TABLE 15. DNA/mass Ratios

Strain of *E.coli* 15	Thymine concentration (μg/ml)	τ (min)	$\frac{\mu g(dA)}{A_{450}}$ ± S.E.	$R_1 \times (\bar{G}/\bar{M})$ ± S.E.	C_1 (min) ± S.E.	$R_2 \times (\bar{G}/\bar{M})$ ± S.E.	C_2 (min) ± S.E.
555-7	5.0	40	1.54 ± 0.08	(0.862 ± 0.045)	(60 ± 7)	(0.927 ± 0.048)	(50 ± 7)
555-7	0.5	38	1.02 ± 0.11	0.571 ± 0.062	117 ± 18	0.614 ± 0.066	106 ± 17
wild type	-	42	1.48 ± 0.11	0.828 ± 0.062	69 ± 11	0.891 ± 0.066	58 ± 11

The strains were grown in M9-glucose unsupplemented or supplemented with the required amino acids (50 μg/ml each) and the indicated concentration of thymine. 25 ml of low density ($0.05 \leq A_{450} \leq 0.40$) cultures were pulled on a Millipore filter (25 mm) and washed with 5 ml cold TCA. Each filter was put into the bottom of a small (6 ml) test tube and cooked with 0.5 ml 0.5 N-perchloric acid at 70° for 30 minutes with a marble on top to prevent evaporation. The tubes were cooled and diphenylamine reagent (1 ml) added (Burton, 1956). Each tube was stirred and its contents decanted into another test tube. This was sealed off with a marble and incubated for 16-20 hr at 30°. Absorbance was determined at 600 nm. The method was standardized against deoxyadenosine. R_1 and R_2 are the normalization factors (see Materials and Methods (3)) assuming the indicated values of C (60 and 50 minutes, respectively).

The time needed for the DNA/mass ratio in the culture to attain a new equilibrium when stepped-up from low to high thymine concentration (Figs. 6 and 7, and see section (4)) gives another estimate of the value of C corresponding to the new thymine concentration. A period shorter than 60 minutes (although longer than 50 minutes) was required when a culture was stepped-up to 5.0 μg/ml thymine.

The time after a nutritional shift-up, when the rate of cell division in the culture increases to the new rate corresponds to the new medium, is a reliable measure of (C+D) in the richer medium (see e.g. Helmstetter et al, 1968). This was achieved when a culture of 555-7 was shifted-up from glycerol medium to glucose medium (section (11)). The value obtained (80 minutes, see Fig. 20) enabled me to calculate an upper limit estimate of 56 minutes for C (in 5.0 μg/ml thymine), if D is 24 minutes (Cooper and Helmstetter, 1968). However, the higher is the assumed value of D, the lower will be the estimate of C. If, indeed, D = 30 minutes in these conditions (Table 14, section (11)) then C = 50 minutes. The real value of C for this concentration probably lies between these two, $50' \leq C$ (5.0 μg/ml thymine) $\leq 56'$.

Finally, the lower estimates for C obtained in M9-glycerol (section (8)) might be the result of reduced rates of exhaustion of the dTTP pool in cells growing slower. This pool is shown later (and see Fig. 22) to determine partly the replication velocity (and see also Beacham et al, in the press). However, since the effect of thymine concentration on cell division in glucose-grown cultures (section (10)) was not observed in glycerol-grown cultures (unpublished results), it is conceivable that the estimates of C obtained in glucose-grown cultures are distorted by other factors whose nature was not revealed in this study.

The possibility remains, therefore, that the glycerol estimations of C are the real ones also for glucose-grown cells, and the similar slopes (Fig. 12) may emphasize this claim.

It would be desirable to try and interpret quantitatively results recorded in the literature with these and other thy⁻ strains of bacteria, and compare them with the results shown in this study. Unfortunately, this cannot be done with most (if not all) of them because (a) the authors did not state the concentration of thymine they used in each experiment, (b) the concentrations of thymine were very often changed in the course of one experiment and (c) it is not obvious that the authors ensured that their cultures were in steady states of exponential growth. However, if we ignore the two last objections, it is possible to find a few observations which can be translated into quantitative terms.

