(10) Another way of estimating C is to measure the ratio between the rate of DNA synthesis after a period of one mass doubling of thymine starvation of a thy⁻ strain and the rate of DNA synthesis in the same culture before the start of this treatment (rate stimulation factor - RSF). This ratio can be expressed in terms of C and τ only (see Appendix 2) if the assumptions (a), (c), (e) and (f) are accepted. Thus it has the form (Fig. 2):

$$RSF = \frac{2^{n+1} - 1}{2^n - 1} \qquad (10)$$

where $n = C/\tau$ (4). Therefore, by measuring this ratio and τ another estimate of C can be obtained.

This method is less sensitive to changes in C (or τ) than the others, as can be seen by comparing Fig. 2 and Fig. 1. It is discussed in Appendix 2 and in the Discussion.

(11) The last method I can see available to estimate C is again tied to the clarification of D. It can be shown (Appendix 3) that the average number of forks per cell (FPC) is a function of C, D and τ such that

$$FPC = 2^{D/\tau}(2^{C/\tau} - 1) \qquad (11)$$

Knowing D, a pair of measurements of FPC and τ uniquely defines C. FPC can be deduced from the distribution of label among the progeny of a pulse-labelled cell (e.g. Chan and Lark, 1969). This method, which is based on assumptions (b), (e) and (g), will also be discussed when results available in the literature are compared to those observed in this study (Discussion).

Four of these methods were used in this study and the close agreement between the results of all four argue for their validity.

