GENETIC ANALYSIS OF PYRIMIDINE MUTANTS OF *SALMONELLA TYPHIMURIUM*[1]

YANHON YAN[2] AND M. DEMEREC

Biology Department, Brookhaven National Laboratory, Upton, New York

Received May 12, 1965

GENETIC and biochemical analyses of pyrimidine-(uracil-)requiring mutants of *Escherichia coli* have shown that at least six gene loci control the pathway of biosynthesis leading to production of uridylic acid (BECKWITH, PARDEE, AUSTRIAN and JACOB 1962). Results of a genetic analysis involving 141 mutants of *Salmonella typhimurium* representing six structural genes will be presented in this paper. They indicate a high degree of homology in gross genomic structure between these two members of the enteric family of bacteria as far as pyrimidine loci are concerned.

MATERIALS AND METHODS

Of the 141 *S. typhimurium* pyrimidine mutants studied, only one or two originated spontaneously; all the others were isolated, by several workers in our laboratory, after treatment of LT-2 and a few LT-7 samples of bacteria with various mutagens (Table 1). Three Hfr strains were used in mapping the mutants: HfrA (SR305, isolated by N. ZINDER), which transfers loci in the order O-*ilv thr try*; HfrB2 (isolated by K. SANDERSON), which transfers loci in the order O-*his str thr*; and, SR315, which transfers loci in the order O-*try his str*. The point of origin and the direction of entry of these Hfr strains, as well as the meaning of symbols, is described by SANDERSON and DEMEREC (1965). The transduction phage was strain P22.

The liquid medium in which bacterial cultures were grown contained 8g dehydrated broth, 5g NaCl, and one liter of distilled water. The minimal agar was composed of 10.5g K_2HPO_4, 4.5g KH_2PO_4, 1.0g $(NH_4)_2SO_4$, 0.05g $MgSO_4$, 15g agar, and 1000 ml distilled water. As carbon source, 4g of either glycerol or glucose was added per liter: glycerol for studies of nutritional properties and syntrophy; glucose for all other experiments. Single- or double-enriched medium was prepared by supplementing minimal medium with 0.01 or 0.02% dehydrated broth. Soft agar medium contained 0.75% agar.

Techniques for making the crosses between Hfr and F^- strains are described in SANDERSON and DEMEREC (1965).

Tests for complete transduction were carried out by mixing directly, on a plate of minimal or enriched-minimal agar medium, recipient bacterial cells from a saturated broth culture with phage grown on a certain donor strain of bacteria, at a multiplicity of 5. Transductants were scored after incubation at 37°C for 48 hours. To observe abortive transduction, about 8×10^8 recipient cells were spread on minimal medium. Each plate was then divided into four to six sections, and a drop of a different phage suspension (about 10^{10} phage particles per ml) was placed on each section. One of the two control sections received phage grown in wild-type bacteria, the other phage grown in the recipient bacteria. After 20 hours incubation at 37°C, abortive transductants were observed under the low-power microscope as minute colonies. Abortive-transduction colonies appeared on the control section that had wild-type phage, and on the experimental sections if the mutants involved complemented each other.

[1] Research carried out at Brookhaven National Laboratory under the auspices of the U.S. Atomic Energy Commission.
[2] Present address: Space Sciences Laboratory, University of California, Berkeley 4, California.

TABLE 1

*Classification of pyrimidine mutants, according to their origin, revertibility and gene loci**

Origin	Revertibility: Spontaneous: Stable	Revertibility: Spontaneous: High or leaky	Revertibility: Inducible by DES and AP: High by DES	Revertibility: Inducible by DES and AP: Low by DES	Revertibility: Inducible by DES only	Revertibility: Not inducible by DES and AP	Total
Spontaneous†	*A160*; *C73*						2
Induced by:							
AP‡		A45,46,47,48	A35,36,39,42,43	A30,32,33,34			45
		B195	B16,28,51	37,38,40,41			"
		D15	C7,8,14,18,19	44			"
		E11,17,50	25,29,193	C21			"
			D13,23,24				"
			E9,20,22,26				"
			F10,52,194				"
DES	D203		C206	D197; E201	D199		5
DBU			C190				1
UV			F1	A2; C55	C53,54		5
NA	*B137*	A147,156	F142,144	A150,152	A149	C145	18
	C138	E141,143		C136,139			"
	D135			F140			"
	F134,146	A147,156					"
X rays	*A78,81*	F61,70,74	B62,65	A79,82	C71	C76	18
	B64		C66	B63			"
	C69.75,77		D67				"
FN	*A102*	B90	A98,107	A99,104,105	A101,103	A100,108,128	44
	B118,124	F114	B88,116,129	109	106,127	B85	"
	C97		C87,94	B92,120	130,131		"
	D95,121		E84,91	C126	B96		"
	E123,125		F115,119,122		C86		"
	F93,117				F89		"

* Mutants in italics cover one or more sites and are presumably deletions (Figure 2).

