1955). Barium KDHP was converted to the sodium salt before use. All other compounds used were obtained from commercial sources.

EXPERIMENTAL RESULTS

General physiological and genetic information regarding arom *mutants:* The mutant *arom-1* (Y7655) has been shown to be unable to convert DHS to shikimic acid (TATUM *et al.* 1954). It accumulates some DHS and large quantities of protocatechuic acid (PCA) in its growth medium. It will grow on shikimic acid as a sole replacement of its requirement for the aromatic amino acids and PAB. Its growth on shikimic acid, both in liquid culture and on agar, attains the rate of the standard strains only after an extensive lag period when sucrose or glucose is supplied as the carbon source. No lag in response to shikimic acid is observed when succinate or aspartate serve as the carbon source (MARY HURD, unpublished observations). This phenomenon closely resembles the glucose inhibition of the induced synthesis of β-galactosidase in *E. coli*, which has been interpreted as being the result of the inhibition of the synthesis of the permeability mechanism for the inducer (COHN 1956). The *arom-1* locus has been found to lie distal to *pe* (peach) and proximal to *fl* (fluffy) on linkage group II (GARNJOBST in BARRATT, NEWMEYER, PERKINS, and GARNJOBST 1954).

Strains bearing *arom-2 Ab* (C161) have been found not to accumulate any of the hydroaromatic precursors of the aromatic ring (METZENBERG and MITCHELL 1958). Taken at face value this implies that a reaction is blocked prior to the synthesis of DHQ. However, it was observed that shikimic acid does not replace the aromatic requirement of *arom-2 Ab*. We have confirmed this and have shown that it also does not respond to DHS or DHQ. Failure to respond to the presumed intermediates and the absence of accumulated compounds related to the hydroaromatic compounds suggested, among other possibilities, that a permeability barrier to hydroaromatic compounds exists in those strains with C (Cal. Tech) genetic background, and/or *arom-2 Ab* is a mutation that leads to the complete loss of the reaction sequence involved in the synthesis of the aromatic ring at least from a step prior to the synthesis of DHQ to a product of shikimic acid.

None of 400 segregants from three successive backcrosses of *arom-2 Ab* to the

standard strains grew on shikimic acid in place of the aromatic requirement. Hence, if a genetically controlled permeability barrier were involved, its determinant must be closely linked to *arom-2 Ab*.

The *arom-3* mutant (C163), unlike *arom-2 Ab* and *arom-1*, is comparatively "leaky" and has only a partial requirement for PAB. It does not respond to shikimic acid for growth. A careful examination of the products accumulated during growth using sensitive chromatographic and bioautographic procedures (for methods employed see TATUM *et al.* 1954) revealed the presence of shikimic acid, DHS, and large amounts of PCA. The accumulation of shikimic acid by *arom-3* apparently was missed by METZENBERG and MITCHELL (1958) because of the relatively small quantity accumulated and the relative insensitivity of the bioassay employed by them. The accumulation pattern observed conforms with that expected for a mutant blocked in a reaction subsequent to the production of shikimic acid.

The physiological behavior of the double mutant *arom-2 Ab arom-3* is identical with *arom-2 Ab*. The *arom-1 arom-3* double mutant secretes the same compounds as does *arom-1*, but like *arom-3*, fails to respond to shikimic acid. Since all attempted crosses of *arom-1* by *arom-2 Ab* have been sterile, the *arom-2 Ab arom-1* double mutant has not been synthesized.

Pairwise tests of functional complementation of the *arom* mutants employing the usual criterion of heterocaryotic growth on minimal medium revealed that combinations of *arom-1* plus *arom-3* and *arom-2 Ab* plus *arom-3* rapidly formed prototrophic heterocaryons while *arom-2 Ab* plus *arom-1* failed to complement each other. Combinations of *pan arom-1* plus *inos arom-2 Ab*, when tested on medium containing the full supplement of aromatic amino acids, grew successfully thus ruling out the possibility of the production of a specific inhibitor of growth in the *arom-2 Ab* plus *arom-1* heterocaryon.

The evidence thus far presented is consistent with the following conclusions: (1) At least one common functional process is impaired in *arom-1* and *arom-2 Ab*. (2) In addition to the disfunction held in common, *arom-2 Ab* is unable to synthesize DHQ and is also unable to convert shikimic acid to some intermediate subsequent to it in the synthesis of the aromatic ring. (3) The metabolic disfunction of *arom-3* involves a reaction subsequent to the synthesis of shikimic acid. This reaction can proceed in both *arom-1* and *arom-2 Ab*.

Genetic analysis of arom *mutants:* The apparent functional "allelism" of *arom-2 Ab* and *arom-1*, as determined by the analysis of complementation, suggested that these mutants should behave as alleles in the strict genetic sense. However, complete sterility of crosses of *arom-1* by *arom-2 Ab* prevented a direct genetic analysis. Instead, a comparative analysis of linkage to linkage group II markers was performed.

Segregants bearing *arom-2 Ab* are strongly selected against in most crosses. Furthermore, *arom-2 Ab* strains are morphologically distinct from the standard type—they are paler in color, and macroconidia production is frequently restricted to the top of the slant. Upon prolonged incubation, microconidia are often produced. Since the diagnosis of pe^{m} depends upon color, conidial distribution and

the production of microconidia, a large error is introduced in scoring random segregants from crosses of *arom-2 Ab* by *pe*^{m}. Problems of allele recovery and scoring were partly avoided by performing a tetrad analysis using the double mutant *pe*^{m} *fl* which produces microconidia exclusively (BARRATT and GARNJOBST 1949) and allows the unequivocal detection of exchanges in the *pe*^{m} *fl* interval by the production of segregants with the characteristic *fl* phenotype.

