MUTATIONAL SITE OF THE GENE CONTROLLING QUANTITATIVE THYMINE REQUIREMENT IN *ESCHERICHIA COLI* K-12

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Received July 12, 1966

NUMEROUS thymineless mutants of *E. coli*, Salmonella, and other bacteria can be obtained by the aminopterin and thymine method (AM-T method) (OKADA, YANAGISAWA and RYAN 1961; OKADA, HOMMA and SONOHARA 1962). However, in order to sustain normal growth, all the thymineless mutants thus obtained require ten to 20 times the concentration of thymine required by *E. coli* 15 T^- (COHEN and BARNER 1954). Similar observations were also reported by BREITMAN and BRADFORD (1964) and by HARRISON (1965). Since a number of mutants with a low thymine requirement, similar to *E. coli* 15 T^-, have been spontaneously obtained from the high thymine requirers, it is very likely that low thymine requirers are derived from high as a result of a second mutation. Thus, if the genes concerned with the first and second mutations were designated as *thy* and *thy-R* respectively, the mutational sequences may be expressed:

$$\text{Wild type} \xrightarrow[\text{mutation at } thy]{} \text{Thy}_{20}^- \xrightarrow[\text{mutation at } thy\text{-}R]{} \text{Thy}_2^-$$

Thy_{20}^- and Thy_2^- are thymineless mutants which require for normal growth about 20 μg and 2 μg thymine per ml, respectively. Thy_2^- is, therefore, genotypically expressed as a double mutant, $thy^-thy\text{-}R^-$. The gene *thy* is supposed to be the structural gene of thymidylate synthetase (BARNER and COHEN 1959), and it is located between the streptomycin (*str*) and histidine (*his*) loci (KITSUJI 1964; ISHIBASHI, SUGINO and HIROTA 1964). Although the precise function of the second gene, *thy-R*, has not yet been elucidated, mutation of *thy-R* apparently results in increased efficiency in the utilization of thymine.

In the present study, the second gene *thy-R* is shown to be far distant from *thy*, and to be linked closely to *thr* on the opposite side from *leu*.

MATERIALS AND METHODS

Bacterial strains: The strains employed were derivatives of *Escherichia coli* K-12. Their characteristics are presented in Table 1. HfrH(3000) was supplied by DR. TSUTOMU WATANABE.

Media: Glucose Simmons (GS) medium, used as minimal medium, was described by OKADA, HOMMA and SONOHARA (1962). The following supplements, when required, were added to the medium: DL-threonine (T), 100 μg/ml; L-leucine (L), 20 μg/ml; L-methionine (M), 20 μg/ml; thiamine (Thi), 1.0 μg/ml; thymine (Thy), 50 μg/ml (Thy_{50}) for Thy_{20}^-, 2.0 or 5.0 μg/ml

TABLE 1

List of bacterial strains

Strain No.	Origin	Genotype	Order of transfer
HfrH(3000)		Hfr, *thi*$^-$	O, *thr*, *leu*, *lac*, ...
HfrP4 X 6		Hfr, *met*$^-$	O, *T1*, *az*, *leu*, *thr*, ...
HfrH(3000)-11	HfrH(3000)	Hfr, *thi*$^-$, *thy*$^-$, *thy-R*$^-$(Thy$_2^-$)	O, *thr*, *leu*, *lac*, ...
HfrP4 X 6-11	HfrP4 X 6	Hfr, *met*$^-$, *thy*$^-$, *thy-R*$^-$(Thy$_2^-$)	O, *T1*, *az*, *leu*, *thr*, ...
Y-70		F$^-$, *thr*$^-$, *leu*$^-$, *thi*$^-$, *lac*$^-$, *str*^{r}	
Y-70-2	Y-70	F$^-$, *thr*$^-$, *leu*$^-$, *thi*$^-$, *thy*$^-$, *thy-R*$^+$, *lac*$^-$, *str*^{r} (Thy$_{20}^-$)	
Y-70-22	Y-70-2	F$^-$, *thr*$^-$, *leu*$^-$, *thi*$^-$, *thy*$^-$, *thy-R*$^-$, *lac*$^-$, *str*^{r}, (Thy$_2^-$)	

