TABLE 3. ΔG as a Function of Thymine Concentration before and after amino acid deprivation

Pre-step up thymine concentration (μg/ml)	ΔG for the indicated post-shift thymine concentrations (%)				$\overline{\Delta G}$ (%)	C (τ = 39 min)
	1.0	2.0	5.0	9.0		
0.25	136	115	131	-	127	110
0.4	102	97	97	-	99	89
0.5	94	93	95	90	93	85
0.6	93	87	84	-	88	81
1.0	73	73	71	74	73	69
2.0	77	67	64	74	70	66
5.0	-	71	62	58	64	61
10.0	-	-	62	60	61	58

The data shown for the pre-step up concentration of 0.5 μg/ml thymine are taken from curves obtained in the experiment shown in Fig. 5. The data shown for the other pre-step up thymine concentrations were obtained from similar experiments. $\overline{\Delta G}$ is the average of all ΔG values shown in each line. Values of C were calculated from the corresponding values $\overline{\Delta G}$ using equations (4) and (6).

time to the external thymine concentration in thy⁻ strains predicts changes in the DNA/mass ratio ($\bar{G}/\bar{M}$) of cultures just by varying the thymine concentration supplied to them if no change in the growth rate accompanies the change in the replication velocity. Since the growth rate seems not to be affected by the thymine concentration (see Section (1)), I expected to find different $\bar{G}/\bar{M}$ for different thymine concentrations. Table 4 shows that this ratio remains constant for at least ten generations of "normal" growth of the culture (see Section (1)) even on a low thymine concentration. Table 5 summarizes seven such experiments using different concentrations of ^{14}C-thymine (with the same specific activity). It demonstrates that there is a progressive decrease in the DNA/mass ratio with decreasing thymine concentrations. On the assumption that the change in this ratio is due only to changes of C it was possible to calculate the corresponding values of C using equation (9). The "reference value" was taken as 60 minutes for C for the culture grown on 5 μg/ml thymine as was estimated by using the ΔG technique. (See Materials and Methods for the calculating procedure.) A graph of C as a function of the reciprocal of the thymine concentration is given in Fig. 10b.

If new cycles of chromosome replication are initiated in a constant cell mass (or volume) (Donachie, 1968; Pritchard, 1968; Pritchard et al, 1969) and the value of this mass as well as the rate of mass increase in the culture are independent of the concentration of thymine present in the growth medium, than by addition of more thymine to the medium of a culture previously grown on lower concentrations ("stepping-up"), one expects a transitional increase in the rate of DNA synthesis in the culture. This is demonstrated in the following experiment described in Fig. 6. A uniformly labelled culture growing on

TABLE 4. DNA per unit mass of a culture in successive generations

Sampling Time (min)	$A_{450} \times 10^3$	Particles/ml $\times 10^7$	$\frac{A_{450}}{10^9 \text{ particles}}$	cpm/ml	$\frac{\text{cpm}}{10^5 \text{ particles}}$	$\frac{\text{cpm}}{A_{450}} \times 10^{-3}$
0	55	1.70	3.24	1075	6.32	19.55
90	130	3.80	4.42	2764	7.27	21.26
130	150	3.65	4.11	3267	8.95	21.78
165	140	3.45	4.06	3243	9.40	23.16
200	145	3.15	4.60	3229	10.25	22.27
230	130	2.55	5.10	2844	11.15	21.88
270	140	2.55	5.50	2945	11.55	21.04
305	140	2.25	6.22	2736	12.16	19.54
340	120	1.80	6.67	2398	13.32	19.98
380	115	1.55	7.42	2447	15.79	21.28
425	120	1.55	7.74	2456	15.85	20.47
460	135	1.65	8.18	2939	17.81	21.77
480	70	0.70	10.00	1563	22.33	22.33

A single colony was suspended in M9 synthetic medium supplemented with ^{14}C thymine (0.4 μg/ml; 0.05 μC/μg) and incubated at 37° with vigorous shaking. Sampling commenced after 3-4 cell doublings and the culture was diluted by half with fresh prewarmed medium after each sample was taken.

TABLE 5. DNA per unit mass as a function of thymine concentration

Thymine concentration (μg/ml)	$\frac{cpm}{A_{450}} \times 10^{-3}$	$\frac{(cpm/A_{450})}{(cpm/A_{450})_{5.0}} \times 0.85$	C (τ = 39 min)
0.25	19.5	0.459	160
0.4	26.8	0.632	104
0.5	28.0	0.661	97
0.7	30.2	0.713	85
1.0	33.7	0.793	70
2.0	35.6	0.838	62
5.0	36.0	(0.850)	(60)

Parallel cultures were grown in M9 containing the indicated concentration of thymine. Specific activity was identical in each case (0.05 μC/μg). Cultures were sampled as described under Table 4. The values for cpm/A_{450} are averages of at least 6 samples taken in successive generations. C was calculated in each case assuming a value of 60 min. for 5.0 μg/ml thymine (see text). This value of C gives $\bar{G}/\bar{M} = 0.85$ when τ = 39 min (Fig. 1).