The results of crosses of *pe*^{m} *fl* by all of the *arom* mutants in addition to *arg-5* are presented in Table 2. Asci which yielded representatives of at least three of the four spore pairs are tabulated as complete tetrads. In view of the poor yield of complete tetrads in crosses involving *arom-2 Ab*, the data obtained from those asci yielding members of two spore pairs from the same half of the ascus are included and referred to as half tetrads. Some multiple exchanges cannot be observed in half tetrads; hence the estimates of interval distances are biased downwards to the extent of the half tetrad frequency.

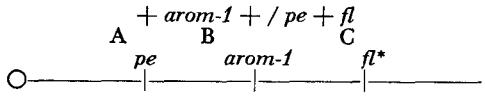
The data obtained are very heterogeneous with regard to interval lengths. This was expected in view of the heterogeneous origin of the mutants employed (STADLER 1956; MITCHELL 1959; PERKINS 1959). However, it can be concluded that: (1) All of the *arom* mutants are located in the right arm of linkage group II. (2) *arom-1* is apparently located between *pe* and *fl* (as observed by GARNJOBST in BARRATT *et al.* 1954). (3) The most probable position of *arom-3* is to the left of *pe* near *arg-5* and centromere.

The data from the *arom-2 Ab* by *pe*^{m} *fl* cross demonstrate an extreme shortening of the centromere to *pe* interval and the absence of exchanges between *pe* and *arom-2 Ab*. However, instead of a corresponding decrease in the *pe fl* region a slight increase in exchange frequency was observed.

The extreme contraction of the centromere to *pe* interval in the *arom-2 Ab*

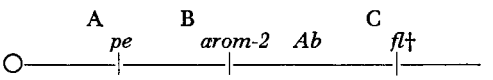
TABLE 2

Tetrad analysis of arom *and* arg-5 × pe fl *crosses*



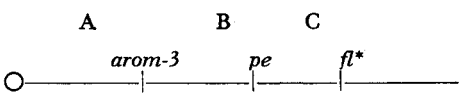
Tetrads isolated = 124	Total exchanges			Complete tetrads = 76 / Half tetrads = 31 Multiple exchanges
Interval	A	B	C	1 A+B (2 or 4), 6A+C (2 or 4), 5A+C (3)
Complete tetrads	24	5	48	2 B+C (2), 2C+C (4),
Nonexchange tetrads = 19				2 A+C+C (2, 3 or 4 (A+C) 4C)
Half tetrads	10	2	7	None
Nonexchange half tetrads = 12				
Total	34	7	55	
Apparent interval length	16.0	3.3	26.0	

$+$ *arom-2 Ab* $+$ / *pe* $+$ *fl*



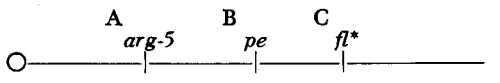
Tetrads isolated = 209			Complete tetrads = 61	Half tetrads = 57
Complete tetrads	1	0	42	1 A+C (2 or 4), 3C+C (4)
Nonexchange tetrads = 22				
Half tetrads	6	0	41	2 A+C (2 or 4), 2C+C (4)
Nonexchange half tetrads = 14				
Total	7	0	83	
Apparent interval length	3.0	0	35.0	

arom-3 $+$ $+$ / $+$ *pe fl*



Tetrads isolated = 107			Complete tetrads = 94	Half tetrads = 12
Complete tetrads	12	34	53	3 A+C (3), 4 A+C (2 or 4), 7 B+C (3) 1 B+C (2), 1 B+C (4), 2C+C (4), 1 A+B+C (2 or 4(A+B) 2(B+C)), 1 A+B+C (2 or 4 (A+B) 4(B+C))
Nonexchange tetrads = 17				
Half tetrads	0	4	8	1 B+C (2), 1 C+C (4)
Nonexchange half tetrads = 2				
Total	12	38	61	
Apparent interval length	5.5	18.0	29.0	

arg-5 $+$ $+$ / $+$ *pe fl*



Tetrads isolated = 97			Complete tetrads = 96	Half tetrads = 1
Complete tetrads	1	17	39	4 B+C (3), 1 B+C (2), 1 B+C (4), 2 C+C (4)
Nonexchange tetrads = 47				
Half tetrads	0	0	0	None
Nonexchange half tetrads = 1				
Total	1	17	39	
Apparent interval length	0.52	8.9	20.0	

* Gene order is derived from the data.
† Assumed order of *arom-2 Ab* and *pe*.
Numbers in brackets refer to the number of strands involved in multiple exchanges.

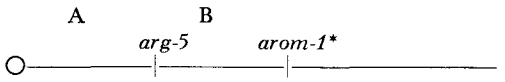
cross was accompanied by the appearance of a high frequency of spore abortion patterns (McCLINTOCK 1945) and poor germination among the normal eight spored asci. The fact that six out of seven of the exchanges in the centromere to *pe* interval were recovered in half tetrads might be indicative of selection against tetrads with exchanges in this region. However, an analysis of strand recovery among the incomplete tetrads failed to reveal any indication of preferential recovery different from that observed in the complete and half tetrads.