Abbreviations: *thr*, threonine; *leu*, leucine; *thi*, thiamine; *met*, methionine; *lac*, lactose; *T1*, T1 phage resistant; *az*, azide resistant; *str*^{r}, streptomycin-resistant; *thy*, the gene controlling the step from wild type to high thymine requirer; *thy-R*, the gene controlling the step between high thymine requirer to low thymine requirer; Thy$_2^-$, the strain requiring thymine at low concentration (ca. 2.0 μg/ml); Thy$_{20}^-$, the strain requiring thymine at high concentration (more than 20.0 μg/ml). All thymineless mutants were obtained by using the aminopterin-thymine method (OKADA, HOMMA and SONOHARA 1962).

(Thy$_2$ or Thy$_5$) for Thy$_2^-$; dihydrostreptomycin (Meiji Seika Kaisha, Japan) (Sm), 200 μg/ml.

For complete medium, nutrient agar having the following composition was used: NaCl, 5.0 g; polypeptone, 10 g; meat extract (Wako's Ehrlich Meat Extract), 5.0 g, in 1,000 ml of distilled water. EMB-Lac-Sm agar described by LEDERBERG (1947) was also used for selecting *lac*$^+$ *str*^{r} recombinants.

Method of obtaining Thy$_{20}^-$ and Thy$_2^-$ mutants: The thymineless mutants isolated by the AM-T method (OKADA, HOMMA and SONOHARA 1962) usually, if not always, grow poorly on nutrient agar, and form thin colonies which are easily distinguishable from wild-type colonies. These *thy*$^-$ mutants require a high concentration of thymine (at least 20 μg per ml) for normal growth. The thin colonies usually give rise to papillae after prolonged incubation; these are usually Thy$_2^-$ mutants, although some of them are wild-type revertants, *thy*$^+$.

Mating procedure: The Hfr and F$^-$ strains were grown overnight in GS(T,L,Thi,M,Thy$_{50}$) or GS(T,L,Thi,Thy$_{50}$) without shaking. They were then diluted approximately 1:50 in fresh medium and reincubated with shaking at 37°C until they were in the exponential phase. Optimal densities of the cultures were measured at 660 mμ with a Shimadzu-Bausch-Lomb Spectronic 20. An OD$_{660}$ of 0.1 was equivalent to about 1.0 × 10^8 cells/ml. Conjugations were carried out by mixing 0.2 ml of the donor and 1.8 ml of the recipient cultures in each of 12 L-shaped tubes which were incubated at 37°C. The cell concentrations of the parent cultures are described in the legend of each figure. Each tube was placed in a horizontal position so as to provide good aeration without shaking. Unless otherwise stated, a 0.5 ml sample was withdrawn from one of the tubes at intervals, and diluted with 4.5 ml of sterile saline. The diluted sample was then subjected to violent agitation (PITTARD and ADELBERG 1964) for 30 seconds on a Thermomixer (Thermonics Co., Japan) to interrupt mating. Then, 0.10 ml of the sample was spread with or without further dilution, on the appropriate selective agar medium. After about 40 hours incubation at 37°C, visible colonies were counted.

Scoring unselected markers: Except where noted, 148 recombinant colonies were transferred to master plates, and after overnight incubation were replica plated on the appropriate selective media (LEDERBERG and LEDERBERG 1952). The results were scored after 10 to 16 hours incubation at 37°C.