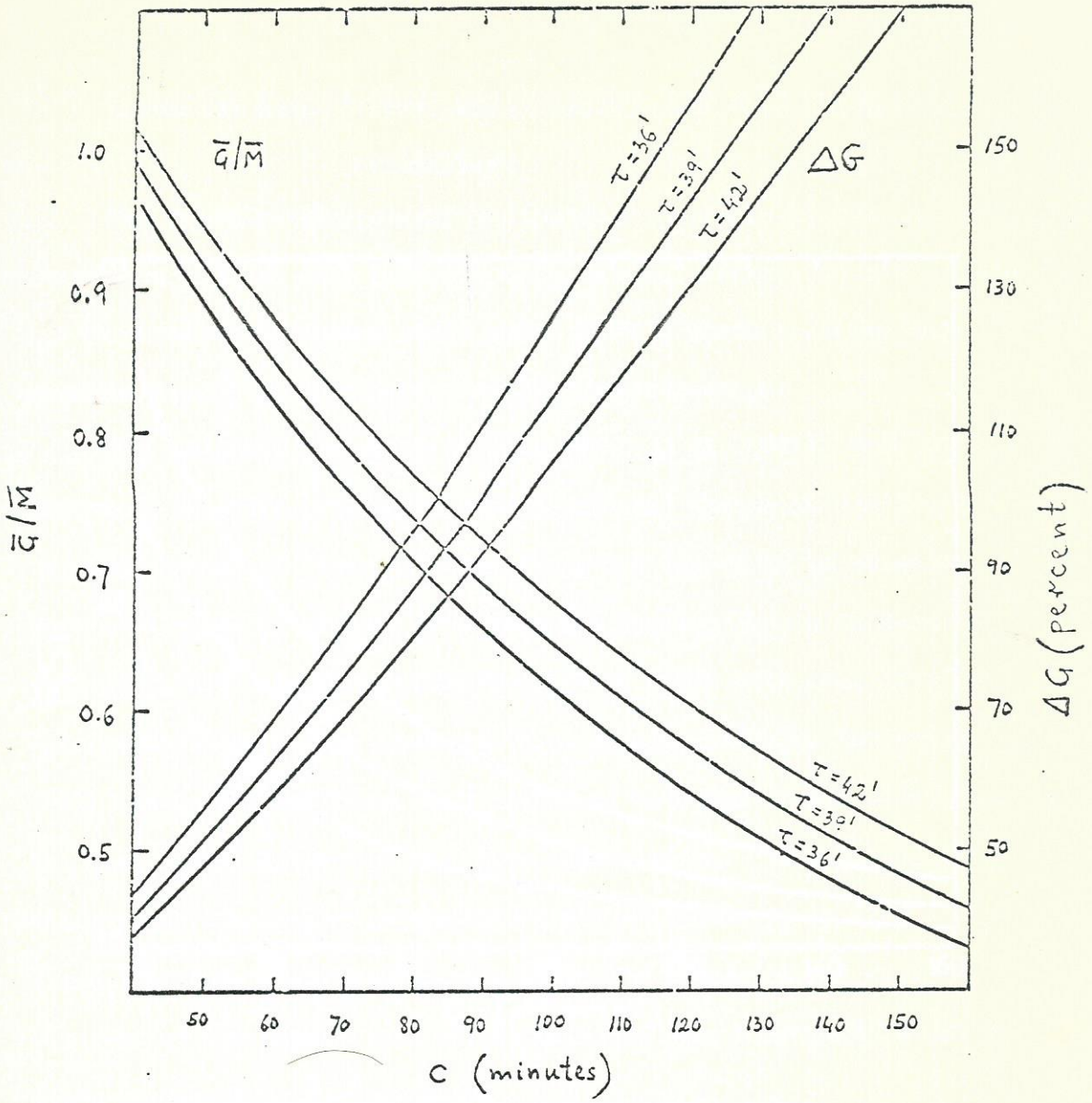

FIG. 1. $\bar{G}/\bar{M}$ and ΔG as functions of C at various growth rates.

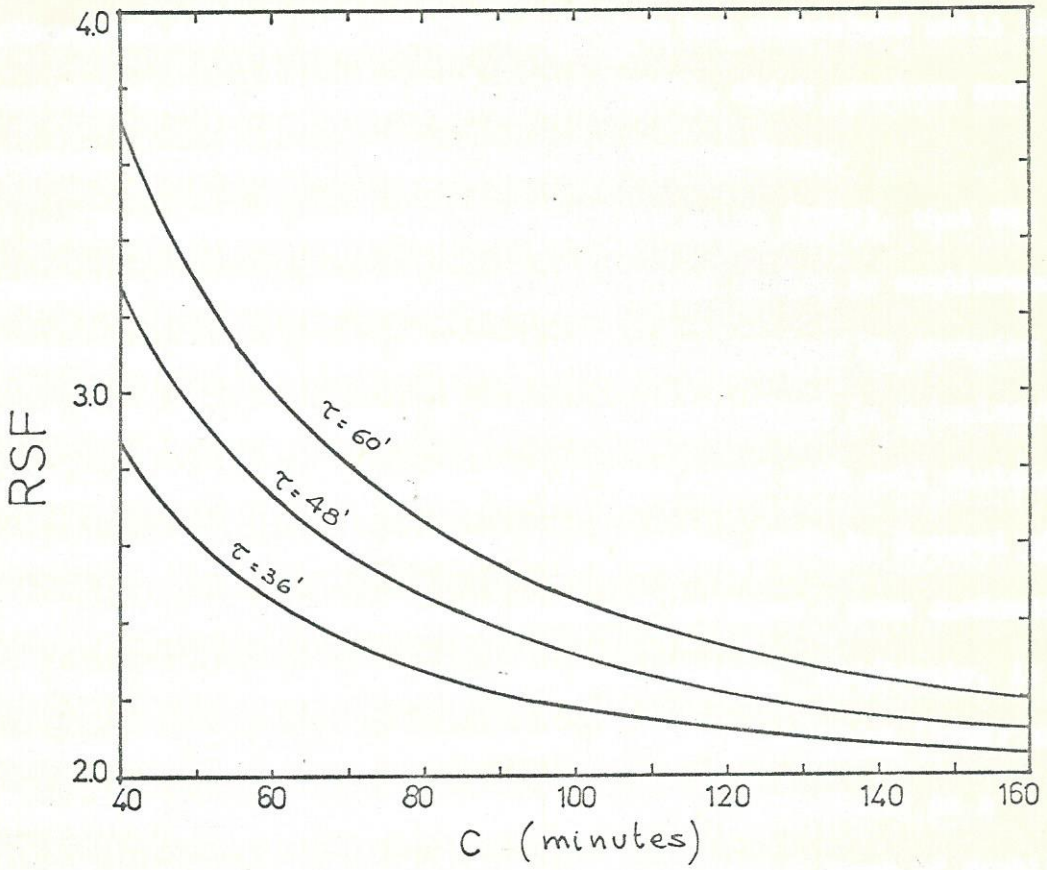

FIG.2. RSF as functions of C at various growth rates.

(IV) Bacterial Cell Division and its Connection with Chromosome Replication

The relationship between the replication cycle of the bacterial chromosome and the cell division cycle was not understood until Cooper and Helmstetter presented their model (1968), which resolves many previous experimental findings and misunderstandings.