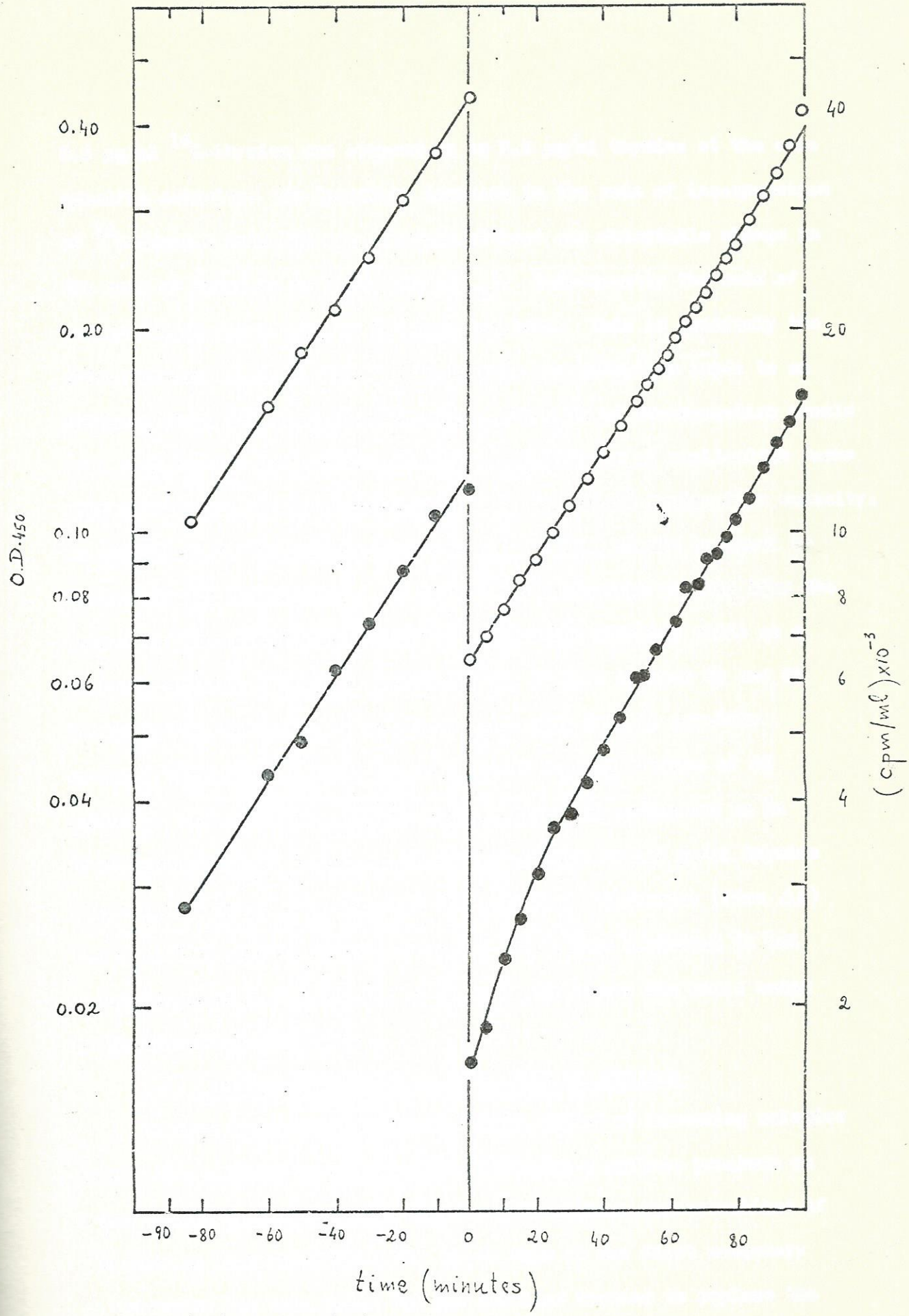

FIG. 6. A_{450} (○) and DNA (●) of a culture before and after a step-up.

0.5 μg/ml ^{14}C-thymine was stepped-up to 5.0 μg/ml thymine at the same specific activity. An immediate increase in the rate of incorporation of ^{14}C-thymine into DNA was observed without any detectable change in the rate of increase of cell mass (A_{450}). Subsequently the rate of incorporation drops back to the pre-step rate. This is precisely the effect predicted if an increase in thymine concentration leads to an increase in replication velocity only. The rate of incorporation should fall back to the previous rate when the distribution of replication forks equilibrates to the new value appropriate to the new replication velocity. As explained in the Introduction - the time taken should be equal to the new replication time C. To facilitate estimation of this time the data have been transformed (Fig. 7) to show the DNA/mass ratio as a function of time. The new equilibrium is reached between 50 and 60 minutes after the step-up, which is consistent with my previous estimate (From ΔG) of C in the presence of 5 μg/ml thymine of 60 minutes. In a similar experiment this period was estimated to be 65-70 minutes after a step-up of such a culture to 1.0 μg/ml thymine which is not very different from the estimated 75 minutes (from ΔG) for C at that concentration. However, since this technique is not accurate enough I did not use it to estimate C over the whole range of thymine concentrations.

(5) Thymine Incorporation Rates after a Step-up

Another independent and more direct method of measuring relative replication velocities was by pulse labelling a culture pregrown on a low concentration of "cold" thymine with various concentrations of ^{14}C-thymine (see Introduction). To do this it was first necessary to determine the time taken for the labelled thymine to replace the unlabelled pool of thymine derivatives present before the addition

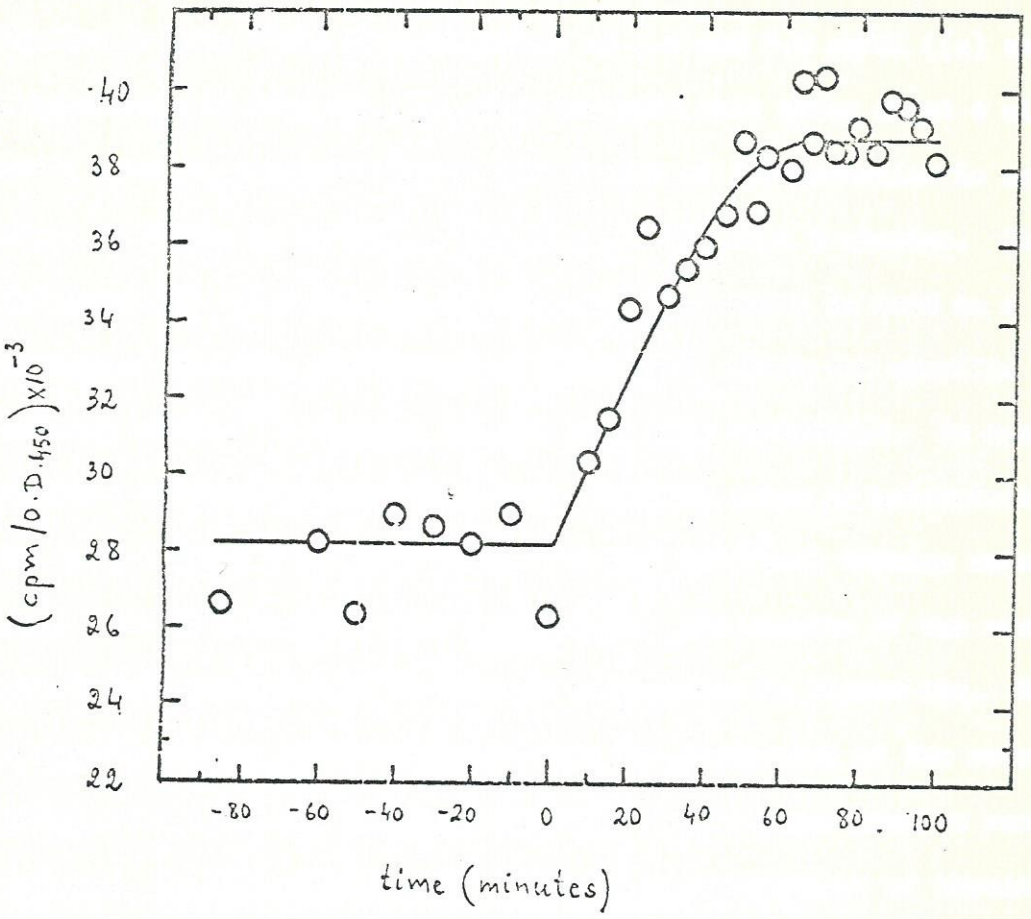

FIG. 7. Change of DNA/mass ratio in a culture due to a step-up.