RESULTS

Effect of thymine concentrations on the growth of Thy$_{20}^-$ and Thy$_2^-$: Figure 1 illustrates the typical thymine requirement of the two types of thymineless

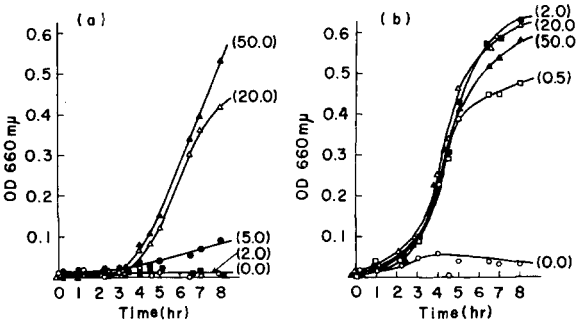

FIGURE 1.—Effect of thymine concentration on growth of Thy_{20}^- and Thy_2^- mutants. (a) Y–70–2 (Thy_{20}^-) was grown overnight in GS(T,L,Thi,Thy_{50}) liquid medium. The cells were washed twice with saline and suspended in GS(T,L,Thi) liquid medium containing various concentrations of thymine, and incubated at 37°C with shaking. At intervals, optical densities of the culture were measured at 660 mμ. (b) Y–70–22 (Thy_2^-) was grown overnight in GS(T,L, Thi,Thy_2) liquid medium. The experimental procedures are identical with those in (a). The numbers in parenthesis indicate the concentration (μg/ml) of thymine.

mutants. More than 20 μg of thymine per ml was necessary for normal growth of Y–70–2, a high thymine requirer (*thy⁻thy-R⁺*) while 2.0 μg thymine per ml was enough to sustain maximum growth for Y–70–22, a low thymine requirer (*thy⁻thy-R⁻*). No growth or slight growth of the high thymine requirer was observed at a concentration of 2.0 to 5.0 μg thymine per ml. The addition of 5.0 μg of thymine per ml in agar medium, therefore, makes it possible to score *thy⁻thy-R⁻* separately from *thy⁻thy-R⁺*.

Kinetics of thy-R⁺ *gene transfer in a cross of HfrP4X6 or HfrH(3000) × Y–70–22:* A preliminary experiment suggested that *thy-R* is probably located near the threonine (*thr*) and leucine (*leu*) loci. In order to determine the exact position of *thy-R* on the linkage map, interrupted mating experiments were carried out. Typical results of the cross HfrP4X6 × Y–70–22 are presented in Figures 2a and b. As expected from the characteristics of the Hfr strain, the frequency of *leu⁺* among *thr⁺* recombinants was almost constant (78%) throughout the mating time, while that of *thr⁺* among *leu⁺* recombinants increased with time and reached a constant value (70%). Few or no *thy⁺*, *thi⁺* and/or *lac⁺* among *leu⁺* or *thr⁺* recombinants were found. The frequency of *thy-R* among *thr⁺* and *leu⁺* increased with mating time, suggesting that *thy-R⁺* may be transferred just later than *thr⁺* with this Hfr strain (JACOB and WOLLMAN 1961).

The above suggestion was confirmed by a cross of HfrH(3000) × Y–70–22, in which chromosome transfer occurs in the opposite direction from the first cross. The results (Figures 3a, b) indicate that chromosome transfer may occur in the order of *thy-R⁺*, *thr⁺*, *leu⁺*, *lac⁺* and that *thy-R* is very closely linked to *thr*.

In Table 2, the distribution of unselected markers among *thr⁺* or *leu⁺* is presented for both crosses. It is noteworthy that no *thr⁻thy-R⁺* is found among *leu⁺* recombinants, although there are significant numbers (8 to 9%) of *thr⁺thy-R⁻* among the *leu⁺*; this indicates strongly that *thr* is located between *thy-R* and *leu*.