The original discovery of Schaechter *et al* (1959), that DNA synthesis in cells growing in glucose minimal medium is continuous throughout most of their division cycle was explained by assuming that initiation of a round of chromoeome replication is coincident with cell birth and the termination of that round with cell division. This concept was widely accepted (e.g. Nagata, 1963a,b; Kuempel *et al*, 1965; Lark, 1966) although many pieces of evidence - some contradicting it, some unexplainable by it - were accumulating. Obviously, it did not explain the earlier observations of Schaechter *et al* (1958) that $\bar{G}$ and $\bar{M}$ of *Salmonella typhimurium* increased exponentially as a function of the growth rate of the cultures over a range of doubling times between 20 and 95 minutes.

On the other hand Forro (1965) concluded, on the basis of previous results (Forro and Wertheimer, 1960) that division in *E.coli* 15T$^-$ did not occur until some time after each of the two chromosomes such a cell contained had initiated a new round of replication. Clark and Maaløe (1967) came to the same conclusion on the basis of their studies with synchronized cultures of *E.coli* B/r, as already described (section II).

Cooper and Helmstetter's model (1968), as summarized in a previous section, makes several predictions, some of them discussed above. They showed (Helmstetter and Cooper, 1968) that a period of

more than 20 minutes (D) after completion of chromosome replication was a prerequisite for the following cell division. It was concluded soon after (Helmstetter and Pierucci, 1968) that completion of a round of chromosome replication in E.coli B/r was not only a necessary condition but also a sufficient condition, for the subsequent cell division (i.e. that the steps leading to cell division, after completion of chromosome replication, do not involve DNA synthesis). A similar conclusion was drawn by Clark (1968a,b).

It was recently shown (Pierucci and Helmstetter, 1969) that a period of protein synthesis which normally occurs concurrent with chromosome replication (but can be uncoupled from it) is required for "initiation" of division. This finding, however, is not incompatible with their previous conclusion.

Looking at the data presented by Helmstetter and Pierucci (1968) as well as by Clark (1968a,b) casts suspicion on this conclusion. These authors invariably found that cultures continue to divide for less than 20 minutes after the start of a period of inhibition of DNA synthesis. This is shorter by ca. 5 minutes than the measured D. It seems, therefore, that concurrent DNA synthesis may be necessary for cell division. This will be considered again in section (11).

MATERIALS AND METHODS

(1) Strains. The following strains were used throughout this work:

P245 : *E.coli* 15

P178 : *E.coli* 15T$^-$ (555-7), *arg*$^-$ *met*$^-$ *try*$^-$ *thy*$^-$ *drm*$^-$

P162 : *E.coli* K12 (CR-34), *leu*$^-$ *thr*$^-$ *thi*$^-$ *thy*$^-$ *dra*$^-$

P162-8 : *E.coli* K12 (CR-34), *drm*$^-$ derivative of P162.

Symbols are those used by Taylor (1970) and follow the conventions suggested by Demerec *et al* (1966).

(2) Media, growth conditions and measurements. M9 synthetic medium (pH 7.0-7.2) containing Na_2HPO_4 - 0.6% NH_4Cl - 0.1%

KH_2PO_4 - 0.3% $CaCl_2$ - 0.002%

NaCl - 0.05% $MgSO_4$ - 0.02%

was used in all the experiments. Either glucose (0.4%) or glycerol (0.4%) was used as carbon source. Required amino acids (50 µg/ml) and thiamine (20 µg/ml) were added as necessary. Thymine was added at the concentration indicated in each experiment, since this was the main variable in this work.

The medium was inoculated with a single colony of the appropriate strain and the culture aerated vigorously at 37^o in a New Brunswick gyrotory shaker.

The absorbance of cultures was measured at 450 nm with a Gilford microsample spectrophotometer, and particle titre was measured with a Coulter electronic particle counter Model B using a probe with a 30 µ orifice. The number of viable cells was measured on samples suitably diluted in phosphate buffer (pH 7) which were spread on plates containing the usual M9 medium (containing 10 µg/ml thymine), solidified with agar (1.5%).

Relative amounts of DNA were determined by using ^{14}C-thymine in the growth medium and measuring the amount of label present in the acid insoluble fraction. Samples were taken from the culture and added to an equal volume of cold (4°) 10% trichloroacetic acid (TCA). At least 20 minutes later each sample (kept in the cold) was filtered through a (Sartorius) membrane filter (0.45 μ, 27 mm) and washed thoroughly 6 times with ca. 5 ml of hot (95°) distilled water. The filteres were dried (using an infrared lamp) and placed with a constant orientation in small vials. Each was counted in a Packard liquid scintillation counter (PPO, 5 gr, POPOP 0.3 gr, in 1 litre of toluene) (Pritchard and Lark, 1964).