The apparent tight linkage of *arom-2 Ab* to centromere was also unexpected since an analysis of random segregants and tetrads from crosses of *arom-2 Ab* by *arg-5* and *arom-3* indicated that *arom-2 Ab* segregated fairly frequently from both loci (ten to 20 percent). The lengths of the *arg-5–arom-2 Ab* and *arg-5–arom-1* intervals were therefore compared by tetrad analysis. In the crosses listed in Table 3, the double mutants, *arg-5 arom-2 Ab* and *arg-5 arom-1* were crossed to

TABLE 3

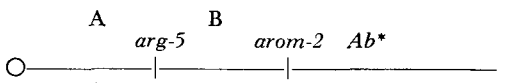
Tetrad analysis of arom arg-5 *crosses*

arg-5 arom-1 / + +



Tetrads isolated=120	Exchanges		Complete tetrads=114	Half tetrads=4 Multiple exchanges
Interval	A	B	1 A+B (2 or 4), 1 A+B (3),	
Complete tetrads	5	31	1 B+B (4)	
Nonexchange tetrads = 81				
Half tetrads	0	2	1 B+B(4)	
Nonexchange half tetrads = 3				
Total	5	33		
Apparent interval length	2.1	14.0		

arg-5 arom-2 Ab/ + +



Tetrads isolated=120			Complete tetrads=73	Half tetrads=26
Complete tetrads	2	28	1 A+B (3), 1 B+B (4)	
Nonexchange tetrads = 45				
Half tetrads	1	1	none	
Nonexchange half tetrads = 24				
Total	3	29		
Apparent interval length	1.5	14.7		

* Gene order is derived from the data.
Numbers in brackets refer to the number of strands involved in multiple exchanges.

the standard + + in order to maximize tetrad recovery. In this cross, as in all of those done with *arom-2 Ab*, many abortive asci were observed. However, the tetrad recovery from completely mature asci was greatly improved. The poor yield of *arg-5 arom-2 Ab* segregants, however, led to the recovery of a large proportion of the nonexchange tetrads as half tetrads. Exchanges in half and complete tetrads are therefore summed in order to avoid bias.

The data obtained for the *arg-5–arom-1* and *arg-5–arom-2 Ab* intervals are surprisingly homogeneous. Although variations of interval lengths observed in other crosses make determinations of confidence limits almost meaningless, the conclusion that *arom-2 Ab* and *arom-1* are at least closely linked is inescapable.

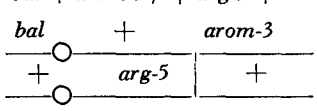
The tetrad analysis of the *arom-3* by *pe*^{m} *fl* cross indicated that *arom-3* was about 18 units proximal from *pe* in the region of *arg-5*. Although the linkage of *arom-3* to *arom-1* and *arom-2 Ab* is loose, in view of the paucity of information with regard to the relation of physical distance with "distance" in the recombinational sense, it seemed of interest to determine whether *arg-5*, a locus controlling an overtly different biochemical function, is located between the loci controlling aromatic biosynthesis.

The orientation of the *arg-5* and *arom-3* loci was determined with the aid of the colonial mutant—balloon (*bal*). PERKINS (1959) showed that *bal* was very close to the centromere of linkage group II and unequivocally established the order *bal* – *arg-5* – *pe*. We have observed *bal* to segregate from centromere independently of *arom-3* (one out of 24 tetrads) which indicates that it is probably in the left arm of linkage group II.

TABLE 4

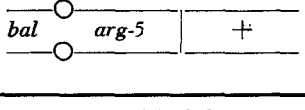
Three point crosses involving bal, arg-5 *and* arom-3

bal + *arom-3* / + *arg-5* +



Supplemented plates			Minimal plates–prototrophs				Interval length
Total viable spores*	Morphology *bal*	+	Total	Morphology *bal*	+	Frequency	*arg-5/arom-3*
8,060	3,660	4,400	22	22	0	0.00273	0.55

bal arg-5 + / + + *arom-3*



Supplemented plates			Minimal plates–prototrophs				Interval length
Total viable spores*	Morphology *bal*	+	Total	Morphology *bal*	+	Frequency	*arg-5/arom-3*
14,700	6,180	8,480	61	3	58	0.00415	0.83

* The viability of the spores plated was about 90 percent in both crosses.

Table 4 contains the results of an analysis of random segregants from crosses of *bal arg-5* by *arom-3* and *bal arom-3* by *arg-5*. Since the great majority of prototrophic segregants from the *bal arg-5* by *arom-3* crosses were morphologically + and from the *bal arom-3* by *arg-5* cross were *bal*, the order *bal–arg-5–arom-3* is established. The orientation of the *arom* mutants is such that no known mutant genes yielding auxotrophic phenotypes lie between *arom-1* (and *arom-2 Ab*) and *arom-3*. However, two loci controlling some morphological properties of Neurospora, *pe* and 584 (PERKINS, personal communication), are apparently located within the *arom-3* – *arom-1* interval.

Reversion of arom *mutants:* A careful examination of the relative reversion frequencies of the *arom* mutants was undertaken using *arom inos* and *arom pan-1* double mutants. Macroconidia were irradiated with doses of ultraviolet light leading to a survival of ten to 50 percent. Complete and partial reversions were selected for on medium supplemented only with calcium pantothenate and inositol and on medium supplemented with the above and PAB plus any two of the three required aromatic amino acids. The reversion frequency of *arom-1* was found to be highest when reversions for the tryptophan requirement were selected (7.5×10^{-5} per nucleus—assuming an average of two nuclei per macroconidium, and 6×10^{-6} per nucleus when selection for complete reversion was employed). Three out of four "reversions" of *arom-1* were found to be due to nonlinked suppressors. *arom-3* shows little variation in reversion frequency on the different selective media and has a mean reversion frequency of 2.5×10^{-7} per nucleus. No reversions of *arom-2 Ab* have been observed under comparable conditions among 5×10^{9} irradiated nuclei. Limitations of the number of conidia that can be plated without interfering with the growth of revertants (GRIGG 1952) prevent a more extensive quantitative search for reversions. However, massive transfers of *arom-2 Ab* conidia to selective media have never been observed to yield revertants under conditions in which contamination could be ruled out.

Enzymatic complements of the arom *mutants:* The growth response and accumulation pattern of the *arom* mutants suggested that *arom-1* is deficient in the ability to convert DHS to shikimic acid and that *arom-3* is unable to carry out some reaction subsequent to the synthesis of shikimic acid. The behavior of *arom-2 Ab*, however, was suggestive of a multiple loss of enzymatic functions in the synthesis of shikimic acid and one of its products. These conclusions were confirmed largely by the direct assay of the specific enzymes present in crude and partially purified extracts of the mutant strains.