of label, and for the internal concentration of dTTP to equilibrate to its new value. A culture was therefore pre-grown on 0.5 μg/ml thymine and the rate of incorporation of label followed after addition of ^{14}C-thymine to give final concentrations of 1.0 and 5.0 μg/ml ((a) and (b) respectively in Fig. 8). The data indicate that after about 4 minutes and 2 minutes, respectively, a linear rate of incorporation is achieved which continues to be linear for at least the next 5 minutes. The big difference in rates between the two cultures cannot be used as a measure of the real difference in velocities, since the specific activity of the ^{14}C-thymine was not carefully controlled. To measure relative replication velocities over a broader range of concentrations the experiment was repeated using a set of different thymine concentrations with identical specific activities. The results of two such experiments are recorded in Table 6 and plotted in Figure 10c. An apparently linear relationship between the reciprocal of the rate of incorporation and the reciprocal of the thymine concentration was obtained. If the addition of increasing concentrations of thymine to a culture does not change the frequency of initiation, then the reciprocal of the rate of incorporation will be directly proportional to the replication time, C. The slope of the line obtained is similar to those calculated from ΔG and $\bar{G}/\bar{M}$.

(6) Rate Stimulation of DNA Synthesis after a Period of One Mass Doubling of Thymine Starvation

One of the predictions that stem from the model of a volume (or mass) controlled initiation of chromosome replication is that, after a period of unbalanced growth of a culture in the sense that synthesis of DNA is blocked but mass increase continues, the rate of DNA synthesis

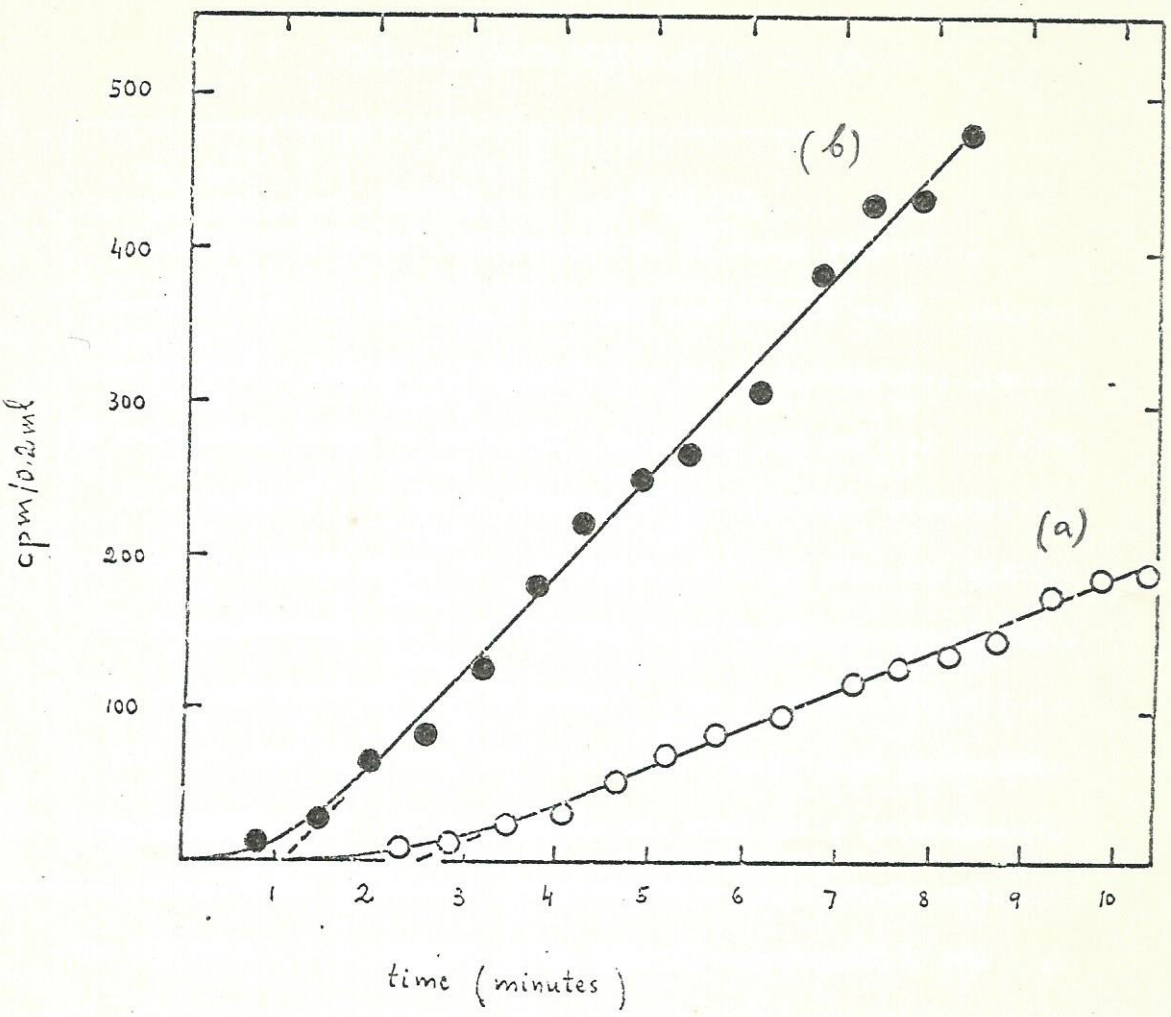

FIG. 8. Incorporation of ^{14}C-thymine after a step-up.