† Mutant *A160* was obtained from cells kept in acetate buffer at pH 4.4 for 30 minutes, from controls for nitrous acid experiments; mutant *C73* is a deletion obtained in LT-7 strain which carries a mutator.

‡ Abbreviations: AP = 2-aminopurine; DBU = 5-deoxybromouridine; DES = diethyl sulphate; FN = fast neutrons; NA = nitrous acid; UV = ultraviolet radiation.

Growth requirements were determined either by streaking suspensions of cells on minimal agar medium containing various pyrimidine compounds (20 μg/ml), or by spreading them on minimal medium and placing crystals or drops of solutions of various compounds on the plates. Syntrophism (feeding) among the mutants was tested by streaking bacterial suspensions at right angles on minimal agar medium. Plates were incubated for 48 hours.

All induced-reversion studies were carried out semiquantitatively by the spot-test method, with diethyl sulphate (DES), 2-amino-purine (AP), and nitrous acid (NA) as the agents. Each plate of enriched-minimal agar medium was spread with about 2×10^8 bacterial cells from a saturated broth culture. In tests with DES, a drop of undiluted DES was placed near the edge of the plate; in tests with AP, 0.1 ml of a 0.2% solution was spread over a rectangular area about 2 cm wide; and with NA, 0.1 ml of a freshly made solution (5 M $NaNO_2$ in 0.2 sodium acetate buffer. pH 4.4) was similarly spread. Revertants were scored after 3 days incubation at 37°C. All chemicals were obtained commercially.

RESULTS

The 141 pyrimidine mutants investigated in this study fall into six groups separable by either transduction, conjugation, syntrophism, or nutritional requirements. Ample evidence supports the conclusion that each group represents at least one gene locus, and so each is identified by the abbreviation specifying pyrimidine mutants (*pyr*) plus a capital letter symbolizing the locus (*pyrA*, *pyrB* . . . *pyrF*). A mutation in any of these six loci is responsible for blocking one step in the pathway of pyrimidine synthesis (Figure 1). Of our mutants 48 are *pyrA* alleles, 18 are *pyrB*, 30 *pyrC*, 12 *pyrD*, 14 *pyrE*, and 19 *pyrF*, as follows:

pyrA: 2, 30, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 78, 79, 81, 82, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 127, 128, 129, 130, 131, 147, 149, 150, 151, 152, 155, 156, 160.

pyrB: 16, 28, 51, 62, 63, 64, 65, 85, 88, 90, 92, 96, 116, 118, 120, 124, 137, 195.

pyrC: 7, 8, 14, 18, 19, 21, 25, 29, 53, 54, 55, 66, 69, 71, 73, 75, 76, 77, 86, 87, 94, 97, 126, 136, 138, 139, 145, 190, 193, 206.

pyrD: 13, 15, 23, 24, 67, 95, 121, 135, 197, 199, 200, 203.

pyrE: 9, 11, 17, 20, 22, 26, 50, 84, 91, 123, 125, 141, 143, 201.

pyrF: 1, 10, 52, 61, 70, 74, 89, 93, 114, 115, 117, 119, 122, 134, 140, 142, 144, 146, 194.