The specific activities of 5-dehydroquinase, DHS reductase and PCA oxidase observed in extracts of the *arom* strains are listed in Table 5. These enzymes could be assayed directly with little error. The specific activities of KDHP synthetase and DHQ synthetase could not be determined accurately because of the presence of dephosphorylating enzymes and the apparent involvement of some unanalysed side reaction of DHQ or DHS. However, the assay procedures are sensitive enough to detect at least ten percent of the activity observed in enzyme preparations obtained from the standard strains (the sensitivity was determined in mixtures of extracts of *arom-2 Ab* and the standard strains). The determina-

TABLE 5

Enzyme complements of arom *and standard strains*

Enzyme	*arom-1*	*arom-2 Ab*	*arom-3*	Standard
KDHP synthetase	+	+	+	+
DHQ synthetase	+	0	+	+
5-dehydroquinase	0.80	0	2.73	0.77
DHS reductase	0	0	28.7	10.6
PCA oxidase				
-not induced	2.4	<0.01	2.5	<0.01
-induced*		0.62		0.36
DHS dehydrase	+	0	+	0

Enzymatic activity is expressed as units of activity per mg protein in fractions described in MATERIALS AND METHODS. Each value is the average specific activity observed in at least two independent preparations. +—Enzymatic activity was observed, but the determination of specific activity is subject to error—see text. 0—No activity observed. *—The synthesis of PCA oxidase was induced by the addition of 100 mg vanillic acid per liter growth medium.

tion of the specific activity of DHS dehydrase is subject to considerable error because of the instability of the enzyme. However, the sensitivity of the assay permits the detection of at least five percent of the minimum activity observed in *arom-1* and *arom-3*. The observed specific activity of DHS dehydrase varied between 0.1 to 0.5 of that found for PCA oxidase. Because of the errors in the determination of the specific activities of DHS dehydrase, KDHP synthetase and DHQ synthetase, only the presence or absence of detectable quantities of these enzymes in the various preparations is indicated in Table 5.

It should be pointed out that the enzymatic conversion of KDHP to DHQ is a rather complex reaction (SRINIVASAN *et al.* 1959). The participation of only one enzyme in the reaction has as yet not been completely established (SRINIVASAN, personal communication). For the sake of simplicity we refer to the enzyme or enzymes as DHQ synthetase.

The data in Table 5 confirm most of the deductions drawn from the information previously presented. DHS reductase is absent in crude extracts of both *arom-1* and *arom-2 Ab*. In addition, *arom-2 Ab* is apparently missing the entire enzymatic apparatus for converting KDHP to DHS while retaining the ability to synthesize KDHP from erythrose-4-phosphate and phosphoenol pyruvate. The entire sequence of reactions studied is present in *arom-3*, thus supporting the notion that *arom-3* is blocked in a reaction subsequent to the synthesis of shikimic acid. The specific activity of dehydroquinase and DHS reductase were found to be considerably higher in *arom-3* than in *arom-1* or the standard strains. However, the probable lack of isogenicity between the strains obfuscates any special significance of these observations.

The enzymatic reactions subsequent to shikimic acid have not as yet been elucidated. However, much can be deduced from the states of induction of DHS dehydrase and PCA oxidase in the various mutants. It has been shown that PCA is derived directly by dehydration of DHS (GROSS 1958). This reaction is irreversible and greatly favored during growth of mutants blocked subsequent to the synthesis of DHS thus leading to the accumulation of PCA as the major metabolic

product (some mutants blocked in the synthesis of phenylalanine and tyrosine have also been found to accumulate PCA). PCA is a substrate and an inducer of the synthesis of PCA oxidase (GROSS and TATUM 1955; GROSS 1959). Extracts of *arom-1* and *arom-3* contain PCA oxidase at the earliest stages of mycelial growth. The accumulation of PCA, the presence of DHS dehydrase and PCA oxidase are then excellent criteria for the involvement of a metabolic block subsequent to the synthesis of DHS. The virtual absence of these two enzymes in extracts of *arom-2 Ab* therefore seems to be a consequence of its inability to synthesize the hydroaromatic compounds. The induction of PCA oxidase synthesis in *arom-2 Ab* is equivalent to that observed in the standard type when vanillic acid or PCA (inducers of PCA oxidase) are added to the growth medium (GROSS and TATUM 1955).

The reactions involved in the synthesis of shikimic acid and the aromatic amino acids are summarized in Figure 1. 5-Phosphoshikimic acid is included in the scheme as a probable intermediate. This compound is accumulated by many *arom* mutants of *E. coli* (DAVIS 1955) and has been implicated in the synthesis of anthranilic acid (SRINIVASAN 1959). Although it has not as yet been demonstrated that 5-phosphoshikimic is derived from shikimic acid by direct phosphorylation, it is provisionally considered to be the immediate product of shikimic acid. Phosphorylated intermediates are usually not accumulated in culture filtrates of Neurospora, presumably because of the presence of active phosphatases (AMES and MITCHELL 1955). Compound "?" is included in the scheme as an hypothetical intermediate to explain the genetic and biochemical information described below.