Cells were grown in M9 supplemented with 0.25 μg/ml thymine. When the culture reached A_{450} = 0.3 it was filtered, washed, resuspended in M9 and distributed almost simultaneousl into six (or ten for experiment 2) pairs of tubes containing M supplemented with different concentrations of ^{14}C-thymine at exactly the same specific activity. An equal volume of TCA w added to one member of each pair after 5 min incubation at 37 and the other after 8 min. Values of cpm/min were derived by subtracting each result for the 5 min tube from that for the corresponding 8 min tube and dividing by 3. These values are function of the replication velocity (V). The relationship b V, C and L (chromosome length) is as follows: V = L/C. If define the length of the chromosome as unity, then V = 1/C. reciprocal of the rate of incorporation will therefore be proportional to C. The values of C in parentheses are the a values assumed for each experiment. They were deduced from line of Fig. 10a and chosen so that the two experiments fall same line.

TABLE 6. Rate of DNA synthesis after a step-up

Thymine concentration (μg/ml)	$\frac{\text{cpm/ml}}{\text{min}}$	$10^{-3}\ \frac{\text{ml min}}{\text{cpm}}$	C (min)
Experiment 1			
0.25	243.1	4.12	135
0.4	263.0	3.80	125
0.5	327.6	3.05	100
1.0	399.1	2.51	83
2.0	504.5	1.98	(65)
5.0	625.2	1.60	53
Experiment 2			
0.25	144.0	6.94	131
0.4	163.8	6.10	115
0.5	234.8	4.26	80
0.7	246.4	4.06	76
1.0	264.4	3.78	71
2.0	265.6	3.76	70
5.0	280.4	3.57	67
10.0	330.0	3.03	(57)
30.0	435.0	2.30	44

will be accelerated. This stimulation was first observed by Pritchard and Lark (1964) and interpreted by them as "premature initiation" and is understandable in terms of the hypothesis that initiation occurs at a defined mass/chromosome origin and is independent of replication itself, since during the period of thymine starvation the culture mass continues to increase. Since Pritchard and Lark (1964) assumed that the bacterial chromosome replication time was equal to the doubling time of the cells growing on glucose synthetic medium ($C = \tau$), the expected value for this rate stimulation factor (RSF) after a period of one doubling of thymine starvation of a thy$^-$ strain was 3, if reinitiation occurs at all chromosome origins. However, Pritchard and Lark (1964) and later P. Barth (1968) performed this experiment with the same strain used in this study (P178) and observed an increase in rate that was closer to two-fold than three-fold. This can be explained if one takes into account the longer replication time suggested by my data in this strain. As stated in the Introduction and shown in Appendix 2, this factor is a function of C and τ only. Therefore, by measuring it and the generation time of the culture one can get an estimate of C. An experiment of this sort conducted on a culture grown on 2 µg/ml thymine is demonstrated in Fig. 9. Table 7 summarizes the results of such experiments, and these are plotted against the reciprocal of the thymine concentration in Fig. 10d. Although two of these were performed in a medium containing glycerol, it seems, in the light of the conclusions drawn from the experiments described later (in Section (8)), that they are comparable to the others, measured in a glucose grown culture.

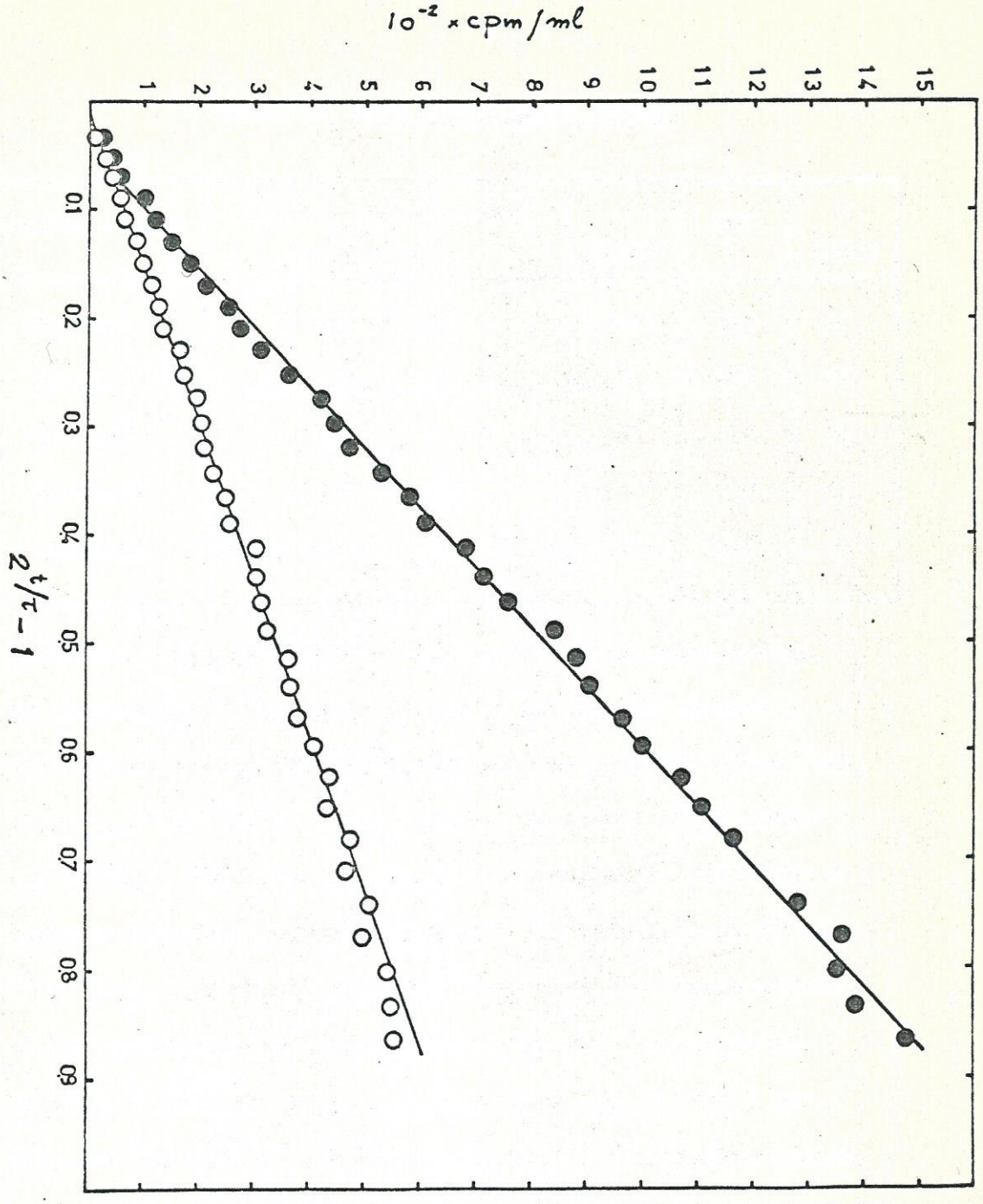

FIG. 9. Incorporation of ^{14}C-thymine before (○) and after (●) a period of one mass doubling of thymine starvation.

