by deoxyguanosine (Beacham et al., 1968). Although it is known now (Beacham et al, 1971) that this metabolite does not significantly raise the internal concentration of dTTP above that obtainable with high concentrations of thymine alone in the growth medium, a substantial reduction in C in P178 was obtained (Tables 12 and 13). The estimates(48 minutes from ΔG and 49 minutes from $\bar{G}/\bar{M}$) are quite close to the estimate of 46 minutes which was obtained by Cooper and Helmstetter (1968) in E.coli B/r. Two additional estimates of C in P178 grown on deoxyguanosine were calculated from measurements of RSF in glucose grown culture (section (6)) or in glycerol grown culture (section (8)). These were 47 and 40 minutes respectively, averaging to 43.5 minutes.

In the case of P162-8, on the other hand, although addition of deoxyguanosine reduces the replication time to a value below that obtained with thymine alone (Tables 8, 12 and 13) it did not fall significantly below that given by the extrapolate for infinite thymine concentrations.

The values of C estimated in the presence of deoxyguanosine in the growth medium were introduced into the appropriate Figures 10a,b,d and 12 on the C axis (where $1/[T] = 0$) and will be discussed in connection with (a) the validity of the absolute values of C given in this study, and (b) the contribution of the internal concentration of dTTP to the determination of the replication velocity, in the Discussion.

Cell Division in E.coli 15T⁻

(10) Problems of Cell Division in thy⁻ Strains

As described in section (1) a definition of a steady state of exponential growth in a glucose grown culture was not simple, and it was necessary to deviate from Campbell's (1957) definition of "balanced growth" due to the phenomenon which was revealed at the start of this study. It was shown (Fig. 4 and uncrecorded results) that the doubling time of cell titre in such a culture of a thy⁻ strain of E.coli (either 15 or K12) is longer than the doubling time of the mass in that culture. It was first thought that a steady state had not been achieved, but a long-term experiment (Table 4) demonstrated that this was not the case. The rate of DNA synthesis paralleled the rate of mass increase (and see Fig. 6), but the rate of cell division lagged behind. The consequence of this is that cell size and its DNA content increases continuously. It was found soon afterwards that basically this phenomenon was independent of the concentration of thymine in the medium, although the concentration did define the rate at which the average cell size increased; the lower concentration of thymine used the faster this process occurred due to a longer inter-division time. Once the culture reached a "normal" growth (see section (1)) the rate of increase in average cell size was uniquely defined by the thymine concentration supplied to the culture. This is shown in Fig. 13.

Although the theoretical as well as the practical difficulties in sizing of cells were known to me (e.g. Grover et al., 1970a,b; Kubitschek, 1969; Harvey and Marr, 1966; Harvey et al, 1967), it

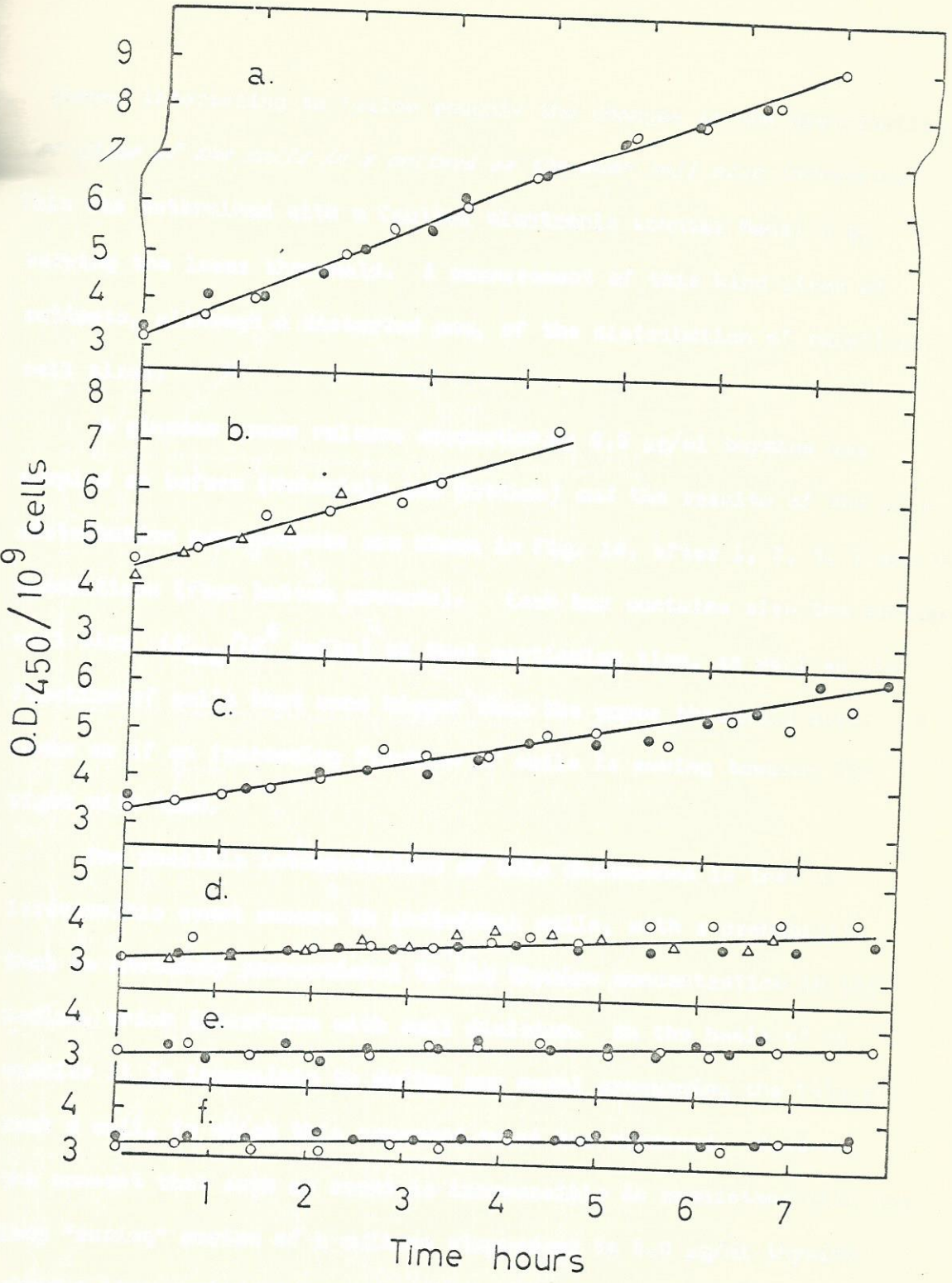

FIG. 13. Average cell sizes in cultures grown exponentially in various concentrations of thymine: (a) 0.4 µg/ml, (b) 0.5 µg/ml, (c) 1.0 µg/ml, (d) 2.0 µg/ml, (e) 5.0 µg/ml, (f) 30.0 µg/ml. The different symbols in each box represent different experiments.

seemed interesting to follow roughly the changes in the distribution of sizes of the cells in a culture as the mean cell size increased. This was determined with a Coulter electronic counter Model B by varying the lower threshold. A measurement of this kind gives an estimate, although a distorted one, of the distribution of relative cell sizes.