Thus it is possible to calculate C from the data of Lark and Lark (1965) for a culture of 555-7 growing on M9 medium with succinate as a carbon source (τ = 70 minutes) and 2.0 μg/ml thymine. They pulse labelled the culture with ^{3}H-thymine for 5 minutes and found that 6% of the cells autoradiographed immediately thereafter were not labelled. Integrating Powell's (1956) age distribution function (1) for cells in a steady state of exponential growth over the range of cell ages from $(\tau-D+5)/\tau$ to $2-(C+D)/\tau$ gives the fraction of unlabelled cells as a function of C and D (see equation (2)). If D is assumed to be 25 minutes, six percent of unlabelled cells corresponds to C = 60 minutes, compared with my value of 65 minutes obtained from ΔG in glucose grown cultures with the same thymine concentration or 58 minutes, when glycerol grown cells were used (Fig. 12a). This value (60 minutes) is an underestimate; the pulse of ^{3}H-thymine must have been effectively shorter than 5 minutes, since 2-4

minutes are required for exhaustion of the internal pools of unlabelled dTTP (Figs. 8 and 9). It should be noted here, that using any other assumed value for D between 20 and 30 minutes does not change this estimate significantly.

The same concentration of thymine (2 μg/ml) was used by Donachie (1969) in a study of DNA replication and cell division in several thy⁻ mutants of E.coli. He found that the ΔG values in a col 15⁻ derivative of E.coli 15T⁻ (JG151; Ishibashi and Hirota, 1965) pregrown on M9 glycerol medium (τ = 60 minutes) fit well with the assumption that C = 60 minutes. Also, he measured RSF to be approximately 3, which is expected when C = τ (see Introduction), thus confirming the findings recorded in this study in a closely related strain.

Qualitatively, there is evidence, that in these and other thy⁻ strains of bacteria, the replication time of the chromosome is longer than in their thy⁺ counterparts and that this time is affected by the thymine concentration in the growth medium in the same manner as was shown in this study. Thus Friesen and Maaløe (1965) performed an experiment similar to that shown in Fig. 10c using a number of unrelated strains of E.coli and obtained similar results in each case. Leakiness of the thy⁻ mutations as an interpretation of their data was ruled out for each strain but no explanation of the phenomenon was offered.

H. Manor (personal communication) measured directly the in vivo step-time for the addition of thymine to a DNA chain growth by a chemical method similar to that used by Manor et al (1969) to measure RNA chain growth rate. His results, measured in E.coli B thy⁻ supplied with 0.2 μg/ml thymine, suggest that the step-time in these conditions is ca. 3-fold longer than it is in E.coli B/r thy⁻ (1400 nucleotides

per second, calculated by Cairns (1963a) from autoradiographic data), supplied with thymidine.

S. Borenstein and E. Elizur (personal communication) obtained higher ratios of early-replicating to late-replicating markers (HOT) in B.subtilis 168 thy$^-$ than in a thy$^+$ variant of this strain. These ratios were higher the lower was the thymine concentration supplied to the cultures. This finding, therefore, extends the conclusion drawn from this study also to other bacterial species than E.coli.

Another bacterial species to which my findings can be extended is S.typhimurium; from their data of segregation of radioactive DNA during growth of a thy$^-$ strain in 2 µg/ml thymine, Chan and Lark (1969) deduced that the average number of forks per cell (F) was 1.2 when τ = 65 minutes and 2.2 when τ = 42 minutes. If the eleventh method to estimate C is valid and equation (11) holds true (see Appendix 3), then these correspond to C values of 61 and 55 minutes, respectively (assuming D = 25 minutes). It is not possible to compare these values with my own since the thymine requirement of the strain used is not known, but the fact that they are higher than in E.coli B/r (although the chromosome length of S.typhimurium is similar to that of E.coli) might suggest a longer C due to the thy$^-$ mutation.

It is more difficult still to extrapolate to species which are not closely related to E.coli. In their study on Lactobacillus acidophilus (R-26) thy$^-$ supplied with 4 µg/ml thymine, Chai and Lark (1967) estimated that the number of radioactive units present after a culture grown with τ = 67 minutes was pulse labelled with radioactive thymine was four. This corresponds (equation (11), using D = 25 minutes) to C = 90 minutes. Much higher values than 40% for the increment of DNA

during amino acid starvation of cultures of this strain, growing in 40 min generation time, also indicating high values for C, were reported by Koukalová *et al* (1969).

It is impossible to evaluate most of the data measured in *E.coli* 15T⁻ (555-7) reported by K.G. Lark and his colleagues, since it was clearly stated in some of their publications that the concentration of thymine was always reduced when incorporation measurements were involved (e.g. Lark *et al*, 1963; Pritchard and Lark, 1964; Chan and Lark, 1969). This treatment changes C as well as disturbing the age distribution of forks along the chromosomes in the culture. However, it seems that in some of their recent work a constant concentration was kept all along the experiment. For instance, Bird and Lark (1968) used the amino acid starvation procedure to label the chromosome origins with ^{3}H-thymine and the termini with ^{14}C-thymine. Then they labelled the DNA with BU at various times and followed the pattern of hybrid DNA formation. They demonstrated that the ^{14}C-DNA (chromosome termini) was replicating ca. 40 minutes after the ^{3}H-DNA (chromosome origins) in glucose grown *E.coli* 15T⁻ (555-7). They concluded that in this strain growing in these conditions (τ = 40 minutes, 2.0 µg/ml thymine) C = 40 minutes. This finding clearly contradicts mine, concluding that for the same set of conditions C is not less than 54 minutes. I can not see at the moment any reasonable interpretation of their data other than theirs. This will have to be explained in the future, perhaps when more is known about the effects of amino acid starvation or of BU on DNA replication. It was clearly demonstrated, for instance, that C = 120-140 minutes when *E.coli* B/r *thy*⁻ is grown in the presence of BU (4 or 8 µg/ml) instead of thymine or thymidine (Pierucci, 1969).