TABLE 7. Rate Stimulation Factor

Carbon source	Thymine concentration (μg/ml)	τ (min)	RSF	C (min)
glucose	5.0 (+ dG)	60	3.4	47
glucose	2.0	40	2.6	57
glucose	1.0	40	2.4	72
glycerol	5.0 (+ dG)	64	3.9	40
glycerol	0.5	61	2.5	96

Cultures of E.coli 15 (P178) were grown on M9 salts medium supplemented by the indicated carbon source and thymine concentration with (200 μg/ml) or without deoxyguanosine (dG). When A_{450} reached 0.2, cells were transferred (see Materials and Methods) into thymine starvation conditions. At times 0 minutes and after one doubling time (τ) a portion was diluted into the same concentration of ^{14}C-thymine and incorporation of the radioactivity into DNA measured. RSF is the ratio between the slopes of the two lines (see Fig. 9) and C was read off the programme output (Fig. 3). The rationale behind the addition of deoxyguanosine (dG) will be described in detail in section (9) and discussed later.

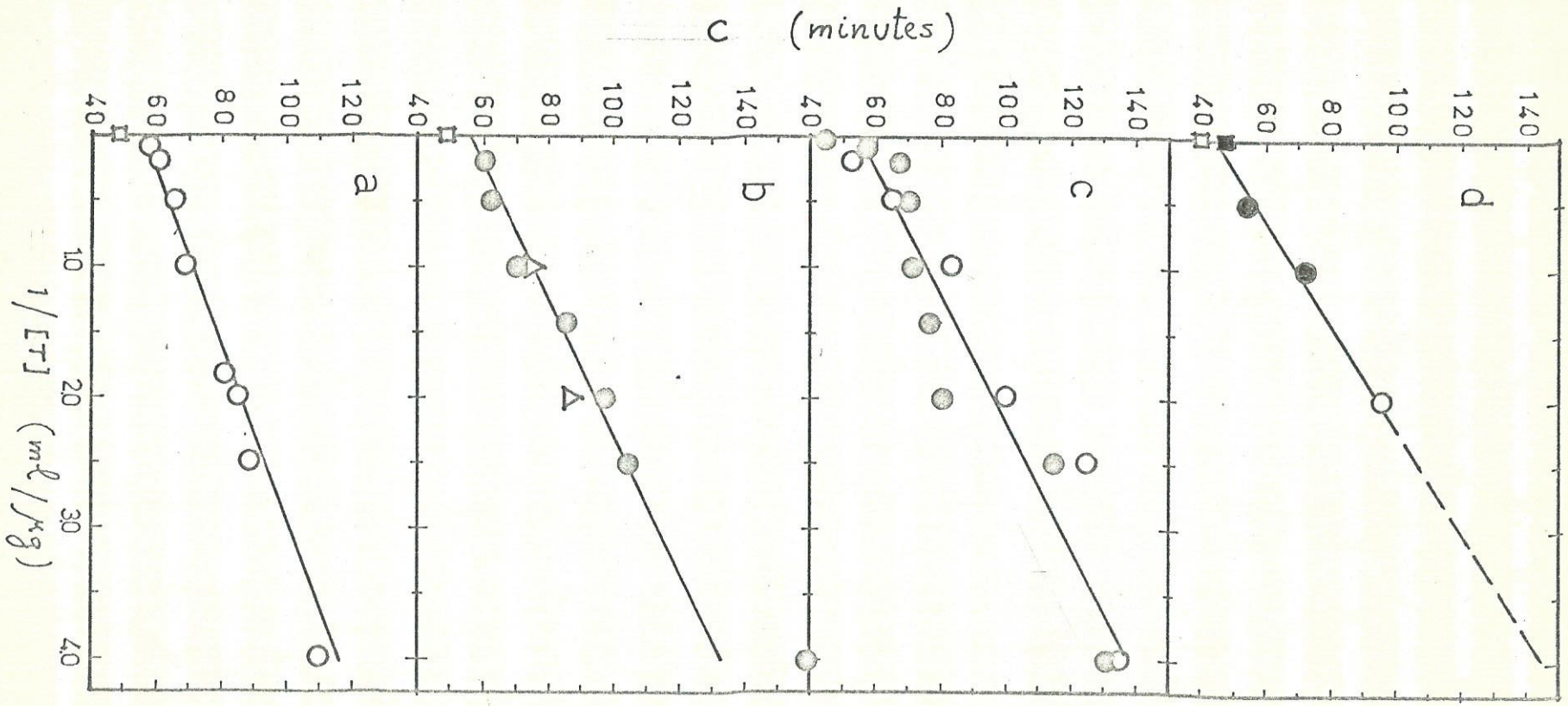

FIG. 10. Relative values of C as a function of the reciprocal of the thymine concentration obtained in four different ways: (a) from ΔG, (b) from $\bar{G}/\bar{M}$ (● , △ , two different experiments), (c) from pulse incorporation of ^{14}C-thymine, (d) from RSF.

□ and ■ : cultures supplemented with 200 µg/ml deoxyguanosine.

In the four preceding sections four independent methods for estimating the replication time of the chromosome of E.coli 15T⁻ are described and discussed. Only two of these procedures gave estimates of C in minutes. The other two methods were dependent on the validity of an assumed reference value for one thymine concentration because of their relative nature. The validity of the actual values of the replication time are therefore in doubt, and will be discussed later. However, all four lines obtained (Fig. 10) are in close agreement to each other with respect to their slopes.