A glucose grown culture supported by 0.5 μg/ml thymine was sampled as before (Materials and Methods) and the results of the size distribution measurements are shown in Fig. 14, after 1, 3, 5, 7 and 10 generations (from bottom upwards). Each box contains also the average cell size ($A_{450}/10^9$ cells) at that particular time, as well as the fraction of cells that were bigger than the upper threshold used. It looks as if an increasing fraction of cells is moving towards the right with time.

One possible interpretation of this phenomenon is that an irreversible event occurs in individual cells, with a probability that is inversely proportional to the thymine concentration in the medium, which interferes with cell division. On the basis of my results it is impossible to define any model concerning the fate of such a cell, in which this presumed event has occurred. However, the concept that such an event is irreversible is consistent with the long "curing" period of a culture stepped-up to 5.0 μg/ml thymine after a long period of growth on 0.5 μg/ml thymine (Fig. 15).

Equation (8) (Introduction) describes how the average cell mass ($\bar{M}$) changes with C, D and τ. It can be seen that one consequence of a step-up, which reduces C, is to decrease the mean cell size. Theoretically, the new cell size will be achieved (C + D) minutes

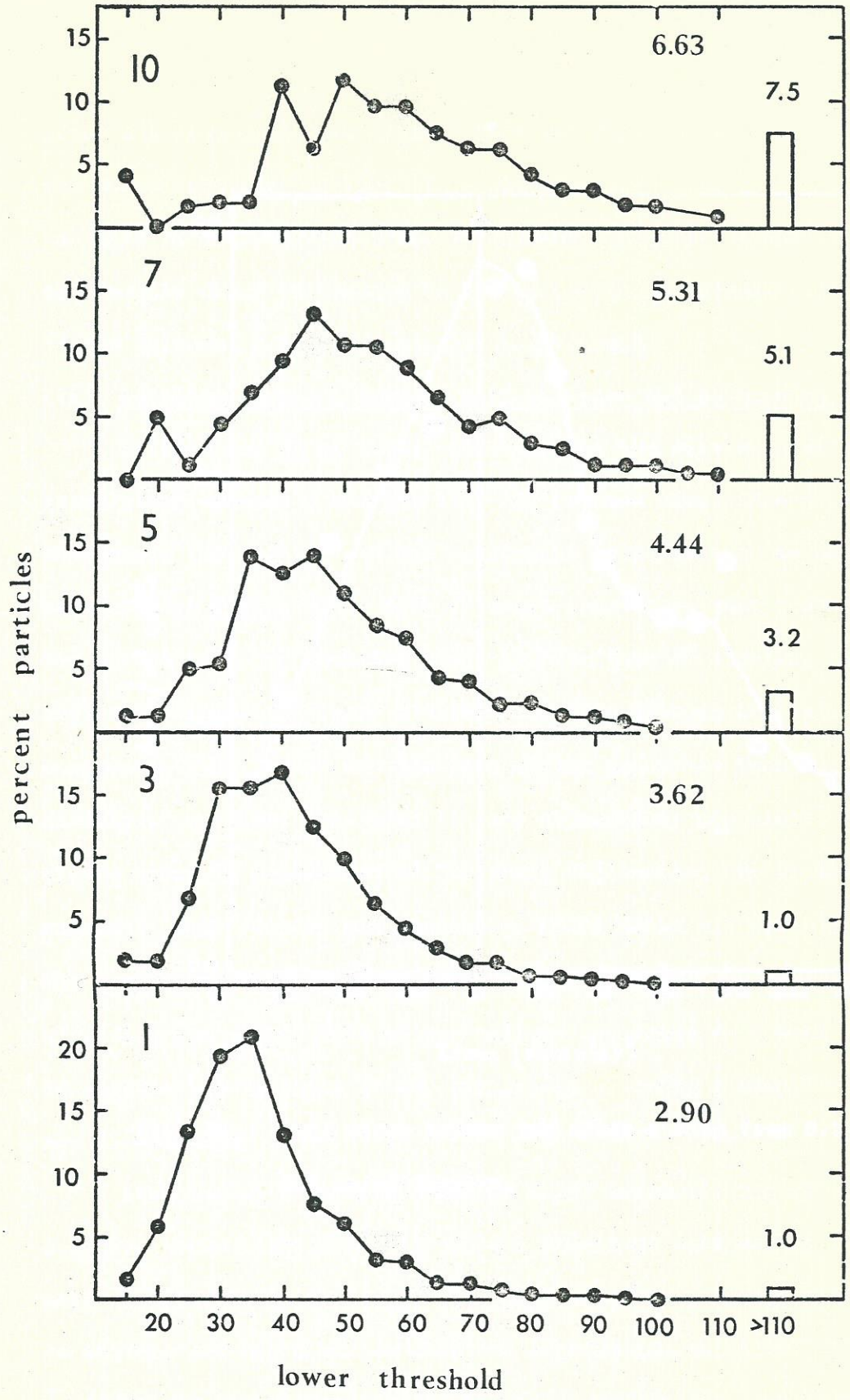

FIG. 14. Size distributions of E.coli 15T⁻ grown exponentially in glucose-M9 supplemented with 0.5 μg/ml thymine for 1, 3, 5, 7 and 10 generations.