Bacteria were transferred from one medium to another by pulling the cells onto a Millipore membrane filter (0.45 μ, 47 mm), washing them on the filter with approximately the same volume of prewarmed unsupplemented M9 medium, and resuspending them in the new medium by rapid shaking. This procedure usually took less than 3 minutes and more than 90% of the bacterial mass (measured at 450 nm) was recovered.

Measurements were performed on cultures whose optical density never exceeded 0.5 at the end of the experiment. This permits at least one more doubling before entry into the stationary phase, and is what is usually termed as "mid-exponential phase". In most of the experiments the optical density of the culture was kept in the range 0.05-0.2 by appropriate dilutions. This was performed by adding fresh prewarmed medium into the culture flask.

(3) Procedures for calculating values of C. In this study four methods were used to estimate replication time, only one of them being direct ((2), Table 1, see Introduction). The other three methods

are based on complicated and laborious calculations. To simplify them a computer programme was developed. This programme, in Fortran language, can be used also for quantitation of other parameters relevant to chromosome replication and cell division in bacteria, as described in detail in the Introduction. The whole programme is reproduced in Appendix 4, but an example of its output is photographed in Fig. 3.

By feeding the programme with a set of values of τ and C one gets in the output, in tabular form, eleven parameters. The first two are τ and C, respectively and only 3 of the others were used in this study: ΔG in column 6; $\bar{G}/\bar{M}$ in column 9; RSF in column 10. (See Introduction for detailed explanation). This output was used as a table against which to read off values of C corresponding to any experimental pair of values of τ and ΔG, on the one hand, or of τ and RSF, on the other.

However, since the values of $\bar{G}/\bar{M}$ are not absolute it was necessary first to assume a value of C for a set of conditions. This was not assumed arbitrarily, but a value obtained from other measurements (either ΔG or RSF) using a particular concentration of thymine was used as a reference value. Thus, for this value of C, I have an observed DNA/mass ratio - $(\bar{G}/\bar{M})_{obs}$ - and the theoretical value given in column 9 of the table - $(\bar{G}/\bar{M})_{calc}$. From these two values a normalization factor $R = (\bar{G}/\bar{M})_{calc}/(\bar{G}/\bar{M})_{obs}$ was obtained and multiplied with each of the other experimentally determined values of $\bar{G}/\bar{M}$. The corresponding value for C is then read off the table using τ and the normalized $\bar{G}/\bar{M}$.

38.000	159.000	4.184	17.179	18.179	206.910	9.177	20.316	0.452	2.058	26.615
38.000	160.000	4.211	17.514	18.514	208.516	9.297	20.690	0.449	2.057	27.133
38.000	161.000	4.237	17.855	18.855	210.124	9.419	21.071	0.447	2.056	27.661
38.000	162.000	4.263	18.202	19.202	211.734	9.543	21.459	0.445	2.055	28.199
38.000	163.000	4.289	18.555	19.555	213.347	9.668	21.854	0.442	2.054	28.747
38.000	164.000	4.316	18.915	19.915	214.963	9.796	22.256	0.440	2.053	29.304
38.000	165.000	4.342	19.282	20.282	216.581	9.925	22.666	0.438	2.052	29.872
38.000	166.000	4.368	19.655	20.655	218.201	10.057	23.083	0.436	2.051	30.451
38.000	167.000	4.395	20.035	21.035	219.824	10.190	23.508	0.433	2.050	31.040
38.000	168.000	4.421	20.422	21.422	221.449	10.325	23.941	0.431	2.049	31.640
38.000	169.000	4.447	20.817	21.817	223.077	10.462	24.381	0.429	2.048	32.251
38.000	170.000	4.474	21.218	22.218	224.706	10.601	24.830	0.427	2.047	32.873