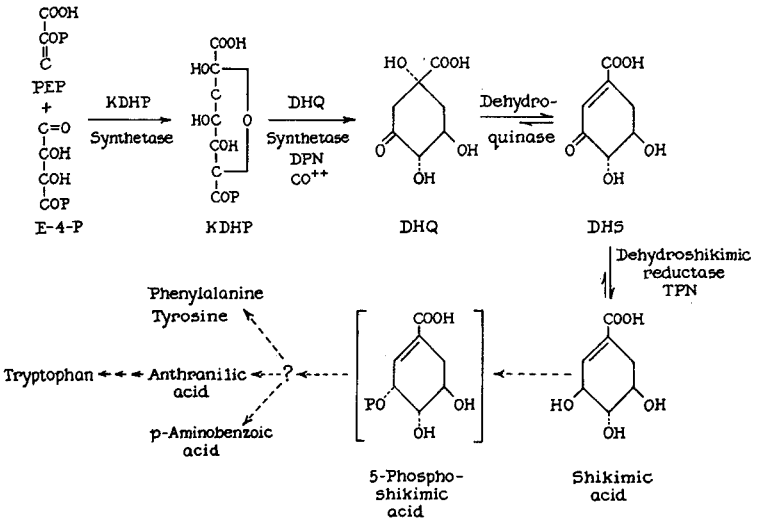


FIGURE 1.—Reaction sequence involved in the synthesis of the aromatic amino acids and PAB. Brackets signify that the compound has not been definitely established as an intermediate. PEP, phosphoenolpyruvate; E-4-P, erythrose 4-phosphate; KDHP 2-keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate; DHQ, 5-dehydroquinic acid; DHS, 5-dehydroshikimic acid; TPN, triphosphopyridine nucleotide.

The isolation of additional arom *mutants:* Although *arom-2 Ab* is phenotypically similar to *arom-1* in its inability to synthesize DHS reductase and is closely linked to *arom-1*, the infertility of crosses between these mutants has prevented the direct demonstration of allelism. The coincidental deficiency of DHS reductase in both mutants and the deficiency in the synthesis of at least two additional enzymes in *arom-2 Ab* strongly suggest that the individual genetic determinants of the synthesis of the deficient enzymes are included within the *arom-2 Ab* region. Support for this conclusion could be obtained by the examination of the linkage relationships of mutants controlling reactions deficient in *arom-2 Ab* other than the conversion of DHS to shikimic acid.

In addition to the above, it was clear that additional support was required for the conclusion that *arom-2 Ab* does not respond to shikimic acid for growth as a consequence of its inability to carry out a reaction subsequent to the synthesis of shikimic acid. As indicated previously, the inability of *arom-2 Ab* to utilize shikimic acid for growth in place of the aromatic supplement could conceivably be due to the loss of a permeability mechanism. Proof of the deficiency in *arom-2 Ab* of a reaction subsequent to shikimic acid synthesis could be obtained if a mutant were found which failed to respond to shikimic acid, did not complement *arom-2 Ab*, but did complement *arom-1* and *arom-3* and, in addition, was tightly linked to *arom-1*.

A search for *arom* mutants was therefore undertaken employing the "inositol-less death" procedure (LESTER and GROSS 1959). After permitting the inactivation of UV irradiated conidia to proceed for four days on minimal sorbose medium or medium supplemented with phenylalanine, tryptophan and PAB, the plates were supplemented with inositol plus either the entire growth factor requirement of the *arom* mutants or tyrosine. Shikimic acid proved to be a poor supplement for the selection of *arom* mutants, for the growth of *arom-1* on shikimic acid in sorbose medium is very slow.

Fourteen *arom* mutants as well as a large number of unclassified auxotrophs were obtained from three experiments. The *arom* mutants fell into two classes. The first of these consisted of 11 mutants which behaved as heteroalleles of *arom-3*. Their behavior, in complementation tests, suggested the existence of five functional subareas of the *arom-3* locus. One of the mutants of this class—R2212, which forms a slow growing prototrophic heterocaryon with *arom-3*, has been examined genetically as well as enzymologically. It falls in the same region of linkage group II (closely linked to *arg-5*) as does *arom-3*, rarely forms prototrophic segregants in crosses with *arom-3* and is biochemically indistinguishable from *arom-3*.

The other group obtained consisted of three mutants which were of the desired phenotype. They did not complement *arom-2 Ab*, but, unlike *arom-2 Ab*, did complement *arom-1* as well as all members of the *arom-3* class. These mutants did not respond to shikimic acid as the sole replacement of their aromatic growth requirement. Shikimic acid, DHS and PCA were secreted during growth, and mycelial extracts contained the full complement of enzymes necessary for the production of shikimic acid from its precursors. These mutants are therefore blocked

in a reaction subsequent to the production of shikimic acid. Their failure to complement *arom-2 Ab* indicates that *arom-2 Ab*, in addition to its deficiency of the three enzymes necessary to convert KDHP to shikimic acid is also deficient in the ability to convert shikimic acid into one of its products. Thus, it is not necessary to invoke the notion of a permeability barrier to explain the failure of *arom-2 Ab* to grow on shikimic acid.

The search for *arom* mutants was by no means exhaustive and, in fact, was terminated upon obtaining the desired class of mutants. DR. R. W. COLBURN (personal communication) obtained a number of *arom* mutants by a modification of the selection procedure of WOODWARD, DEZEEUW, and SRB (1954) after X-ray treatment. Four of these were found to be alleles of *arom-1*.

Genetic analysis of arom-4 *mutants:* The locus designation *arom-4* was assigned to the new class of *arom* mutants, and one of them, R2204, (referred to hereafter as *arom-4*) was used in the genetic analysis.

An analysis of random segregants from crosses of *arom-4* by *arg-5* pe^m *fl* and *arom-3* revealed that *arom-4* is within the same area of linkage group II as is *arom-1*—approximately four units from *pe* between *pe* and *fl* and about 20 units from *arg-5*. The direct determination of allelism of *arom-4* with *arom-2 Ab* could not be done because crosses of the two, like crosses of *arom-1* by *arom-2 Ab*, have been completely sterile. However, a critical estimate of the linkage of *arom-4* to *arom-1* and the order of the loci with respect to *arg-5* was obtained from the three point cross—*arg-5 arom-1* by *arom-4*—summarized in Table 6.