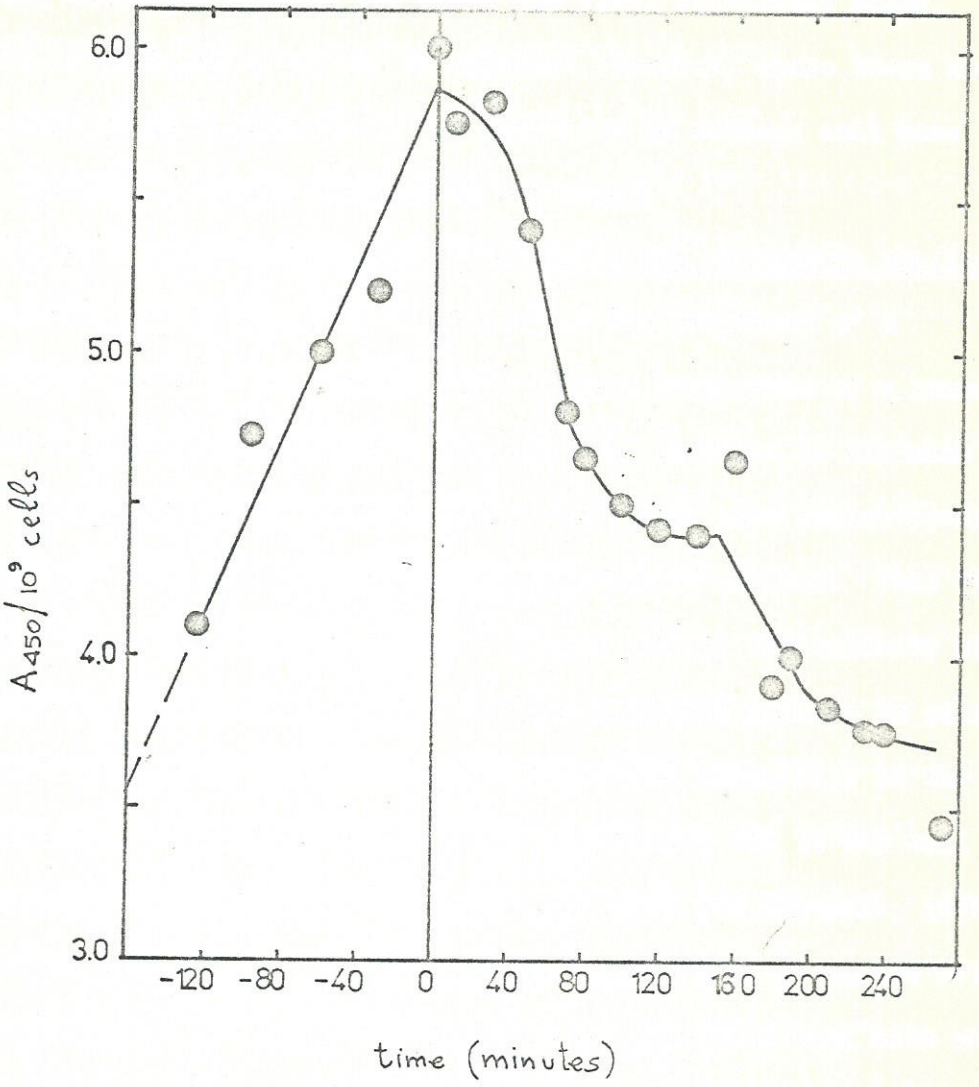

FIG. 15. Cell sizes before and after a step from 0.5 µg/ml to 5.0 µg/ml thymine.

after the step-up is performed. This may contribute to the shape of the curve described in Fig. 15, since the first relatively quick response is achieved after ca. 80 minutes. However, since there are two factors which contribute to the reduction in average cell mass in the culture, a proper analysis is difficult.

(11) Possible coupling between cell division and chromosome replication.

Helmstetter et al (1968) demonstrated the existence of a correlation between C and the time (D) between completion of a round of chromosome replication and the subsequent cell division in cultures of slow growing E.coli B/r. Both of these parameters were proportional to the growth rate for doubling times greater than 65 minutes. This finding does not necessarily imply a direct coupling between the velocity of the chromosome replication and the rate of the processes leading to cell division. However, this idea is very attractive, especially in the light of the possible connection of the newly replicating DNA to the cell membrane (e.g. Ganesan and Lederberg, 1965; Smith and Hanawalt, 1967). The system described in this study, which enables C to be varied without affecting the growth rate of the culture can serve as a tool with which to analyse the validity of any hypothesis assuming a coupling between C and D.

Stepping-up a culture grown on a low concentration of thymine to a higher concentration should cause a transient increase in the rate of cell division in the culture. The pattern of this change will depend on whether and how D varies with the replication velocity. If D is constant and independent of the replication velocity, then after a step-up the rate of cell division should stay constant for D minutes and then accelerate. It will fall back to the old rate after a period

equivalent to the new (post step-up) value of (C+D). If increasing the thymine concentration enhances the processes that determine D, one expects to find an immediate and gradual increase in the rate of cell division after the step-up which will fall back to the pre-step-up rate also after the new (C+D) minutes. If this were the case then the value D could be estimated in a way analogous to that used for estimating C after a step-up, by determining the time required to achieve the new mean cell size in the culture.

The data in Fig. 16 show that the rate of cell division remains constant at the pre-step-up rate for a finite but variable time after the step-up, the delay depending on the post-step thymine concentration. One possible interpretation for this observation is that the length of D is in some way coupled to the velocity with which the chromosome was replicated prior to completion. This hypothesis was not tested in the present study. It should be emphasized here that the combination of factors involved in cell division (described in the previous section) could lead to a false conclusion about the values of D. A series of experiments like those described in Fig. 16 was repeated using various post-step thymine concentrations. These are summarized in Table 14 and the apparent values of "D" are plotted against the reciprocal of the post-step thymine concentration in Fig. 17. The six points fall on a straight line, indicating a correlation between "D" and the concentration of thymine used. Five of these values are plotted in Fig. 18 against the values of C in the corresponding thymine concentrations estimated by $\bar{G}/\bar{M}$ (see section (4) and Table 5). The values of Helmstetter and Cooper (1968) for a glucose grown culture of E.coli B/r (C = 46 min, D = 24 min) are added (○). There is a clear correlation