(7) Changes of C in another thy⁻ Strain

The main conclusion drawn from the results described in the sections (1) - (6) is that the replication velocity is affected by the concentration of thymine in the growth medium of E.coli 15T⁻ (555-7) and that a substantial change in velocity occurs without any detectable effect on the growth rate of the culture. This discovery introduces a potentially serious source of error in the interpretation of data involving measurements of rates of DNA synthesis and determination of macromolecular composition of cells in thy⁻ mutants since these parameters are sensitive to changes in the replication time of the chromosome as well as to changes in growth rate. In the case of strain 555-7, this problem is probably not a serious one since only a small increase in the replication velocity occurs by increasing the thymine concentration above 1.0 µg/ml and in most published work with this strain concentrations greater than this have been used. However, this strain, as other 15T⁻ strains of E.coli, is exceptional in being able to grow normally on very low concentrations of thymine. Concentrations greater than 0.2 µg/ml will support normal growth (Maaløe and Rasmussen, 1963; Lark et al,

1963; Pritchard and Zaritsky, 1970) and the major change in replication velocity occurs over the range 0.25 - 1.0 μg/ml. Most Tlr (thymine low requirement) strains of E.coli require about ten times this concentration for normal growth (I. Beacham, personal communication). The nature of this difference in the minimal requirement is unknown yet. It was also necessary to show that the phenomenon described in the previous sections is not unique to the 555-7 strain. For these reasons I chose a thy⁻ derivative of E.coli K12 strain CR34, widely used in studies of DNA synthesis (e.g. Caro and Berg, 1968; Wolf et al, 1968). Since it requires at least 2.5 μg/ml thymine for normal growth (see section (1)) in glucose minimal medium it was anticipated, by comparison with 555-7, that significant changes in the chromosome replication time would occur over the concentration range 2.5 - 10.0 μg/ml thymine, used in most experiments with this and other Tlr strains.

Two methods were employed to estimate C in P162, the agreement between which supports their validity. Values for ΔG obtained with this strain grown in M9-glucose medium containing 2.83 and 8.0 μg/ml thymine are given in Table 8 together with the estimates of C calculated from them. As was found with P178 (section (3)) ΔG is increased when the thymine concentration in the growth medium is reduced. Values of $\bar{G}/\bar{M}$ were obtained for cultures growing on several concentrations of thymine (Table 9) and C calculated from these assuming a value of 77 minutes at 8.0 μg/ml thymine (Table 8). Using this reference value, experiments 1 and 2 in Table 9 supply four additional estimates of C. One of these values (101 minutes for 4 μg/ml thymine) was used in the same way as reference point in experiment 3 (Table 9) to obtain a fifth estimate for C. These five values, as well as the two obtained from the ΔG

TABLE 8. ΔG as a function of thymine concentration before amino acid starvation in P162

Thymine concentration (μg/ml)	τ (min)	$\overline{\Delta G}$ (%)	C (min)
8.0	54	57.6	77
2.83	60-61	78.5	114

Procedure as in Table 3.

TABLE 9. $\bar{G}/\bar{M}$ as a function of thymine concentration in P162

Thymine concentration (μg/ml)	τ (min)	$\bar{G}/\bar{M}$ (10^{-4} x cpm/A_{450})	R x $\bar{G}/\bar{M}$	C (min)
Experiment 1				
2.0	56	0.596	0.593	164
4.0	48	0.734	0.730	101
8.0	49.5	0.853	(0.848)	(77)
16.0	50	0.916	0.911	65-66
Experiment 2				
8.0	46.5	0.830	(0.825)	(77)
2.5	55.5	0.722	0.718	120
Experiment 3				
4.0	49	0.750	(0.738)	(101)
5.0	49	0.795	0.782	90-91

See text and legend to Table 5.

experiments (Table 8) are plotted in Fig. 11 against the reciprocal of the thymine concentration. As previously found in similar experiments with P178 (sections (3) - (6), Fig. 10 and Pritchard and Zaritsky, 1970), these values fall on a straight line apart from that obtained for a culture growing on 2.0 μg/ml, which is below the minimum concentration required for normal growth (see section (1)). The data for P162 are similar to those obtained for P178 also in that there is agreement between two independent methods of calculating C.

Comparison between the response of each strain to changes in the external thymine concentration and the validity of the absolute values of C calculated will be presented in the Discussion.

(8) Experiments Performed in M9 Glycerol Medium

Helmstetter et al (1968) showed that replication velocity is constant over a wide range of growth rates (22 minutes $\leq \tau \leq$ 65 minutes). Conversely, it has been demonstrated here (and see Pritchard and Zaritsky, 1970; Zaritsky, 1970; Zaritsky and Pritchard, 1971) that the growth rate remains constant while varying the replication velocity over a similarly wide range (48 minutes $\leq C \leq$ 130 minutes). It was important for the picture to be complete to show that the change of C with the variation of the external thymine concentration in a culture grown with one growth rate is similar to the corresponding change in a culture of the same strain grown at a different rate. A condition that must be fulfilled is that the doubling times of both cultures should fall in the limits defined by Helmstetter et al (1968). A culture of P178 growing in M9-glycerol medium could be compared with M9-glucose culture of this strain, since its doubling time was found to be 60 $\pm$ 6 minutes.