T	C	EN	EF	HOT	DG	GB	MB	GOM	RSF	PFC
39.000	36.000	0.923	0.896	1.896	35.380	2.146	2.095	1.024	3.116	1.373
39.000	37.000	0.949	0.930	1.930	36.458	2.167	2.133	1.016	3.075	1.425
39.000	38.000	0.974	0.965	1.965	37.541	2.188	2.171	1.008	3.037	1.478
39.000	39.000	1.000	1.000	2.000	38.629	2.210	2.210	1.000	3.000	1.532
39.000	40.000	1.026	1.036	2.036	39.723	2.232	2.250	0.992	2.965	1.587
39.000	41.000	1.051	1.072	2.072	40.821	2.254	2.290	0.984	2.933	1.643
39.000	42.000	1.077	1.110	2.110	41.924	2.277	2.331	0.977	2.901	1.700
39.000	43.000	1.103	1.147	2.147	43.032	2.300	2.373	0.969	2.872	1.758
39.000	44.000	1.128	1.186	2.186	44.146	2.323	2.416	0.962	2.843	1.817
39.000	45.000	1.154	1.225	2.225	45.264	2.347	2.459	0.954	2.816	1.877
39.000	46.000	1.179	1.265	2.265	46.387	2.370	2.503	0.947	2.791	1.938
39.000	47.000	1.205	1.306	2.306	47.515	2.394	2.548	0.940	2.766	2.000
39.000	48.000	1.231	1.347	2.347	48.648	2.419	2.594	0.933	2.742	2.063
39.000	49.000	1.256	1.389	2.389	49.786	2.443	2.640	0.926	2.720	2.128
39.000	50.000	1.282	1.432	2.432	50.928	2.468	2.687	0.919	2.698	2.194
39.000	51.000	1.308	1.475	2.475	52.076	2.494	2.736	0.912	2.678	2.260
39.000	52.000	1.333	1.520	2.520	53.228	2.519	2.785	0.905	2.658	2.328
39.000	53.000	1.359	1.565	2.565	54.386	2.545	2.835	0.898	2.639	2.398
39.000	54.000	1.385	1.611	2.611	55.548	2.572	2.885	0.891	2.621	2.468
39.000	55.000	1.410	1.658	2.658	56.715	2.598	2.937	0.885	2.603	2.540
39.000	56.000	1.436	1.706	2.706	57.886	2.625	2.990	0.878	2.586	2.613
39.000	57.000	1.462	1.754	2.754	59.063	2.652	3.043	0.872	2.570	2.687
39.000	58.000	1.487	1.803	2.803	60.244	2.680	3.098	0.865	2.555	2.763

FIG. 3. An example of the computer's output

RESULTS

(1) Criteria for "Normal" Exponential Growth of Bacterial Cultures

Two necessary conditions for the quantitation of C are that the bacterial culture used is in a steady state of exponential (balanced) growth, and that an accurate measure of its doubling time can be obtained. Initially Campbell's (1957) definition for balanced growth was used ("Growth is balanced over a time interval if, during that interval, every extensive property of the growing system increases by the same factor"). However, careful measurements of four parameters, that were made on both strains used extensively in this study and grown in M9-glucose minimal media, revealed that these (and other thy$^-$) strains never reached a steady state of exponential growth, since the doubling time of cell mass (optical density) was consistently less than the doubling time of cell number in the culture. This phenomenon is illustrated in Fig. 4, and will be described in greater detail in a following section (10). In this context it is important to point out that the phenomenon makes it impossible to get a meaningful estimate of $\bar{M}$ and $\bar{G}$. Fortunately, it was found (see for example Fig. 6) that the doubling time of the DNA in such a culture was equal to that of the mass, and the DNA/mass ratio remained constant for at least 10 generations (see Table 4). Therefore new criteria, by which "normal" growth of a culture was defined, were (and see Zaritsky and Pritchard, 1971):

(a) The culture's mass grows exponentially at a rate, which is not increased significantly by increasing the thymine concentration in the growth media;

(b) The DNA/mass ratio is constant and does not change with time;