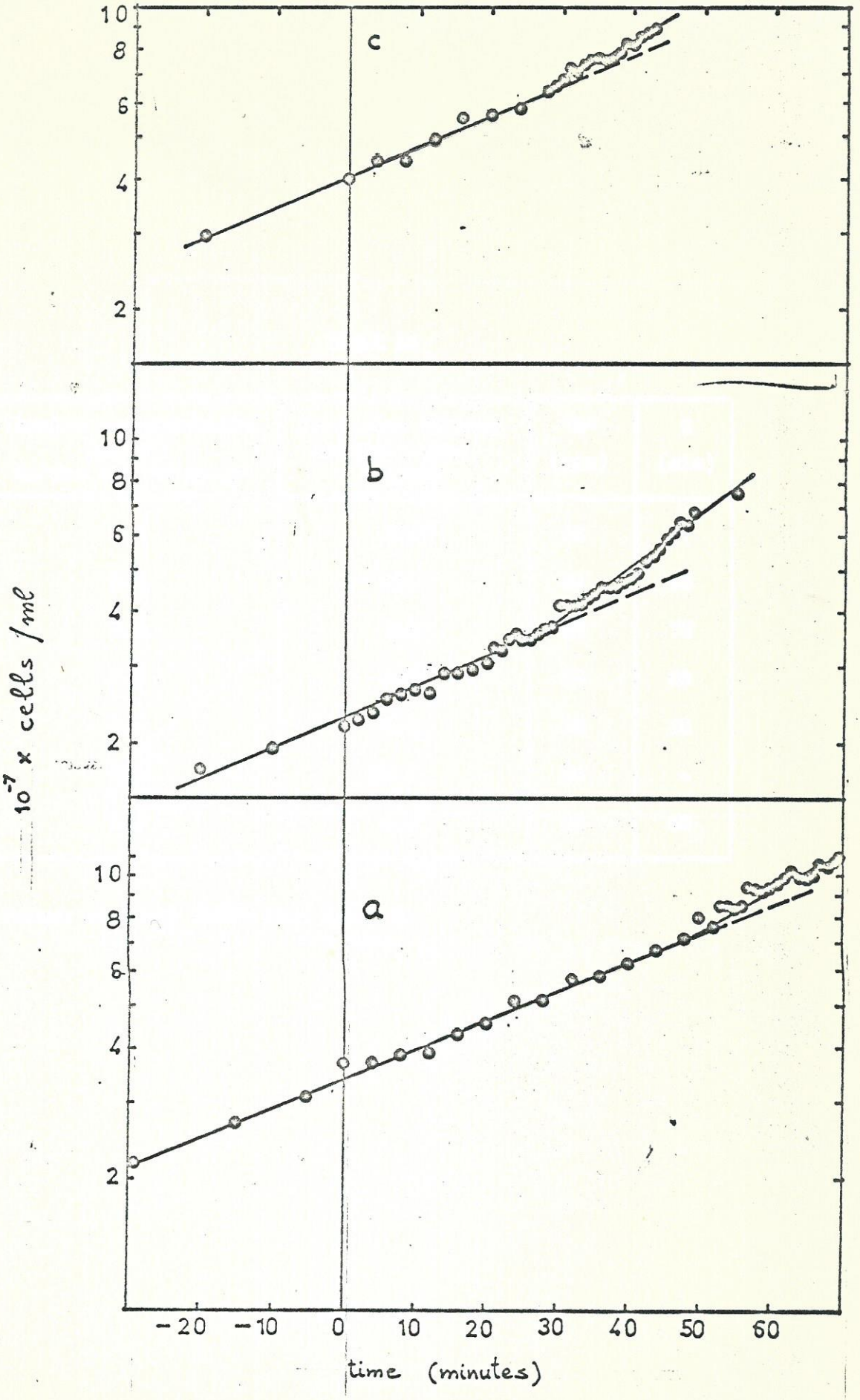

FIG. 16. Cell titres before and after step-up experiments. (a) from 0.5 to 0.7 µg/ml, (b) from 0.5 to 5.0 µg/ml and (c) from 1.0 to 5.0 µg/ml.

TABLE 14

Thymine concentration (μg/ml) before the "step-up"	Post-step thymine concentration (μg/ml)	"D" (min)	C (min)
0.4	0.5	65	97
0.4	0.7	53	85
0.4	1.0	45	70
0.4	1.9	38	62
0.4	5.0	30	60
0.4	50.0	25	-
1.0	5.0	30	60

Values of C are taken from Table 5.

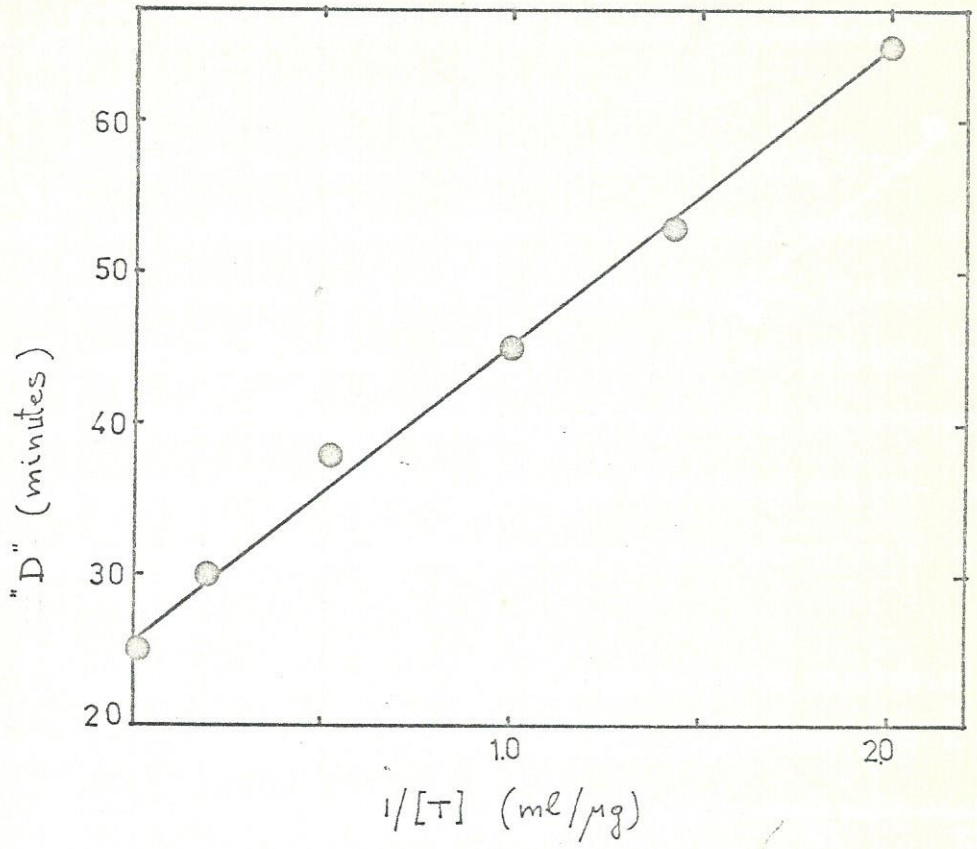

FIG. 17. "D" against the reciprocal of the thymine concentration.