Since there was no obvious selection against the recovery of *arg-5* segregants, and all of the segregants prototrophic with respect to the aromatic requirement did not require arginine for growth, the order *arg-5* – *arom-1* – *arom-4* is established.

TABLE 6

Three point cross–arg-5 arom-1 + / + + arom-4

	arg-5	*arom-1*	+ *
	+	+	*arom-4*
Spores plated on arginine only†			11,700
Prototrophs for aromatic requirement			19
Genotype of prototrophs	*arg-5* + +		0
	+ + +		19
Frequency of recombinants			0.00162
Apparent interval length			0.32
Spores plated on arginine and aromatics			835
Number of segregants isolated			200
Phenotypes‡	*arg arom*		107
	+ *arom*		92
	+ + (genotype = + + +)		1

* Gene order is derived from the data.
† Approximately 84 percent of the spores plated were viable.
‡ No distinction was made between *arom-1* and *arom-4*.

The interval between *arom-1* and *arom-4* was found to be quite small (0.32 units). If one assumes that the *arom-1* — *arom-4* interval measured is reasonably close to the average interval length for the postulated loci controlling the synthesis of the hydroaromatic intermediates, then an approximate length of one unit is obtained for the entire cluster.

The physiological deficiencies of the *arom* mutants are illustrated in Figure 2, and the linkage relationships are illustrated in Figure 3.

There is no direct evidence that the reaction controlled by *arom-4* precedes that of *arom-3* in the biosynthetic sequence. However, the association of the determinants of the three prior reactions in the sequence with *arom-4* within the *arom-2 Ab* aberration is prejudicial in favor of the order illustrated. Compound "?" is postulated as a common intermediate for all of the aromatic compounds as the simplest explanation for the quadruple requirement of *arom-3*.

DISCUSSION

Linkage and function: The data presented above indicate that the genes determining the synthesis of at least four enzymes involved in the sequential production of precursors of the aromatic amino acids are clustered in a rather small region of linkage group II of Neurospora. This constitutes the first demonstration of *extensive* linkage of the genetic determinants of sequential biosynthetic reactions in an organism other than *E. coli* and Salmonella. Gene clustering of this sort can therefore no longer be considered a result of a selective mechanism peculiar to the mode of physiological activity of the enteric bacteria. However, since most of the linked sequences studied in the enteric bacteria have been found to be quite randomly distributed in the genome of the fungi, serious restrictions

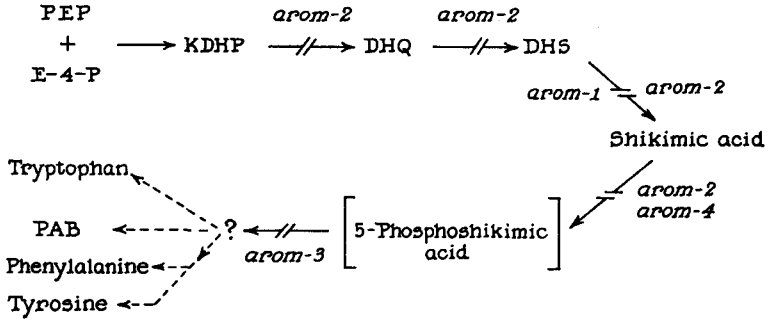


FIGURE 2.—Physiological deficiencies of the *arom* mutants. See legend of Figure 1 for definitions of abbreviations.

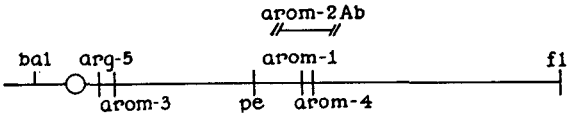


FIGURE 3.—Most probable linkage relationship of *arom* loci.

must be placed on general hypotheses concerning the maintenance of linkage in the two groups of organisms.

There appears to be no selective advantage for the concerted heterocatalytic functions of the linked genes in terms of the ultimate synthesis of essential metabolites. Heterocaryons of *arom-1* and *arom-4* are indistinguishable from the standard strains with regard to morphology and rate of growth. Furthermore, the significance of the relationship between linkage and the similarity of the compounds involved in the various enzymatic reactions is obscure. Each of the enzymes displays a different mechanism of catalysis and must, in addition to different binding sites for the various cofactors involved, have at least configurational differences about the substrate binding site. It was, therefore, no surprise to find that the separation of 5-dehydroquinase from DHS reductase could be almost completely accomplished simply by ammonium sulfate fractionation and/or ion exchange chromatography on diethylaminoethylcellulose (GROSS, unpublished).

JACOB (1959) has proposed that the rates of the heterocatalytic syntheses of a gene cluster are controlled at the chromosomal level by "repressers" (VOGEL 1957) which may, in some cases at least, be synthesized as a consequence of the specific reaction sequence involved. According to this view no *a priori* restrictions are placed on the nature of the reactions involved. They may be related in terms of a specific biosynthetic sequence as well as participation in a common physiological process. Thus, the genes determining λ coli-phage synthesis in *E. coli* K-12 are linked and are subject to repression (JACOB 1959) in a manner similar to that of the gene cluster determining the biosynthesis of tryptophan (COHEN and JACOB 1959). The selective advantage for clustering, then, would be derived not from the efficiency that might be obtained from an "assembly line" production of substrates but from the control of reactions by the alteration in the relative rates of synthesis of specific proteins during growth. According to this view, no intuitive restrictions are placed on the most advantageous arrangements of genes for any particular organism, and it becomes just as easy to envision the linkage of genes determining morphogenetic properties (e.g., *T*, *Ki*, *Fu* in the mouse; DUNN 1956) as the linkage of those involved in the production of essential metabolites. Hence, the different linkage pattern in the enteric bacteria and the fungi might not represent a tendency toward randomness in the fungi but, more interestingly from a physiological and developmental point of view, the requirement for different patterns of control of growth and differentiation in the two groups of organisms.