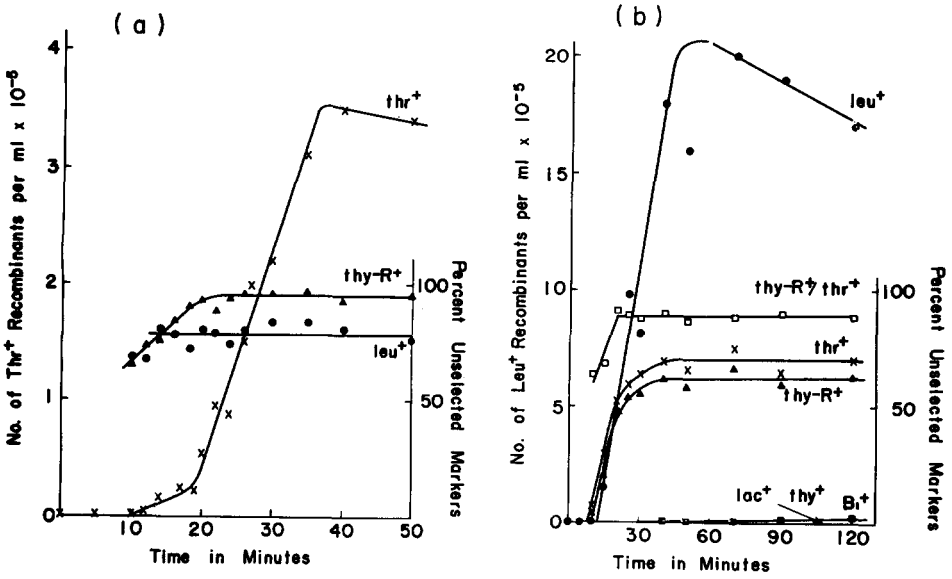

FIGURE 2.—Kinetics of chromosome transfer from HfrP4 X 6 (order of chromosome transfer *leu*, *thr*, *thi*, ----) to Y-70-22. Cells in the exponential phase of growth were mixed in the proportion, 0.2 ml male and 1.8 ml female cultures. Conjugation was interrupted by violent agitation for 30 sec. by a Thermomixer at periodic intervals. (a) The selected markers were *thr*+*str*^{r} and the unselected markers were *thy-R*+, *leu*+, *thi*+, *thy*+ and *lac*+. The final concentration of males in the mating mixture at 0 time was 4.3×10^7/ml and of females, 4.8×10^8/ml. (b) The selected markers were *leu*+*str*^{r} and the unselected markers were *thy-R*+, *thr*+, *thi*+, *thy*+, and *lac*+. The final concentration of males in mating mixture at 0 time was 3.8×10^7/ml and of females, 4.6×10^8/ml. In both (a) and (b), the unselected markers were scored by replica plating with appropriate selective media for 148 recombinant colonies at each time interval. The distributions of the various unselected markers per 100 recombinants are plotted against the time of sampling and exposure to mechanical agitation. The ratio of *thy-R*+/*thr*+ is also plotted against mating time in (b).

Kinetics of thy-R$^-$ *gene transfer and segregation of* thy-R$^-$: Although *thy-R*$^+$ cannot be used as a selected marker, *thy-R*$^-$ associated with *thy*$^-$ can be utilized as a selected marker with medium containing 5.0 μg thymine per ml. Strain HfrP4X6–11 or HfrH(3000)–11, both of which are *thy*$^-$*thy-R*$^-$, was used as donor

TABLE 2

Frequencies of occurrence of unselected markers in thr+ *or* leu+ *recombinants**

Selected markers	*leu*+				*thr*+			
Unselected markers	*thr*$^-$ *thy-R*$^-$	*thr*+ *thy-R*$^-$	*thr*$^-$ *thy-R*+	*thr*+ *thy-R*+	*leu*$^-$ *thy-R*$^-$	*leu*+ *thy-R*$^-$	*leu*$^-$ *thy-R*+	*leu*+ *thy-R*+
Donor	%	%	%	%	%	%	%	%
HfrP4 X 6	33.8	8.8	0	57.5	0.7	4.7	23.0	71.6
HfrH(3000)	12.3	8.2	0	79.8	2.7	4.1	25.0	68.3

* The conditions were identical with those in Figures 2 and 3. The proportions were calculated from the results at 50 minutes mating. The total number of colonies tested was 148 in each case. Recipient strain was Y-70-22.