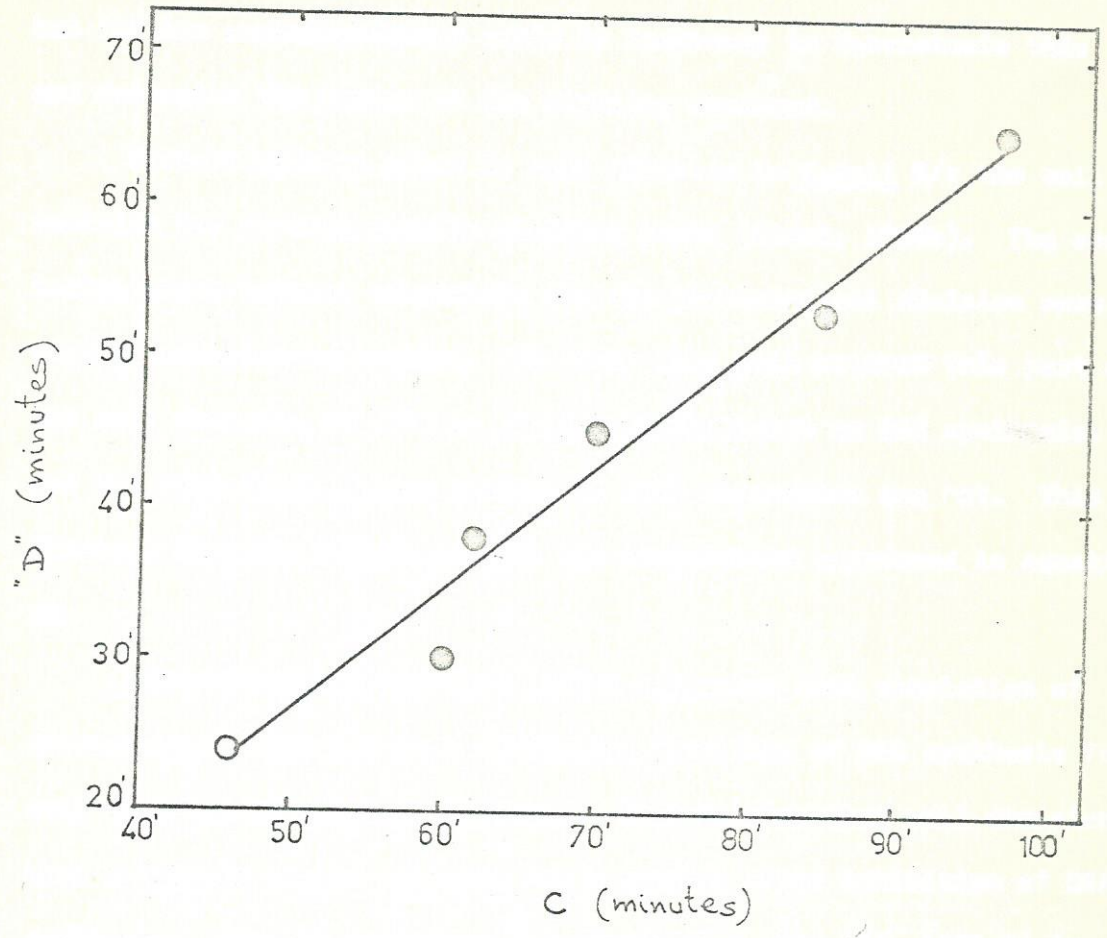

FIG. 18. "D" against C. (●), Values of C obtained by $\bar{G}/\bar{M}$.

between the two parameters, calculated to have the form

$$\text{"D"} = 0.83C - 16 \qquad (12)$$

This relationship suggests that C and D are coupled in some way, but further experimentation would be necessary to demonstrate this and determine the nature of the coupling observed.

Fifteen minutes prior to cell division in E.coli B/r the cell contains two physiologically-distinct parts (Clark, 1968b). The events taking place between completion and the physiological division were termed compartmentalization. It could very well be that this part of the cell division process is completely dependent on DNA synthesis, but the subsequent events leading to cell separation are not. This finding, however, cannot explain the correlation expressed in equation 12.

Helmstetter and Pierucci (1968) suggested that completion of chromosome replication is a sufficient condition of DNA synthesis for cell division in s.s.e.g. cultures. This implies that cell division in such a culture should continue for D minutes after inhibition of DNA synthesis. Fig. 19 describes the growth of E.coli $15T^-$ (555-7) in M9-glucose containing 5.0 µg/ml thymine before and after treatment with nalidixic acid (NAL). This agent blocks primarily DNA synthesis (Goss et al, 1965). It can be seen that although the mass continues to increase exponentially for almost one doubling, the cells continue to divide for a period of 15-18 minutes only. This period is approximately the same as that found in E.coli B/r treated similarly (Helmstetter and Pierucci, 1968; Clark, 1968a,b). Cell numbers increased by a factor of 1.2. Similar experiments in which DNA replication was inhibited by thymine deprivation gave only ca. 10%