Repression of the synthesis of enzymes involved in related reactions by the products of the reactions has been primarily observed in *E. coli* and Salmonella (VOGEL 1957; PARDEE 1959; AMES and GARRY 1959). Although the activity of DHS dehydrase was found to be considerably higher in *arom-3* than in the standard strain, the variations observed could be due to genotypic effects other than repression. Repeated attempts to demonstrate repression of the enzymes of shikimic acid synthesis with the standard and mutant strains of Neurospora have failed. However, as AMES and GARRY (1959) have suggested, this might be a consequence of the inability at present to control the growth of Neurospora in such a way that it is not always maximally repressed.

The autoinduced biosynthesis of enzymes: That at least some enzymes in Neurospora are subject to wide variations in synthetic rate is obvious from the data presented with regard to PCA oxidase and DHS dehydase. The standard strain and *arom-2 Ab* produce very little of these enzymes during growth in the usual media. The synthesis of PCA oxidase, at least, can be induced in both of these strains by the addition of PCA or a number of analogues to the growth medium (GROSS and TATUM 1955). Of particular interest here is the fact that both DHS dehydrase and PCA oxidase are present in high concentration in *arom-1*, *arom-3* and *arom-4* as part of the constitutive complement of enzymes. Thus, the presence of these enzymes in the mutant strains apparently stems from the accumulation of their inducer-substrates as a consequence of interference in the normal biosynthesis of the aromatic ring. This enzymatic pleiotropy leads to the diversion of the precursors of aromatic biosynthesis into a constitutive series of reactions leading ultimately to their total catabolism (GROSS 1959).

Thus, whether or not one wishes to view enzymatic induction as a reversal of "repression" (VOGEL 1957), the heterocatalytic activity of genes of Neurospora are subject to wide variation. It is important to note in this respect that although DHS is an intermediate in aromatic biosynthesis the presence of DHS dehydrase could only be demonstrated under condition of DHS accumulation. Apparently the concentration of DHS in mycelia of the standard strains never reaches inducing concentrations.

Some aspects of arom-2 Ab: Failure of *arom-2 Ab* to complement *arom-1* and *arom-4* in heterocaryons apparently rules out the possibility that the enzymatic deficiencies observed in *arom-2 Ab* result from a primary block in the synthesis of DHQ, which is, in turn, required to induce the synthesis of the enzymes involved in subsequent reactions in aromatic biosynthesis. Furthermore, the abnormal segregation of *arom-2 Ab* and its failure to revert suggest that the genetic determinants of the enzymes responsible for the conversion of DHQ to a product of shikimic acid are involved in some relatively small chromosomal aberration.

Except for its anomalous segregation from *pe*, the segregation of *arom-2 Ab* from all linkage group II markers was found to be approximately the same as that of *arom-1*. In view of this and the apparent independent segregation of *arom-2 Ab* from linkage group I, IV and V markers (GROSS, unpublished observations), it does not seem likely that *arom-2 Ab* is an *extensive* inversion or translocation. It is possible that the spore abortion patterns observed in crosses *arom-2 Ab* did not result from chromosomal missegregation (MCCLINTOCK 1945) but instead, as a consequence of a deficiency in the spore maturation process. The lack of exchanges between *arom-2 Ab* and *pe*, as well as the suppression of exchanges in the *pe* to centromere interval observed in the cross of pe^{m} *fl* by *arom-2 Ab*, could be explained on the assumption that each of the mutants involves a very short inversion or deletion. If this were true, serious pairing difficulties would arise only in crosses involving the heterologous aberrations.

Neither the failure to revert nor the absence of several enzymatic activities can be taken as sufficient evidence for the specific involvement of a deletion in *arom-2 Ab*. First of all, reversion frequencies per nucleus vary quite widely, and the methods of detection in Neurospora become exceedingly crude below fre-

quencies of less than 10^{-9}. Secondly, the assumption that the loss of a number of heterocatalytic functions can only be interpreted as a consequence of a deletion does not seem at all necessary. Until further genetic and cytogenetic work can be done with *arom-2 Ab*, it seems best to consider *arom-2 Ab* some sort of small chromosomal aberration in the immediate vicinity of *arom-1* and *arom-4*.

SUMMARY

The genetic and physiological properties of several *arom* mutant strains of Neurospora are described. The most likely interpretation of the evidence presented is that the genes responsible for the production of at least four enzymes involved in the synthesis of the aromatic ring are closely linked in linkage group II. Another gene involved in the same reaction sequence, while also on linkage group II, is loosely linked to the others. The inability of *arom-2 Ab* to carry out four reactions in the sequence appears to be due to an aberration in the *arom-1* and *arom-4* region of linkage group II.

The problem of linked genes controlling related physiological reactions has been discussed in terms of the involvement of common control mechanisms for protein synthesis.

Autoinduced enzyme synthesis is proposed as the mechanism leading to the appearance of two enzymes, as part of the constitutive enzymatic complement of *arom-1*, *arom-3* and *arom-4*, that are present, if at all, only in extremely low concentrations in *arom-2 Ab* and the standard strains.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Drs. D. D. Perkins, Mary B. Mitchell and H. K. Mitchell for their generous contribution of mutant strains, to Drs. D. B. Sprinson and P. R. Srinivasan for their gifts of several compounds required for this investigation and to Mary Hurd and Elsie Wagner for technical assistance.

LITERATURE CITED

Ames, B. N., and B. Garry, 1959 Coordinate repression of the synthesis of four histidine biosynthetic enzymes by histidine. Proc. Natl. Acad. Sci. U.S. **45**: 1453–1461.

Ames, B. N., and H. K. Mitchell, 1955 The biosynthesis of histidine. J. Biol. Chem. **212**: 687–696.

Ballou, C. E., H. O. L. Fisher, and D. L. MacDonald, 1955 The synthesis and properties of D-erythrose 4-phosphate. J. Am. Chem. Soc. **77**: 5967–5970.