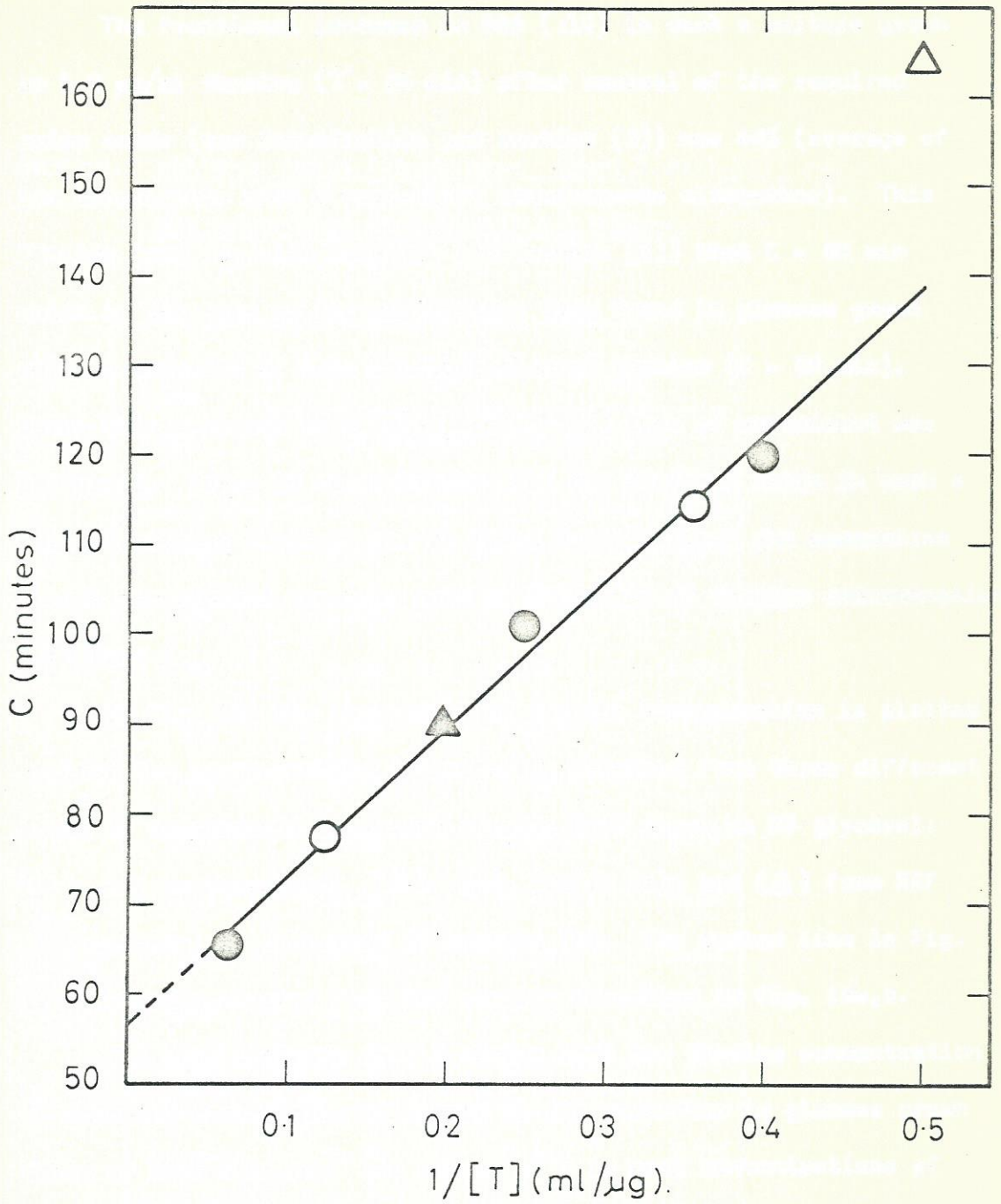

FIG. 11. Relative values of C in P162 as a function of the reciprocal of the thymine concentration obtained in two different ways: (○), ΔG; (▲), (●), (△), $\bar{G}/\bar{M}$

The fractional increase in DNA (ΔG) in such a culture grown on 5.0 μg/ml thymine (τ = 55 min) after removal of the required amino acids (see Introduction and section (3)) was 44% (average of two experiments with 6 post-shift measurements altogether). This implies (and see also Materials and Methods (3)) that C = 60 min which is the same as the replication time found in glucose grown culture supplied by this concentration of thymine (C = 60 min). Using this estimate as a reference the $\bar{G}/\bar{M}$ type of experiment was performed. Table 10 demonstrates that the DNA/mass ratio in such a culture remains constant for at least 3 generations and summarizes 3 such experiments covering most of the range of thymine concentrations affecting C.

In Fig. 12a the reciprocal of thymine concentration is plotted against the corresponding values of C, obtained from three different sorts of experiments done with strains P178 grown on M9 glycerol: (•) ΔG, as described; (O) $\bar{G}/\bar{M}$, (Table 11); and (Δ) from RSF measurements, as described in section (6). The dashed line in Fig. 12a was reconstructed from the results described in Fig. 10a,b.

The data indicate that C varies with the thymine concentration in glycerol grown cells similarly to its variation in glucose grown cells of both strains. The values of C in most concentrations of thymine are slightly lower for glycerol grown cells than the values obtained from measurements in glucose. This could be due to the smaller demand on internal dTTP concentration in slow growing cells than in fast growing cultures.

This possible explanation is supported by similar results obtained for P162-8 cultures grown on M9 glycerol (τ = 80 $\pm$ 8 min).

TABLE 10. DNA/mass ratio for glycerol grown E.coli 15T⁻ and the calculated corresponding C values.

Time (min)	$\bar{G}/\bar{M}$ (10^{-4} x cpm/A_{450}) in thymine concentration		
	0.4	1.0	5.0
0	2.31	-	-
20	1.86	-	-
40	2.46	2.69	2.87
60	2.46	2.65	2.75
80	2.39	2.60	-
100	2.50	2.69	2.79
120	2.31	2.70	2.84
140	2.31	2.73	2.83
160	2.31	2.78	2.92
180	2.31	2.73	-
Average	2.32	2.70	2.83
τ (min)	58	61	62
R x $\bar{G}/\bar{M}$	0.828	0.964	(1.01)
C (min)	96	69	60