Complementation was observed in all crosses between the different groups, except for *pyrA* and *B*, where recipients suitable for detecting abortive transduction are not available. Reciprocal crosses were made between representative

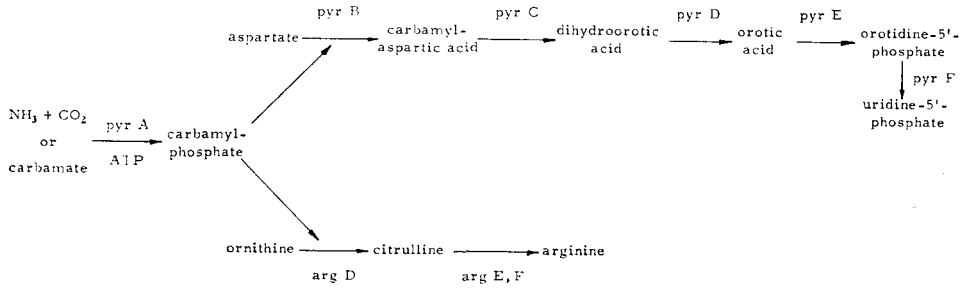

FIGURE 1.—Pathway of biosynthesis of uridine-monophosphate and its relation to biosynthesis of arginine (Adapted from CROSBIE 1960).

markers of all the other loci; *pyrA* and *B* mutants were used as donors only. Numbers of stable transductants were invariably lower in crosses between alleles of the same locus than in crosses between mutants of different loci.

The growth requirement of *pyrA* mutants was satisfied by uracil or orotic acid plus arginine or citrulline (but not ornithine). Slight growth occurred when dihydroorotic or carbamyl aspartic acid was substituted for uracil. Several alleles (*pyrA32, 42, 43, 104, 151, 155* and *156*) were able to grow partially on arginine or citrulline alone. Another group of alleles (*pyrA45, 46, 48*) grew slowly on the minimal medium supplemented with a low concentration of (1–5 μg/ml) of arginine (but not citrulline); with a high concentration of arginine (20 μg/ml), they required uracil as well. These are leaky mutants. None of the *pyrA* mutants responded to supplements of carbamyl phosphate.

When grown on double-enriched medium supplemented with arginine (or citrulline), five *pyrA* mutants (*30, 35, 38, 104* and *127*) out of 15 tested (*2, 30, 33, 34, 35, 36, 37, 38, 39, 41, 78, 81, 104, 127* and *131*) gave rise to large- and small-colony revertants. The large-colony revertants were wild type, while small-colony revertants could grow on medium supplemented with arginine (or citrulline) but not on minimal medium or medium supplemented with uracil or ornithine. Thus the change that had occurred in these mutants affected their phenotypic expression only partially, modifying the requirement from arginine plus uracil to arginine. Further tests by transduction (Table 2) showed that suppressor mutations were responsible for the appearance of the small colonies; and that a suppressor originating in a culture of one mutant (*pyrA30*) could affect another mutant that gave rise to suppressor mutations (*pyrA35*), but not a mutant in whose cultures suppressors did not appear (*pyrA2, 78*).

Nutritional requirements of the other five groups of pyrimidine mutants are shown in Table 3. None of the mutants responded to orotidylic acid, probably because the compound could not penetrate the cells. All the groups responded to uracil. By their responses to the other three compounds tested, all except *E* and *F* are nutritionally distinguishable.

Syntrophism tests showed that *pyrF* mutants feed *B*, *C*, and *D*; *pyrE* feed *B*, *C* and *D*;*pyrD* feed *B* and *C*; and *pyrC* feed *B*.

Approximate locations of the *pyr* loci were determined by conjugation experiments. The average times of entry, in minutes, of various loci for HfrA were: *pyrB*, 24; *serB*, 26; *pyrA*, 28; *pyrC*, 65; *pyrD*, 66; *purB*, 68; *tryD*, 77. For HfrB2 the times of entry were *his*, 13; *pyrE*, 85. According to tests with the donor strain SR315 the *pyrF* locus is close to *try*.

The position of *pyrD* in relation to neighboring *gal* and *purB* loci was determined by a cross between F⁻ *gal*⁺ *pyrD197 purB210 his*⁺ and HfrA *gal50 pyrD*⁺ *purB*⁺ *his* 23, in which the histidine was used for counter-selection of the Hfr. By selection for *purB*⁺ and subsequent testing for the unselected markers (*gal* and *pyrD*), the following recombinant classes were obtained: *gal*⁺ *pyr*, 119; *gal pyr*, 17; *gal*⁺ *pyr*⁺, 65; and *gal pyr*⁺, 71. The *gal pyrD purB*⁺ class occurs with a frequency of $17/272 = 6.3\%$, considerably lower than any of the other three classes. Under the assumption that the order of the loci is *gal pyrD purB*, this is