Barratt, R. W., D. Newmeyer, D. D. Perkins, and L. Garnjobst, 1954 Map construction in *Neurospora crassa*. Advances in Genet. **6**: 1–93.

Barratt, R. W., and L. Garnjobst, 1949 Genetics of a colonial microconidiating mutant strain of *Neurospora crassa*. Genetics **34**: 351–369.

Cohen, G. N., and F. Jacob, 1959 Sur la répression de la synthèse des enzymes intervenant dans la formation du tryptophane chez *Escherichia coli*. Compt. Rend. **248**: 3490–3492.

Cohn, M., 1956 On the inhibition by glucose of the induced synthesis of β-galactosidase in

Escherichia coli. pp. 41–48. *Enzymes: Units of Biological Structure and Function*. Edited by O. H. GAEBLER. Academic Press. New York.

DAVIS, B. D., 1955 Biosynthesis of the aromatic amino acids. pp. 799–811. *Amino Acid Metabolism*. Edited by W. D. MCELROY and H. B. GLASS. Johns Hopkins Press. Baltimore, Maryland.

DEMEREC, M., and P. E. HARTMAN, 1959 Complex loci in microorganisms. Ann. Rev. Microbiol. **13**: 377–406.

DUNN, L. C., 1956 Analysis of a complex gene in the house mouse. Cold Spring Harbor Symposia Quant. Biol. **21**: 187–194.

GRIGG, G. W., 1952 Back mutation assay method in microorganisms. Nature **169**: 98–100.

GROSS, S. R., 1958 The enzymatic conversion of 5-dehydroshikimic acid to protocatechuic acid. J. Biol. Chem. **233**: 1146–1151.

1959 Enzymatic autoinduction and the hypothesis of intracellular permeability barriers in Neurospora. Trans. N.Y. Acad. Sci. **22**: 44–48.

GROSS, S. R., R. D. GAFFORD, and E. L. TATUM, 1956 The metabolism of protocatechuic acid by Neurospora. J. Biol. Chem. **219**: 781–796.

GROSS, S. R., and E. L. TATUM, 1955 Structural specificity of inducers of protocatechuic acid oxidase synthesis in Neurospora. Science **122**: 1141.

JACOB, F., 1959 Genetic control of viral functions. Harvey Lectures, Ser. **53**: (In press).

LESTER, H. E., and S. R. GROSS, 1959 Efficient method for selection of auxotrophic mutants of Neurospora. Science **129**: 572.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL, 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265–275.

MCCLINTOCK, B., 1945 Preliminary observations of the chromosomes of *Neurospora crassa*. Am. J. Botany **32**: 671–678.

METZENBERG, R. L., and H. K. MITCHELL, 1958 The biosynthesis of aromatic compounds by *Neurospora crassa*. Biochem. J. **68**: 168–172.

MITCHELL, M. B., 1959 Genetic recombination in Neurospora. Genetics **43**: 799–813.

MITSUHASHI, S., and B. D. DAVIS, 1954 Conversion of 5-dehydroquinic acid to 5-dehydroshikimic acid by 5-dehydroquinase. Biochim. et Biophys. Acta **15**: 54–61.

NEWMEYER, D., 1954 A plating method for genetic analysis in Neurospora. Genetics **39**: 604–618.

PARDEE, A. B., 1959 Mechanisms for control of enzyme synthesis and enzyme activity in bacteria. pp. 295–304. *Ciba Foundation Symposium on the Regulation of Cell Metabolism*. Edited by G. E. W. WOLSTENHOLME and C. M. O'CONNOR. Little Brown and Co. Boston, Massachusetts.

PERKINS, D. D., 1959 New markers and multiple point linkage data in Neurospora. Genetics **44**: 1185–1208.

PONTECORVO, G., 1958 *Trends in Genetic Analysis*. Columbia University Press. New York.

SALAMON, I. I., and B. D. DAVIS, 1953 The isolation of a precursor of shikimic acid. J. Am. Chem. Soc. **75**: 5567–5571.

SRINIVASAN, P. R., 1959 Biosynthesis of anthranilic acid from 5-phosphoshikimic acid and L-glutamine. Federation Proc. **18**: 329.

SRINIVASAN, P. R., M. KATAGIRI, and D. B. SPRINSON, 1959 The conversion of phosphoenolpyruvic acid and D-erythrose 4-phosphate to 5-dehydroquinic acid. J. Biol. Chem. **234**: 713–715.

SRINIVASAN, P. R., and D. B. SPRINSON, 1959 2-Keto-3-deoxy-D-*arabo*-heptonic acid 7-phosphate synthetase. J. Biol. Chem. **234**: 716–722.

STADLER, D. R., 1956 A map of linkage group VI of *Neurospora crassa*. Genetics **41**: 528–543.

TATUM, E. L., S. R. GROSS, G. EHRENSVÄRD, and L. GARNJOBST, 1954 Synthesis of aromatic compounds by Neurospora. Proc. Natl. Acad. Sci. U.S. **40**: 271–276.

VOGEL, H. J., 1956 A convenient growth medium for Neurospora. Microbial Genetics Bull. **13**: 42–43.

1957 Repressed and induced enzyme formation: a unified hypothesis. Proc. Natl. Acad. Sci. U. S. **43**: 491–496.

WEISS, U., B. D. DAVIS, and E. S. MINGIOLI, 1953 Identification of an early precursor as 5-dehydroquinic acid. J. Am. Chem. Soc. **75**: 5572–5576.

WOODWARD, V. W., J. R. DEZEEUW, and A. M. SRB, 1954. The separation and isolation of particular biochemical mutants of Neurospora by differential germination of conidia, followed by filtration and selective plating. Proc. Natl. Acad. Sci. U.S. **40**: 192–200.