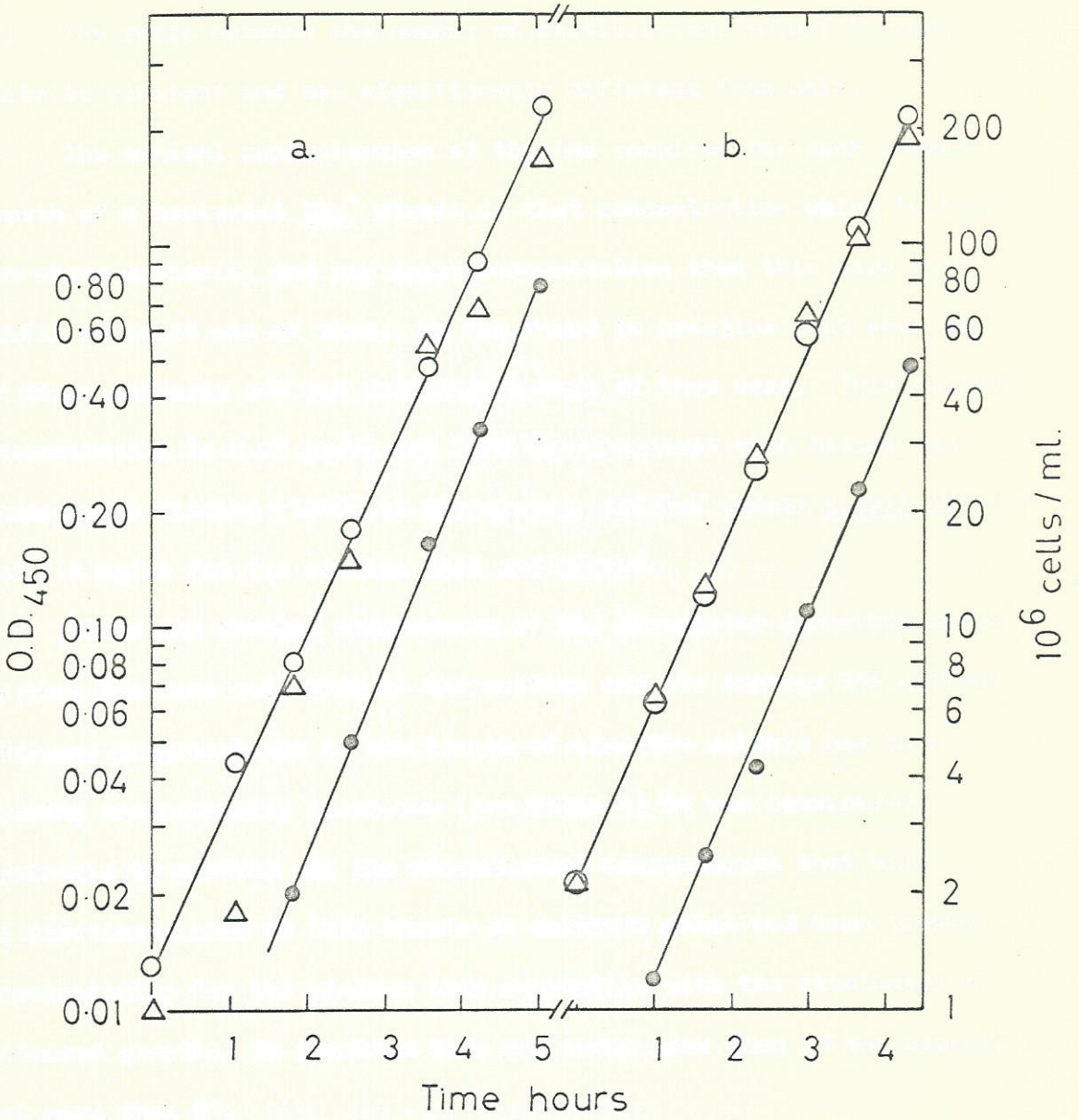

FIG. 4. Absorbance and cell titre of exponentially growing cultures of E.coli 15T⁻, supplemented with (a) 0.5 μg/ml, and (b) 2.0 μg/ml thymine. (•), A_{450}; (○), particles/ml; (△), cells/ml.

(c) The ratio between the number of particles and colony forming units is constant and not significantly different from unity.

The minimal concentration of thymine required for such normal growth of a bacterial thy⁻ strain is that concentration which fulfils the above criteria, and any lower concentration than this does not fulfil at least one of them. It was found in practice that when one of these criteria was not fulfilled - none of them were. This minimal concentration is 0.25 μg/ml for P178 (in agreement with Maaløe and Rasmussen's finding (1963) in the closely related strain E.coli 15TAU⁻) and 2.5 μg/ml for the strains P162 and P162-8.

Since one obvious consequence of the phenomenon described above is that the mean cell size in the culture and its average DNA content increase progressively as the length of time the culture has been growing exponentially increases (see Table 4) it was considered desirable to perform all the experimental measurements that will be described later in this thesis on cultures which had been growing exponentially for the minimum time comptabile with the attainment of balanced growth. In practice this was never less than 3τ and usually not more than 5τ.

(2) Leakiness of thy⁻ Mutations

As described in Materials and Methods, DNA estimations were determined by measuring the incorporation of ^{14}C-thymine present in the growth medium into the cold TCA insoluble fraction of the cells. Since my main interest was in the relative DNA content of cultures grown on media supplemented with varying concentrations of thymine,

it was crucial for the arguments which follow to show that the strains I used are not leaky with respect to the thy⁻ mutations they contain. More specifically, if they are leaky, the proportion of endogeneously synthesized ^{12}C-thymine incorporated into DNA will vary with the concentration of labelled thymine present in the growth medium.