TABLE 2

Numbers of large (L) and small (S) colonies appearing on minimal medium (min) and minimal supplemented with arginine (min + arg) in transduction experiments made with phages grown on pyrA30, pyrA35, *and bacteria derived from the small colonies of these strains*

	Recipients															
	pyrA30				*pyrA35*				*pyrA2*				*pyrA78*			
	min		min+arg		min		min+arg		min		min+arg		min		min+arg	
Donors	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S
pyrA30	1	0	1	1	4	0	5	1	.	.	.	.	.	.	.	.
35	3	0	8	4	1	0	2	7	.	.	.	.	.	.	.	.
Small colonies from:																
pyrA30-1	2	0	3	32	4	0	3	35	.	.	.	.	.	.	.	.
-2	5	0	2	80	2	0	4	62	4	0	6	0	6	0	4	0
pyrA35-1	8	0	7	~350	1	0	1	~350	3	0	6	0	4	0	5	0
-2	2	0	3	~150	2	1	3	~300	.	.	.	.	.	.	.	.

TABLE 3

Growth requirements of pyrB, C, D, E *and* F *mutants*

Locus	CAA*	DHOA	OA	U
pyrB	+	+	++	+++
C	—	+	+++	+++
D	—	—	+++	+++
E	—	—	—	+++
F	—	—	—	+++

* Abbreviations: CAA=carbamyl aspartic acid; DHOA=dihydroorotic acid; OA=orotic acid; U=uracil. None of the mutants responded to orotidylic acid.

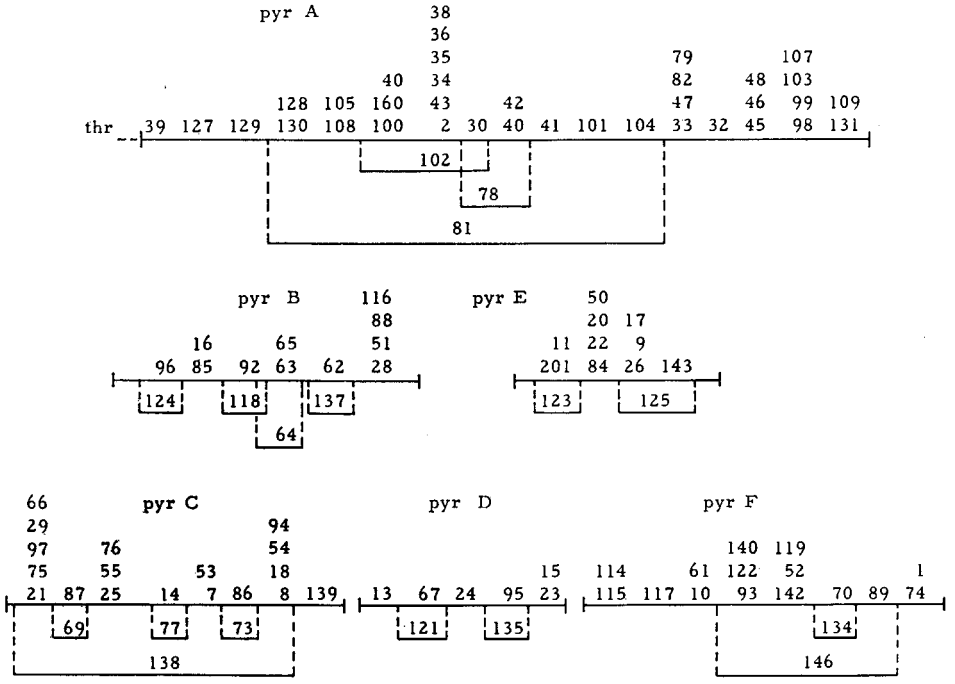

FIGURE 2.—Genetic maps of six pyrimidine loci.

a quadruple-crossover class whereas the other classes require only two crossover events. The low frequency of this class supports the order *gal pyrD purB*. These data suggest the following order of loci: *pyrB serB pyrA gal (pyrC pyrD) (pyrF try) his pyrE*. The order of loci within brackets is not known. The location of these loci on an over-all linkage map of *S. typhimurium* is shown by SANDERSON and DEMEREC (1965).

Genetic maps of the *pyr* loci (Figure 2) were prepared by first establishing which mutant sites are included in each of several multisite deletions found in every locus, and then approximately determining the order of sites within and between deletions from the frequencies of recombinants obtained in transduction intercrosses. The relative order of particular mutant sites is definite only when determined by deletions, otherwise it is approximate. Mutants listed one above the other are located close together rather than at the same site.