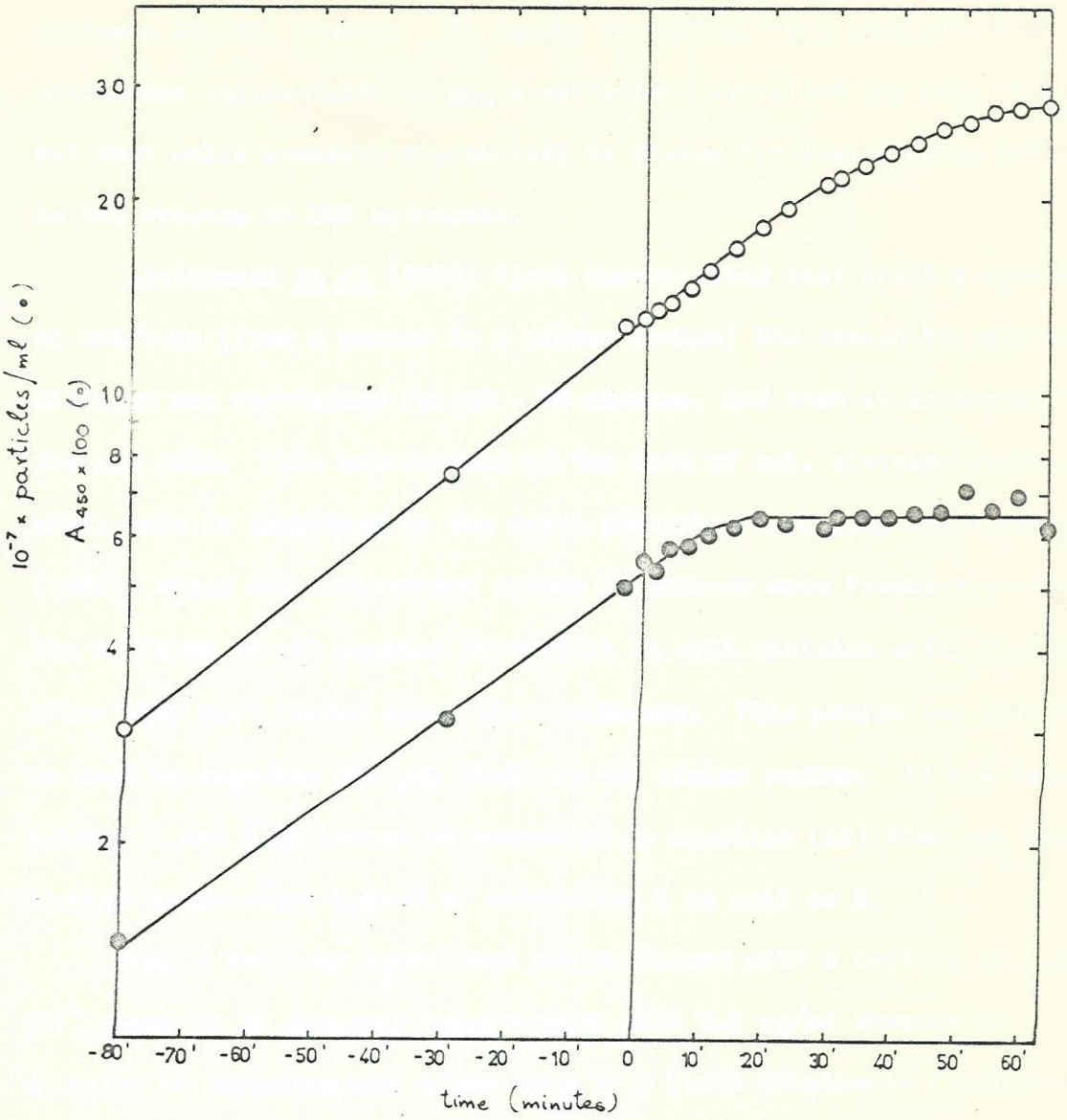

FIG. 19. Absorbance (○) and cell titre (●) of a culture before and after a treatment with NAL (50 µg/ml).

increase in cell number. It seems, therefore, that completion of chromosome replicationn is not a sufficient condition for cell division, but that cells preserve the ability to divide for less than 20 minutes in the absence of DNA synthesis.

Kjeldgaard et al (1958) first demonstrated that after a nutritional shift-up (from a poorer to a richer medium) the pre-shift rate of cell division was maintained for 60 - 70 minutes, and then it increased to the new rate. This maintenance of the rate of cell division at the pre-shift rate is explained by the model presented by Cooper and Helmstetter (1968), since new replication points introduced more frequently after the shift-up do not express themselves in cell division until D minutes after they have passed along the chromosome. This period can therefore be used to estimate the sum (C+D) in the richer medium. If the relation between C and D is indeed as described in equation (12) then one has another independent method of estimating C as well as D.

Such a shift-up experiment was performed with a culture of E.coli 15T$^-$ grown on M9 glycerol supplemented with 5.0 µg/ml thymine by diluting it approximately eight-fold to a fresh M9-glucose (containing the same thymine concentration) at time 0. Absorbance and particle number were followed before and after this time and recorded in Fig. 20. It can be seen that the mass doubling time decreased from 70 minutes to 47 minutes immediately after the shift-up, while the doubling time of cell numbers remained at 70 minutes for a period of 80 minutes. This suggests that under these conditions (M9-glucose, 5.0 µg/ml thymine) $C + D = 80$ minutes. Assuming that equation (12) is valid, one has a set of two equations with two variables, $C + D = 80$ and $D = 0.83C - 16$. The solution for this pair of equations is given by $C = 52$ minutes and