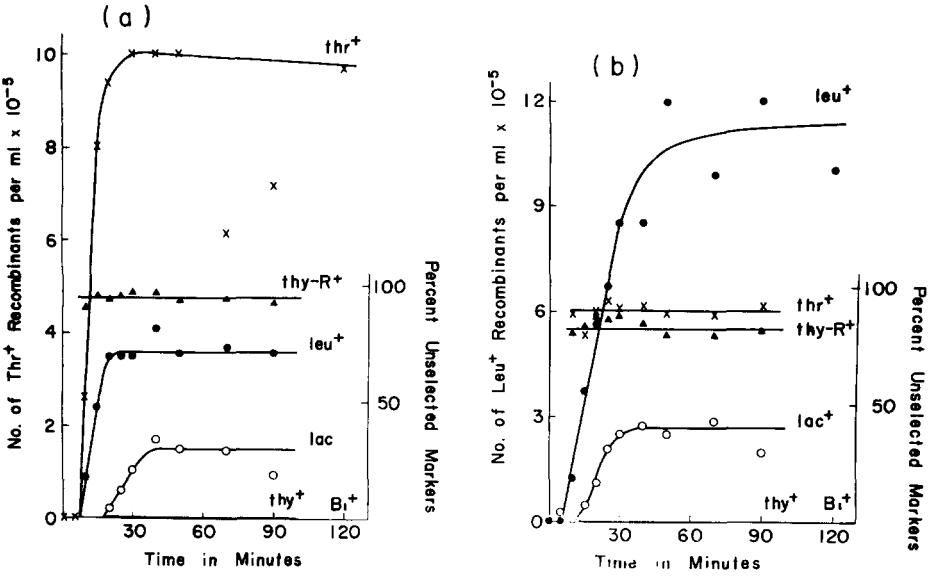

FIGURE 3.—Kinetics of chromosome transfer from HfrH(3000) (order of chromosome transfer: O, *thr*, *leu*, *lac*, ---) to Y-70-22. Cells in the exponential phase of growth were mixed in the proportion, 0.2 ml male and 1.8 ml female cultures. Conjugation was interrupted by being subjected to violent agitation for 30 sec by a Thermomixer at periodic intervals. (a) The selected marker was *thr*+*str*^{r}, and the unselected markers were *leu*+, *thy-R*+, *lac*+, *thy*+ and *thi*+. The final concentration of males in the mating mixture at 0 time was 2.2×10^7/ml, and of females, 4.7×10^8/ml. (b) The selected marker was *leu*+*str*^{r} and the unselected markers were *thr*+, *thy-R*+, *lac*+, *thy*+, and *thi*+. The final concentration of males in the mating mixture at 0 time was 2.2×10^7/ml, and of females, 4.4×10^8/ml. In both (a) and (b), the unselected markers were scored by replica plating with appropriate selective media for 148 recombinant colonies at each time interval. The distributions of the various unselected markers per 100 recombinants are plotted against the time of sampling and exposure to mechanical agitation.

and Y-70-2 (*thy*$^-$*thy-R*+*str*^{r}) was used as recipient (see Table 1). Since *thr* and *thy-R* are very closely linked, their kinetics of transfer were expected to be similar. As seen in Figures 4a and b, *thy-R*$^-$ entered the zygote simultaneously with *thr*$^+$ and *leu*$^+$. Since 90% of *thr*$^+$ and 60% of *leu*$^+$ recombinants carry *thy-R*$^+$ (Table 3), the low frequency of *thy-R*$^-$ recombinants is obviously attributable to unfitness of the selective media for the isolation of *thy-R*$^-$ recombinants.

Thus an aliquot of mating mixture at 40 minutes was diluted tenfold in fresh GS(T,L,Thi,M,Thy$_{50}$,Sm) liquid medium and agitated to interrupt the mating. This culture was incubated further without shaking. During this incubation period, the numbers of *thr*$^+$, *leu*$^+$ and *thy-R*$^-$ recombinants in the culture were measured at various intervals. The results are shown in Table 4. The number of *thy-R*$^-$ recombinants increased sharply, while *thr*$^+$ or *leu*$^+$ remained constant or decreased slightly; hence the ratios of *thy-R*$^-$/*thr*$^+$ and of *thy-R*$^-$/*leu*$^+$ increased with incubation time.