DISCUSSION

Six gene loci controlling the synthesis of pyrimidine in *S. typhimurium* (*pyrA*, *B*, *C*, *D*, *E*, *F*) have been identified by analyses of 141 mutants in terms of nutritional properties, complementation, syntrophism and location on a genetic map. By every one of these criteria the *pyr* gene loci of Salmonella are homologous to six similar loci analyzed in *E. coli* (BECKWITH *et al.* 1962; TAYLOR *et al.*

1964; A. L. TAYLOR, personal communication, who obtained evidence that *pyrF* is located close to *try*).

Two of the loci (*pyrC, D*) are located very close together, probably forming a cluster. Cotransduction tests, however, could not be carried out between them, and so it was not possible to determine whether they are in the same or different transducing fragments.

Almost all the pyrimidine-requiring mutants used in this study had been induced by mutagens (see Table 1). Originating in a number of different experiments, they were not suitable for comparison of frequencies of induction of pyrimidine mutants by different mutagens. The number of mutants of spontaneous origin, on the other hand, was so small as to merit attention. Only two were found (see Table 1), and since one of them (*pyrA160*) was isolated from cells suspended for 30 minutes in acetate buffer at pH 4.4 (a control treatment in nitrous acid experiments) it may or may not be a spontaneous mutant. The other was found in strain LT-7 which carries the mutator gene.

Most of the pyrimidine mutants were obtained in experiments designed to study the frequency of deletions among spontaneous and induced mutations in the *cysC* region of *S. typhimurium*. In those experiments all the auxotrophs detected in treated and untreated (control) cultures were isolated, identified, and kept in our stock collection. The numbers of *cysC*-region mutants found in the treated materials were: 2-aminopurine, 58; ultraviolet light, 21; nitrous acid, 52; X rays, 51; and neutrons, 37. From the untreated material 59 spontaneous *cysC* mutants were collected. The most striking divergence between these values and those for pyrimidine mutants is in the small number of spontaneous pyrimidine-requiring mutants. Even the two spontaneous pyrimidine mutations may be due to complex changes, since both are stable and one of them (*pyrC73*) covers another single-site mutation (Figure 2) and is probably a multisite (deletion) change. Since the pyrimidine and the *cysC*-region mutants were collected in the same set of experiments, with similar techniques of isolation and identification, it is very unlikely that some technical variation caused the observed difference. It must be due to some other factor or factors, responsible either for a very low frequency of spontaneous occurrence of mutations in pyrimidine loci or for elimination of the mutants under our experimental conditions.

The mutability pattern of the pyrimidine loci shows no other unusual features. Multisite mutations (deletions) are not rare; they may even be more frequent than among most other groups of mutations. They appear spontaneously, and our data are sufficient to show that they are also induced by X rays, fast neutrons and nitrous acid, but not by 2-aminopurine. This is a pattern similar to that observed in studies of *cysC*-region mutants (DEMEREC 1960 and unpublished material). The pattern of reverse mutability of *pyr* mutants is also similar to that observed in work with cysteine mutants (EISENSTARK and ROSNER 1964), and agrees with predictions of the model proposed by FREESE (1963). Mutants induced by AP, all of which are supposedly transition changes, can all be reverted by AP; whereas among the other mutants, which may be either transitions or

transversions, some cannot be reverted by AP. Seven of the induced pyrimidine mutants are mutagen stable; that is, they may revert spontaneously but the frequencies of reversion cannot be increased by mutagens that have been tested (DEMEREC and CAHN 1953).

Biochemical analyses of pyrimidine mutants in *E. coli* have shown that a mutation in the *pyrB*, *C*, *D*, *E* or *F* locus leads to loss of activity of the corresponding enzyme (YATES and PARDEE 1957; BECKWITH *et al.* 1962) but that a mutation in locus *pyrA* does not result in any significant loss of activity of carbamyl phosphokinase, which is presumably controlled by that gene (BECKWITH *et al.* 1962; THORNE and JONES 1963; KANAZIR *et al.* 1959). In Neurospora, it has been observed that two loci control the synthesis of carbamyl phosphate, mutations in one causing an arginine requirement, those in the other a uracil requirement. Thus there are two carbamyl phosphate pools, which under special circumstances may feed into each other (DAVIS 1963). Although more extensive experiments are necessary for proof, it seems probable that such a scheme may operate in *S. typhimurium* as well as in Neurospora. This conclusion is based on the observation that some *pyrA* alleles (*pyrA32*, *42*, *43*, *104*, *151*, *155* and *156*) can grow weakly on arginine-supplemented minimal medium, and that in others a mutation in a suppressor locus is able to eliminate their pyrimidine requirement.