Each of the strains was, therefore, grown in two batches of M9 medium containing ^{14}C-glucose and ^{3}H-thymine; one was supplemented with a *low concentration of thymine (0.5 µg/ml for P178 and 2.0 µg/ml for* P162) and the other with a 10-fold higher concentration. When each of the exponentially growing cultures attained an optical density 0.6-0.7 it was centrifuged, washed twice with Tris EDTA buffer and resuspended in 5 ml of this buffer. The suspension was incubated with 200 µg/ml lysozyme at 37° for 5 minutes, when sodium lauryl sulfate (0.5%) was added. Five minutes later 200 µg/ml pronase was added (preincubated for 2 hr. at 37°), and the mixture was further incubated (37°) for 30 minutes. The DNA was purified twice by fractionation ofa CsCl gradient centrifugation (48 hr. x 35,000 rpm). After an overnight dialysis of the DNA-containing fraction against distilled water, it was lyophilised and hydrolysed with concentrated formic acid (175° x 30 minutes) in order to hydrolyse the DNA to release free bases (Vischer and Chargaff, 1948). This was performed by inserting a sealed tube containing the DNA-containing fraction into an oil bath prewarmed to 175° for half an hour. After cooling the tube, the acid was evaporated and the hydrolysate redissolved in 18 µl of a solution of unlabelled thymine, uracil and cytosine (200 µg/ml each). The mixture was chromatographed on a Polygram cellulose thin layer (MN 300, cel 300, 0.1 mm cellulose, 20 x 20 cm) using distilled

water (adjusted to pH 9 with NH_4OH) in the first dimension and *n*-butanol-H_2O (86:14) in the second (Smith, 1960, p.237). The spots were detected by UV absorption and each cut into several pieces before counting in a liquid scintillation counter. The ratio of $^3H/^{14}C$ counts in various pieces of each spot was variable presumably due to contamination with other ^{14}C-containing materials. The pieces with the highest $^3H/^{14}C$ ratio were used to obtain the values recorded in Table 2.

TABLE 2. Relative contribution of ^{14}C-glucose to DNA-thymine in thy$^-$ mutants grown on different thymine concentrations

Strain	Thymine concentration (μg/ml)	Contribution of ^{14}C-glucose (percent)
P178	0.5	5.0
P178	5.0	3.0
P162	2.0	3.5
P162	20.0	4.2

The data show that in both strains the contribution of ^{14}C-glucose to DNA-thymine is not more than 5% even when the external concentration of ^3H-thymine is the minimum compatible with normal growth. These values are in close agreement with other estimates obtained for this strain and other strains carrying the same thy$^-$ mutation (Cohen and Barner, 1954; Pritchard and Lark, 1964; Friesen and Maaloe, 1965). No corrections were made for this small contribution in subsequent experiments.

Estimations of the Chromosome Replication Time.

(3) DNA Increment after Removal of Amino Acids from the Cultures of E.coli 15T⁻

It was originally pointed out by Maaløe and Hanawalt (1961) that the increment in DNA predicted to occur in a culture deprived of a required amino acid is 39 percent if no further initiation occurs and all replication forks progress to the chromosome terminus. This increment is not usually found in practice in cultures of thy⁻ mutants growing in synthetic media with glucose as carbon source (e.g. Billen and Hewitt, 1966; Donachie, 1969; Pritchard and Zaritsky, 1970). An increment of 39 percent is only predicted, however, for cultures in which $C = \tau$ (i.e. when the average number of forks per chromosome is 1). *On the other hand, as described in the Introduction, measurement of this increment in DNA can provide an estimate for the average number* of forks per chromosome in the culture, and hence of C. One problem associated with this method is that the capacity to replicate DNA declines during amino acid starvation (Doudney, 1966) and therefore not all chromosomes necessarily complete replication (which is one of the assumptions essential for the quantitation required). The following experiment was conducted to demonstrate this phenomenon and to define the minimal concentration of thymine required to complete chromosome replication in the absence of amino acids.