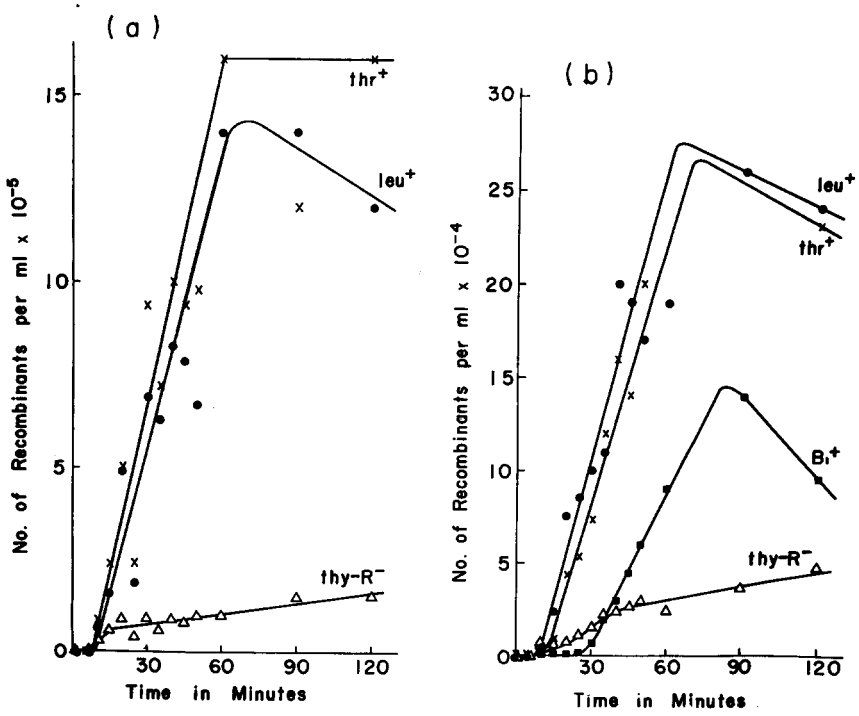

FIGURE 4.—Interrupted mating experiment between (a) HfrH(3000)–11 or (b) HfrP4X6–11 (*thy*⁻, *thy-R*⁻, *str*^{s}) and Y–70–2 (*thy*⁻, *thy-R*+, *str*^{r}). Cells in the exponential phase of growth were mixed in the proportion, 0.2 ml male and 1.8 ml female. Conjugation was interrupted by being subjected to violent agitation for 30 sec with a Thermomixer at periodic intervals. Recombinant selection was achieved by counterselection of streptomycin. (a) The final concentration of males in the mating mixture at 0 time was 4.3×10^7/ml, and of females, 4.9×10^8/ml. (b) The final concentration of males in the mating mixture at 0 time was 4.7×10^7/ml, and of females, 4.4×10^8/ml.

The frequencies of unselected markers in *thr*+, *leu*+ or *thy-R*⁻ recombinants at 90 minutes in the kinetic experiment are given in Table 3.

The order of frequencies of unselected markers in each kind of recombinant is completely compatible with the conclusion that *thy-R*, on the opposite side from *leu*, is linked to *thr* more closely than is *leu* (Figure 5).

TABLE 3

Frequencies of occurrence of unselected markers in thy-R⁻, thr+ *or* leu+ *recombinants**

Time (min)	Selected markers	Unselected markers (%)				
		thi+	*thy-R*⁻	*thr*+	*leu*+	*thy*+
90	*thy-R*⁻	1.8	100.0	80.2	56.7	0
	thr+	9.9	92.8	100.0	68.5	0
	leu+	5.4	60.2	64.8	100.0	0

* The total number of recombinant colonies tested was 111 in each case. Donor, HfrP4X6–11; Recipient, Y–70–2; Mating medium, GS(T,L,Thi,M,Thy_{50}).