The authors wish to express appreciation to DR. K. E. SANDERSON for many stimulating discussions and comments, and to MISS AGNES C. FISHER for help in editing the manuscript.

SUMMARY

Genetic analyses of 141 pyrimidine (*pyr*) mutants have shown that at least six gene loci control the pathway of biosynthesis leading to production of uridylic acid. Two of these loci, *pyrC* and *D*, are close together on the genetic map and are probably members of a cluster of genes controlling related functions. The other four are located in different portions of the chromosome. With respect to function and position on the genetic map, these six *pyr* loci of *S. typhimurium* appear to be homologous to six *pyr* loci described in *E. coli* (BECKWITH *et al.* 1962; TAYLOR *et al.* 1964).

Mutants of the *pyrA* locus require uracil or orotic acid plus arginine or citrulline, but several of the alleles are able to grow partially on arginine or citrulline. A suppressor has been found that modifies the requirement in certain other *pyrA* mutants from arginine plus uracil to arginine alone.

Genetic mapping within individual loci indicates that multisite mutations are not rare.

Pyrimidine mutants are readily induced by 2-aminopurine, diethyl sulfate, ultraviolet radiation, X rays, fast neutrons and nitrous acid, but they very rarely occur spontaneously. Multisite mutations can occur spontaneously, and are found among the mutants induced by all the mutagens tested except 2-aminopurine.

LITERATURE CITED

BECKWITH, J. R., A. B. PARDEE, R. AUSTRAIN, and F. JACOB, 1962 Coordination of the synthesis of the enzymes in the pyrimidine pathway of *E. coli*. J. Mol. Biol. **5**: 618–634.

CROSBIE, G. W., 1960 Biosynthesis of pyrimidine nucleotides. pp. 323–348. *Nucleic Acids*, Vol. 3. Edited by E. CHARGAFF and J. N. DAVIDSON. Academic Press, New York.

DAVIS, R. H., 1963 Neurospora mutant lacking an arginine-specific carbamyl phosphokinase. Science **142**: 1652–1654.

DEMEREC, M., 1960 Frequency of deletions among spontaneous and induced mutations in Salmonella. Proc. Natl. Acad. Sci. U. S. **46**: 1075–1079.

DEMEREC, M., and E. CAHN, 1953 Studies of mutability in nutritionally deficient strains of *Escherichia coli*. J. Bacteriol. **65**: 27–36.

EISENSTARK, A., and J. L. ROSNER, 1964 Chemically induced reversions in the *cysC* region of *Salmonella typhimurium*. Genetics **49**: 343–355.

FREESE, E., 1963 Molecular mechanism of mutations. pp. 207–269. *Molecular Genetics*, Vol. 1. Edited by J. H. TAYLOR. Academic Press, New York.

KANAZIR, D., H. D. BARNER, J. G. FLAKS, and S. S. COHEN, 1959 Some physiological and genetic properties of a strain of *Escherichia coli* requiring thymine, arginine and uracil. Biochim. Biophys. Acta **34**: 341–353.

SANDERSON, K. E., and M. DEMEREC, 1965 The linkage map of *Salmonella typhimurium*. Genetics **51**: 897–913.

TAYLOR, A. L., J. R. BECKWITH, A. B. PARDEE, R. AUSTRIAN, and F. JACOB, 1964 The chromosomal location of the structural gene for orotidylic acid pyrosphosphorylase in *Escherichia coli*. J. Mol. Biol. **8**: 771.

THORNE, K. J. I., and M. E. JONES, 1963 Carbamyl and acetyl phosphokinase activities of *Streptococcus faecalis* and *Escherichia coli*. J. Biol. Chem. **238**: 2992–2998.

YATES, R. A., and A. B. PARDEE, 1957 Control by uracil of formation of enzymes required for orotate synthesis. J. Biol. Chem. **227**: 677–692.