A culture was grown for at least five generations in ^{14}C-thymine at a concentration of 0.5 µg/ml and the required amino acids were removed by filtration when it reached A_{450} = 0.4. The subsequent increment of DNA was determined by further incubation in medium lacking amino acids and containing ^{14}C-thymine at different

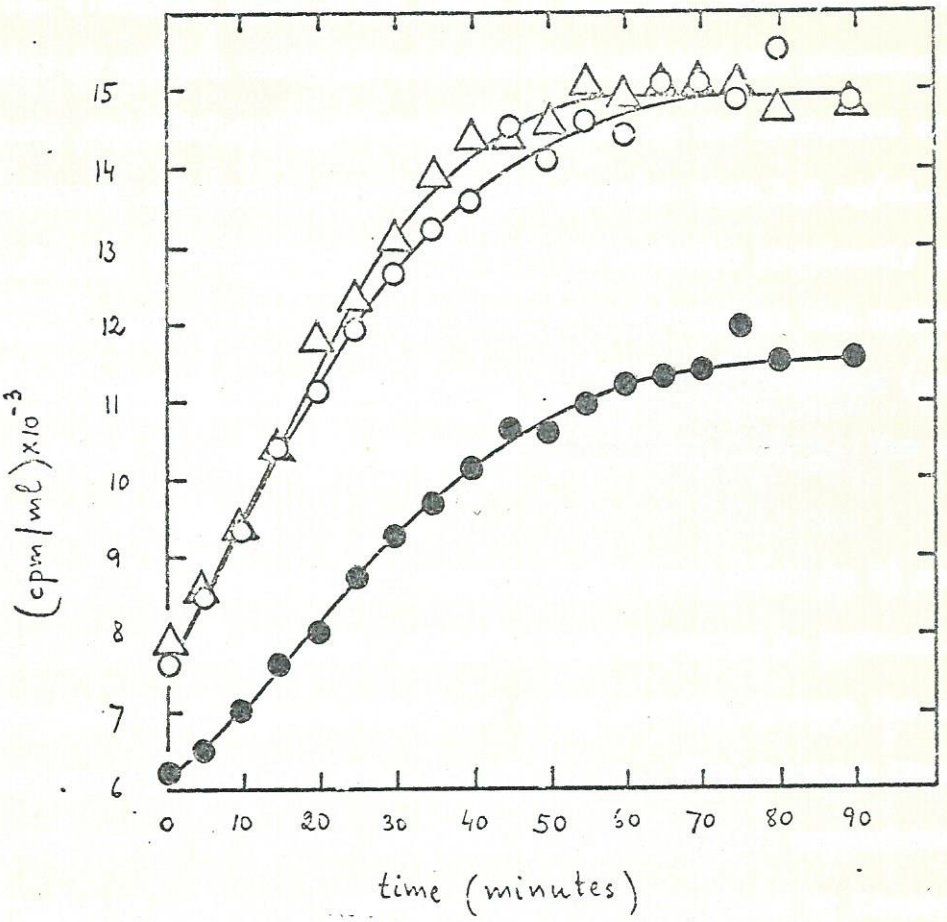

FIG.5. Increment in DNA during amino-acid starvation.

The following post-shift thymine concentrations were used:

(●), 0.5 µg/ml; (o), 1.0 µg/ml; (Δ), 9.0 µg/ml.

concentrations but with the same specific activity. The curves shown in Fig. 5 suggest that, after about 70 minutes, replication ceases whether or not all replication forks have reached the chromosome terminus. However, there is no significant difference in the increment of DNA in the presence of thymine concentrations greater than 1.0 μg/ml.

△G was then measured in a series of cultures previously grown on different concentrations of thymine. In each case it was determined in a set of cultures containing various thymine concentrations (not lower than 1.0 μg/ml) during the amino acid starvation period. The results are given in Table 3.

There is a progressive increase in △G (and hence in C) as the thymine concentration in the previous growth medium is reduced. In Figure 10a the calculated values of C are plotted against the reciprocal of the corresponding thymine concentrations. The straight line obtained extrapolates to 57 minutes for C at infinite thymine concentrations. This value is higher by more than 20% than that found by Cooper and Helmstetter (1968) in thy^+ strains of E.coli B/r. The validity of this difference and its possible reasons will be discussed later.

(4) Changes of DNA/Mass Ratio in Cultures Supplied with Different Concentrations of Thymine

Maaløe (1961) first suggested that "the capacity for DNA synthesis in a culture is correlated with the DNA/mass ratio". This concept was later extended by Donachie (1968) and Donachie et al (1968) and emphasized by Copeland (1969), Helmstetter et al (1968) and others. They relate the initiation event to a definite DNA/mass ratio. The concept that this ratio is constant for any given growth rate can be true only in cases where the replication velocity of the bacterial chromosome is constant. The hypothesis that correlates the replication