TABLE 4

Segregation of thy-R⁻*

Experiment No.	Time (min)	Selective markers			Ratio		
		leu⁺ (×10⁵)	*thr*⁺ (×10⁵)	*thy-R*⁻ (×10⁵)	*thy-R*⁻/*thr*⁺	*thy-R*⁻/*leu*⁺	*leu*⁺/*thr*⁺
1†	0′	3.3	4.2	0.7	0.17	0.21	0.78
	60′	2.4	3.2	1.6	0.50	0.67	0.75
	110′	3.0	2.4	3.2	1.33	1.06	1.25
	140′	3.5	4.1	3.3	0.81	0.94	0.85
2‡	0′	8.3	10.0	0.9	0.09	0.11	0.83
	40′	5.8	6.1	2.3	0.38	0.40	0.95
	70′	7.4	6.7	4.8	0.76	0.65	1.10
	110′	5.4	6.2	3.5	0.57	0.65	0.87

* The mating mixture at 40 minutes incubation was diluted tenfold with GS(T,L,Thi,Thy_{50}, Sm) liquid medium, vibrated violently for 30 seconds with a Thermomixer, and then incubated at 37°C. At intervals, samples were withdrawn and plated on appropriate selective agar media.
† Experiment 1: HfrP4X6–11 × Y–70–2.
‡ Experiment 2: HfrH(3000)–11 × Y–70–2.

DISCUSSION

From the kinetics of *thy-R* transfer and the frequencies of unselected markers in four similar experiments (Figures 2a, b, 3a, b), it was concluded that *thy-R* is located very near *thr* on the opposite side from *leu*. The kinetics of each selected marker (Figure 4a, b) and the frequencies of unselected markers in each recombinant class (Tables 2, 3) also agreed with this conclusion. *thy-R* is thus far from *thy*, which is between *str* and *his* (KITSUJI 1964) and very near arg_2 (ISHIBASHI, SUGINO and HIROTA 1964). A similar observation was also reported by S. ALIKHANIAN, T. ILJINA, E. KALIAEVA, S. KAMENEVA and V. SUKHODOLEC (personal communication).

From the kinetic experiment on *thy-R*⁻ transfer, the phenotypic expression of *thy-R*⁻ appears to be delayed. Since the high thymine requirers (*thy*⁻*thy-R*⁺) undergo thymineless death at low thymine concentration (HARRISON 1965), the delayed expression of the *thy-R*⁻ phenotype may result in a lower yield of *thy-R*⁻ recombinants on the selective medium employed. This delayed phenotypic expression can be explained either by *thy-R*⁻ being recessive to *thy-R*⁺, or if *thy-R*⁻

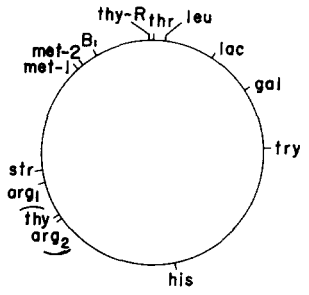

FIGURE 5.—Location of *thy-R* on the circular linkage group of *E. coli* K-12.

is dominant by a period of time being required for appearance of the product which is synthesized under its control, that converts the cells into low thymine requirers. The available data (Table 4) are consistent with either of these explanations, but are not sufficient to determine whether *thy-R*$^-$ is recessive or dominant.

The expert assistance of Mrs. Yoshiko Kaneko and Mr. Shinichi Matsudaira is gratefully acknowledged. I would also like to thank Dr. Sigeru Kuno for his critical advice and for reading the manuscript.

SUMMARY

Low-thymine requiring thymineless strains (Thy_2^-) are double mutants (genotype *thy*$^-$*thy-R*$^-$) derived from high-thymine requiring thymineless single mutants (Thy_{20}^-), (genotype: *thy*$^-$*thy-R*$^+$), which are the type originally selected by use of aminopterin and thymine. By kinetic experiments and the rate of segregation, *thy-R*$^-$, which is concerned with the second mutational event, was shown to be linked to *thr* on the opposite side from *leu*. Recombinants that have received the *thy-R*$^-$ gene must be incubated for some time in fresh medium before they express the *thy-R*$^-$ phenotype.

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