See text and Tables 4 and 5

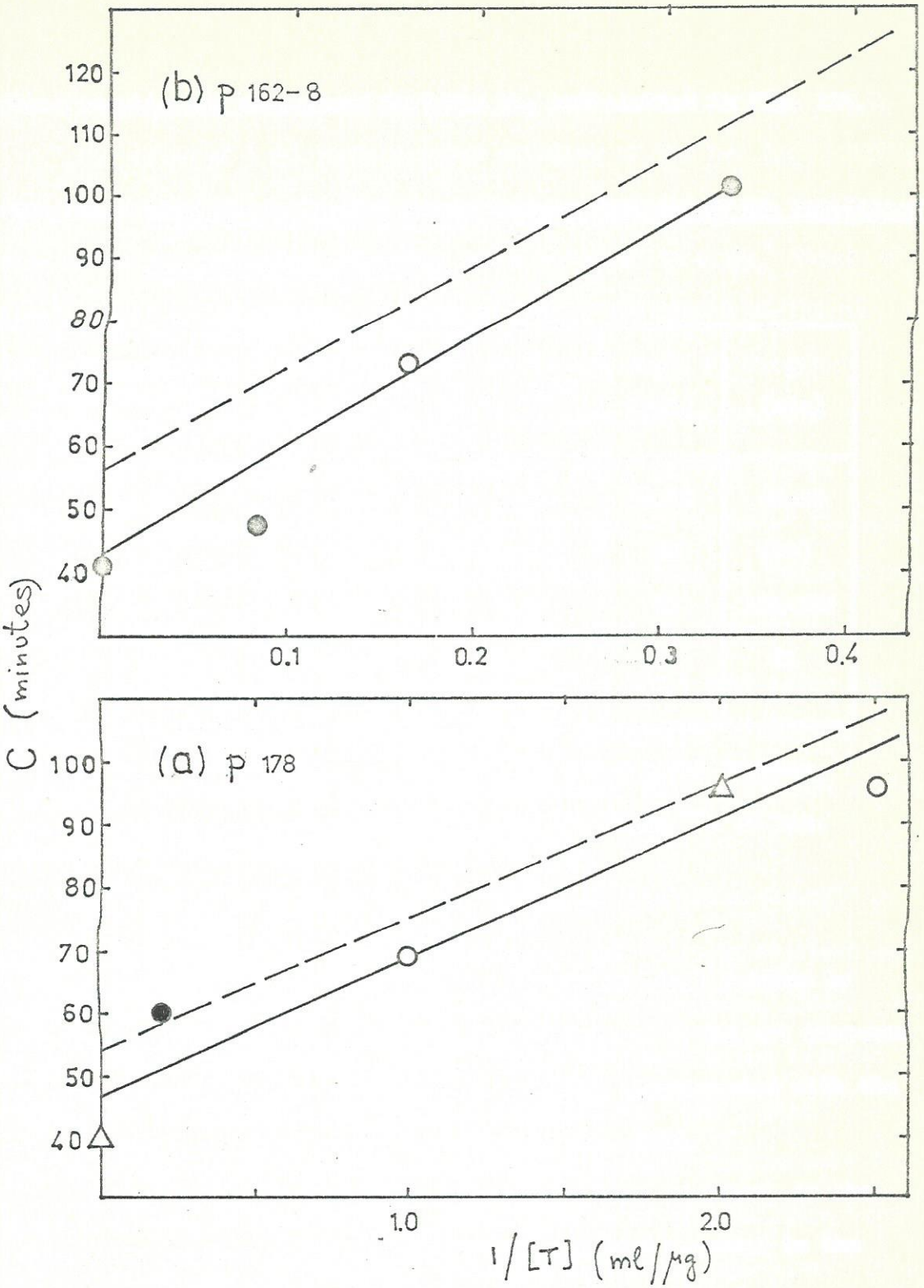

FIG. 12. C against the reciprocal of the thymine concentration in glycerol grown cultures of (a) P178, (b) P162-8 (see text).

These results are summarized in Table 11, and plotted in Fig. 12b.

TABLE 11. DNA/mass ratio for glycerol grown P162-8 and the calculated corresponding C values.

Thymine concentration (μg/ml)	$\bar{G}/\bar{M}$ $10^{-4} \times \frac{cpm}{A_{450}}$	R x $\bar{G}/\bar{M}$	τ (min)	C (min)
12.0	1.75	1.16	72-73	47-48
6.0	1.60	(1.02)	78	(73)
3.0	1.46	0.93	82	101-102
1.5 + deoxy-guanosine	1.83	1.17	80	41

See text and Table 4. C = 73 minutes for 6 μg/ml thymine, since ΔG for that concentration was 33%, where τ = 84 min.

Although the growth rate is slower than the lower limit defined by Helmstetter et al (1968) for constant C, values of C did not increase above those measured in glucose grown cultures supplemented with the same thymine concentration (dashed line in Fig. 12b).

A more thorough discussion considering this question, in the light of my results as well as other observations recorded in the literature, will be presented in the Discussion.

(9) Effects of Deoxyguanosine on the Bacterial Replication Velocity

The value of C in glucose grown cultures of the two thy⁻ strains extrapolates to 57 minutes for saturating concentrations of thymine. This is nearly 30% higher than the value obtained in a thy⁺ strain of E.coli (B/r) by Helmstetter and Cooper (1968) who used a different method to estimate C. Although this difference might be due to a systematic error in my estimates of C (see previous section and Discussion), it could be real. If real, it might reflect either a difference between the replicating system of B/r and those of K12 and 15, or might be due to the fact that both strains have low intracellular thymidine triphosphate (dTTP) concentrations even at saturating concentrations of thymine (Beacham et al, 1971), since they carry thy⁻ mutations. In an attempt to test this last possibility I obtained new estimates of C (Tables 12 and 13) for P162-8 and P178 in glucose cultures supplemented with deoxyguanosine (200 µg/ml).

T1r strains were shown to have lesions in the genes specifying deoxyribomutase (drm) or deoxyriboaldolase (dra) first by Breitman and Bradford (1967) and later by others (Breitman and Bradford, 1968; Barth et al, 1968; Munch-Petersen, 1968). These mutations block further degradation of deoxyribose-1-phosphate (dRib-1-P). The higher concentration of this compound so maintained permits strains carrying these mutations to grow on lower concentrations of thymine than their corresponding dra⁺ drm⁺ strains. It was assumed that deoxyguanosine would raise the dTTP concentration by providing an additional source of deoxyribose-1-phosphate through its breakdown. Strain P162-8 is a drm⁻ derivative of P162. It was necessary to use it for this experiment since growth of the latter strain, being dra⁻, is inhibited

TABLE 12. ΔG in cultures grown in the presence of deoxyguanosine before amino acid deprivation

Strain	Thymine concentration (μg/ml)	τ (min)	ΔG (%)	C (min)
P178	5.0	47.5	38	48
P162-8	8.0	52.5	39	53

See text and Table 3.

TABLE 13. $\bar{G}/\bar{M}$ in cultures grown in the presence of deoxyguanosine

Thymine concentration (μg/ml)	deoxy-guanosine (200 μg/ml)	τ (min)	$\bar{G}/\bar{M}$ (10^{-4} x cpm/A_{450})	R x $\bar{G}/\bar{M}$	C (min)
Experiment 1: P178					
5.0	-	37	1.765	(0.833)	(60)
5.0	+	41.5	2.00	0.945	49
Experiment 2: P162-8					
8.0	-	48	1.01	(0.837)	(77)
8.0	+	49	1.17	0.970	54
Experiment 3: P162-8					
8.0	-	46.5	0.830	(0.825)	(77)
1.25	+	49	0.967	0.961	55

See text and Table 5.