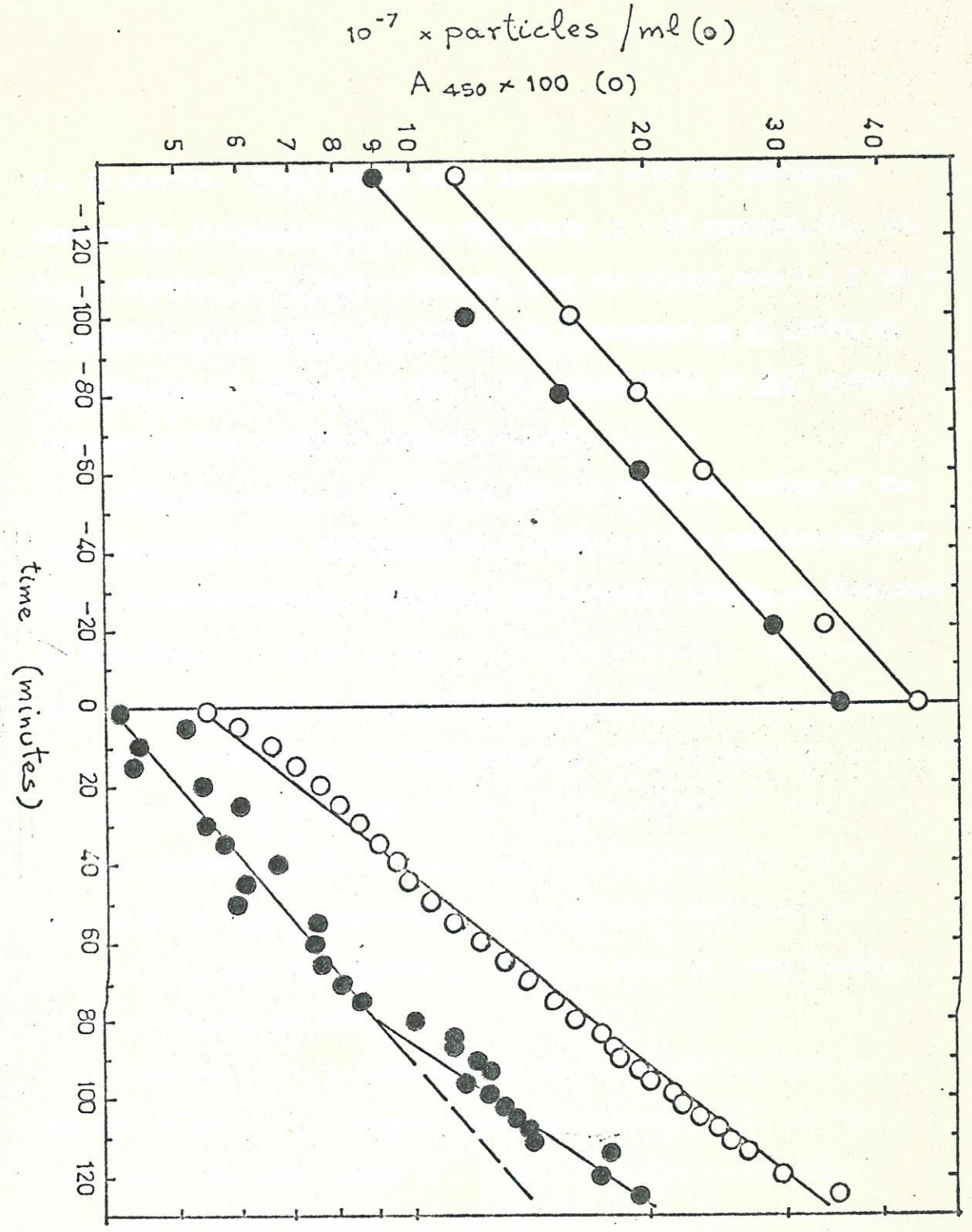

FIG. 20. Absorbance (O) and cell titre (●) of a culture before and after a shift-up.

D = 28 minutes. These are in close agreement with the estimates obtained by different methods in this study (C = 60 minutes, Fig. 10; D = 30 minutes, Table 14). However, they are higher than C + D (60 - 70 minutes) measured in a similar way by Kjeldgaard *et al* (1958) in *S.typhimurium*. This may be due to the longer (C+D) in *thy*$^-$ strains compared to this period in the prototrophs.

It is premature to try to describe the molecular nature of the processes leading to bacterial cell division and the coupling between them and the chromosome replication, if it really exists, as indicated by Fig. 18.

DISCUSSION

In this study four methods, more or less independent of each other, were used to demonstrate changes in the replication velocity of the chromosome of *thy*$^-$ strains of *E.coli*. None of them by itself proves unequivocally that the changes in cell composition which are observed when cultures are grown in the presence of different thymine concentrations arise solely from changes in replication velocity. The simplest alternative possibility would be that, contrary to the assumption that the mass/chromosome origin ratio at initiation is constant, it is affected by the thymine concentration. However, this does not explain the variation of ΔG with the thymine concentration. Therefore, if this were the explanation it would be necessary to invoke an additional effect to account for the changes found in ΔG. Since all the changes observed are consistent qualitatively with an effect on C alone and are in good quantitative agreement with each other and to some other results recorded in the literature, it seems reasonable to favour this hypothesis.

Only two of the methods used in this study (ΔG and RSF) can give absolute values of C in minutes (see Introduction), both are open to criticism. The quantitation of C that is based on the fractional increment in DNA (ΔG) in a culture after shifting it down to conditions of amino acid starvation depends on the assumption that this treatment inhibits instantaneously further initiations in the culture but does not interfere with completion of those rounds of chromosome replication which were initiated before the shift-down. It was found in other strains that during this treatment not all chromosomes necessarily

terminate (Wolf et al, 1968; Caro and Berg, 1968) and the cells gradually lose their capacity to replicate DNA (Doudney, 1966). The consequence of this may be smaller values of ΔG at all thymine concentrations which give underestimates of the corresponding values of C. The main results of this effect would be to decrease the whole set of values of C and lower the line plotted in Fig. 10.

On the other hand, inhibition of protein synthesis by amino acid deprivation may not inhibit further initiations for a short time, thus increasing the measured ΔG values and hence overestimating the calculated values of C over the whole range. The main result of this effect would be to raise the line plotted in Fig. 10. If each of these two effects is acting preferentially on cells growing on different thymine concentrations the consequence of this would be to change the slope of the line.

The steeper slope and the lower C values for high thymine concentrations obtained when the rate stimulation factor (RSF) was used (Fig. 10d) are consistent with the possibility that estimates of C from ΔG are distorted by one or more of the factors described above although it must be pointed out that estimates of C from the RSF are themselves subject to potential errors. Thus it is conceivable that after a period of thymine starvation equal to the time required for one mass doubling, the velocity of replication, after restoration of the same concentration of thymine, might be changed. Since this treatment was shown to disturb the internal concentrations of the nucleotide triphosphates (Neuhard, 1966), it can be expected that the disturbed balance of the four alters the replication velocity compared to that in a s.s.e.g. culture. However, the straight line obtained when ^{14}C-incorporation into DNA is plotted against $2^{t/\tau} - 1$, where t is the time after addition of ^{14}C-thymine into

the medium of a starved culture as described (Fig. 9, line b), suggests that this technique and its quantitative interpretation are valid. The expected line on such a plot is straight for a period of time C, after which its slope is expected to decrease abruptly due to the synchronous completion of those forks initiated presumably in a synchronous fashion when thymine was restored to the medium.

Therefore, the line drawn in Fig. 10d probably gives a more reliable estimate of C than that described in Fig. 10a (from ΔG experiments). This is supported by the slopes of the lines (b, c in Fig. 10) obtained from the two other methods used in this study to estimate relative values of C. One of these (DNA/mass ratio; $\bar{G}/\bar{M}$) is probably the most reliable method of all four used in this study since it involves measurements of macromolecular composition of cultures in a steady state of exponential growth where the cell metabolism is not interfered with by any treatment as is the case with the other methods. Unfortunately it does not give C in absolute terms and the slope of the line obtained when plotting C against the reciprocal of the thymine concentration (Fig. 10) depends slightly on the position of the reference value (see also Materials and Methods (3)). The line plotted in Fig. 10b is based on values of C calculated from different DNA/mass ratios where C was assumed to be 60 minutes for 5.0 µg/ml thymine. Since this value was derived from the ΔG experiment - it is open to the potential errors inherent in this method, as discussed. When C values are calculated on the assumption that C = 50 min (for 5.0 µg/ml thymine) the line constructed has virtually the same slope as that plotted in Fig. 10b. This supports the validity of the slope obtained by this method, which is bigger than that described in Fig. 10a.

A similar slope is obtained by the fourth method used in this study (Fig. 10c) measuring the relative rates of incorporation of ^{14}C-thymine into DNA after a step-up to various thymine concentrations (section (5)). This supports the conclusion that the rate of change of C with the reciprocal of the thymine concentration is underestimated when measurements of ΔG are used.

As was argued before, the balance of internal pools of the four nucleotide triphosphates does not appear to be disturbed to such an extent as to alter significantly the replication velocity as soon as four minutes after a period of thymine starvation equivalent to one mass doubling time (Fig. 9). Since a step-up condition does not follow such an extreme treatment as thymine deprivation, it might be expected that this balance adjusts itself to the new concentration of thymine in less than four minutes after the step-up is performed. Moreover, the cells used in each pulse labelling experiment were taken from one batch culture and the incorporation of ^{14}C-thymine into DNA after the step-up was followed for a few minutes only and the experiment completed in less than a quarter of a generation time. These two points argue for and support the validity of the relative C values in P178 obtained by this method (section (5)).

The three methods discussed last agree closely in the relative estimates of C they produce. Since this invalidates the other method (ΔG) it means that I am left with one valid method only to estimate absolute values of C. The fact that the estimates obtained by RSF measurements when the cultures were supplemented with deoxyguanosine are equivalent to the extrapolate of the line obtained with this method (Fig. 10d), supports the conclusion that RSF is a reliable method for

estimating C in minutes. This allowed me to change the scale of the relative values of C obtained by the two other methods used in this study ($\bar{G}/\bar{M}$, and pulse incorporation). Using the value C = 43.5 minutes, for a culture supplemented with deoxyguanosine, as a reference value (average of two different measurements), I obtained a new set of C values for the DNA/mass measurements (Tables 5 and 13). These are plotted (●) in Fig. 21, with the points (○) obtained by RSF measurements. The third set of points in this figure (△) were taken from the pulse incorporation data (experiment 1 in Table 6; and see also Pritchard and Zaritsky, 1970), using C = 54 minutes for 2.0 μg/ml thymine (since this was not measured after stepping up into deoxyguanosine-supplemented medium). The three sets of points fall on a straight line, thus supporting quantitatively each other.

Experiments of RSF in P162-8 have not been done in this study. Therefore such a correction, as was done in Fig. 21 for P178, could not be done for CR34. The other line (a) in this figure represents the line (Fig. 11) for this strain. This is probably also shifted upwards, since for glycerol-grown cultures of this strain too, all values of C are lower than they are for its glucose grown cultures (Fig. 12b).

Several of my other results, as well as others recorded in the literature for this strain (555-7) strengthen my suspicion about the validity of the actual values of C given in Fig. 10a, b, c. A rough comparison of DNA/mass ratio in s.s.e.g. cultures indicates (Table 15) that in 5.0 μg/ml thymine it is not significantly different from that in *E.coli* $15T^+$. This is expected if C for this concentration is similar to C in the *thy*$^+$ strain.

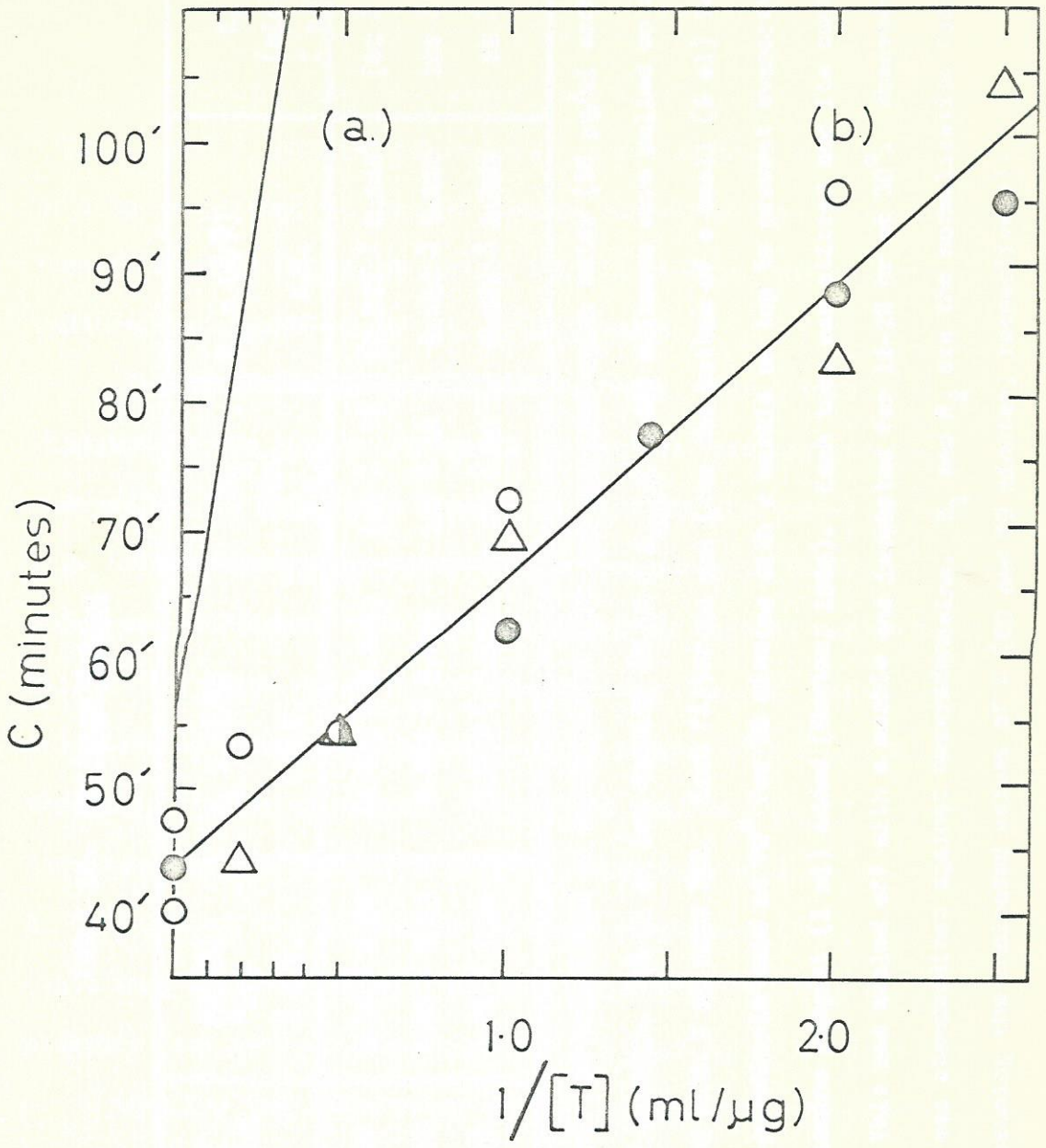

FIG. 21. C against the reciprocal of the thymine concentration in (a) P162 and (b) P178.