The discovery that the replication time is affected by the concentration of thymine in the growth medium, and that a substantial change in velocity occurs without any detectable effect on growth rate, introduces a potentially serious source of error in the interpretation of data involving measurements of rates of DNA synthesis and determination of the 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 and others "super low" thymine requiring strains (Ahmad and Pritchard, 1971) concentrations greater than this have been used. Such strains are exceptional, however, 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 strains of E.coli require about ten times this concentration for normal growth (see sections (1) and (7)).

As anticipated from the minimal concentration of thymine required for normal growth of CR34, significant changes in replication velocity are found in cultures maintained on concentrations in the range 2.5 - 10.0 µg/ml typically used in studies with this and other thy⁻ (Tlr) strains of E.coli K12. Figure 21 also compares the slopes obtained when C is plotted against the reciprocal of thymine concentration in both strains used in this study. The lines constructed as already

discussed (a) for CR34, replot of part of the line in Fig. 11, and (b) for 15T⁻ from part of the measurements of RSF (○), $\bar{G}/\bar{M}$ (●) and pulse incorporation (△). The very striking difference in slopes is probably due to differences in the way in which the intracellular concentrations of dTTP respond to changes in the external thymine concentration, as will be discussed later (Beacham et al, 1971).

It will be noticed that the degree of scatter in the estimates of C from measurements of $\bar{G}/\bar{M}$, RSF and pulse incorporation (Fig. 21) as well as from ΔG measurements (Fig. 10a) in P178 is much greater than in P162 (Fig. 11), estimated from ΔG and $\bar{G}/\bar{M}$ measurements. This may be due to the difficulty of maintaining a constant concentration of thymine in the growth medium of P178 since the concentration range over which there are substantial changes of C is such that a significant proportion of the added thymine will be metabolised during the experiments.

On the grounds of a possible variation of internal dTTP concentration with the external thymine concentration (Beacham et al, 1968) it was suggested (Pritchard and Zaritsky, 1970) that the changes in replication velocity associated with changes in the concentration of thymine in the growth medium were determined by changes in the internal dTTP concentration only or by accompanying variations in the intracellular pools of other deoxynucleotides. Since the dTTP pools in the two strains studied here grown in the same conditions were recently measured directly by Beacham et al (1971) it is possible to try and correlate this pool to the corresponding replication times. Although in both strains increase in the external thymine concentration raised the internal dTTP concentration - the rates of change are completely different; a smaller increase of the thymine concentration in P178 causes the same raise of C that is observed in P162 when the thymine

concentration is increased by a bigger amount (Beacham et al, 1971). In Fig. 22, C is plotted against the intracellular dTTP concentration in P178 and P162 (and P162-8). The curves were constructed using the real values of dTTP concentrations with the lines described in Fig. 21 for the corresponding C values (except for the values in the presence of thymine and deoxyguanosine in P162-8, taken from Tables 12 and 13). It is clear that the replication time changes much more steeply with changes in the dTTP concentration in P162 than it does in P178. Although it is possible that the DNA polymerases in the two strains differ in their response to changes in the concentration of dTTP, it seems more likely that the difference reflects differences in the response of the other deoxynucleotides to changes in the dTTP pool and that, as might be expected, the replication velocity is determined by the relative concentrations of all four deoxynucleotide substrates of the polymerase. It has previously been also shown (Munch-Petersen and Neuhard, 1964; Neuhard 1966) that a reduction in the dTTP pool is associated with increase in the concentrations of dATP and dCTP but the relative increase in the concentrations of these two deoxynucleotides differs markedly under identical conditions in different strains. Such differences may also exist between P178 and P162.

A number of puzzling observations in the literature are resolved when these long and variable replication times, measured especially in CR34, are taken into account.

As originally pointed out by Maaløe and Hanawalt (1961) the increment in DNA predicted to occur in a culture deprived of a required amino acid is 39% if no further initiation occurs and all replication forks progress to the chromosome terminus. This increment is not usually

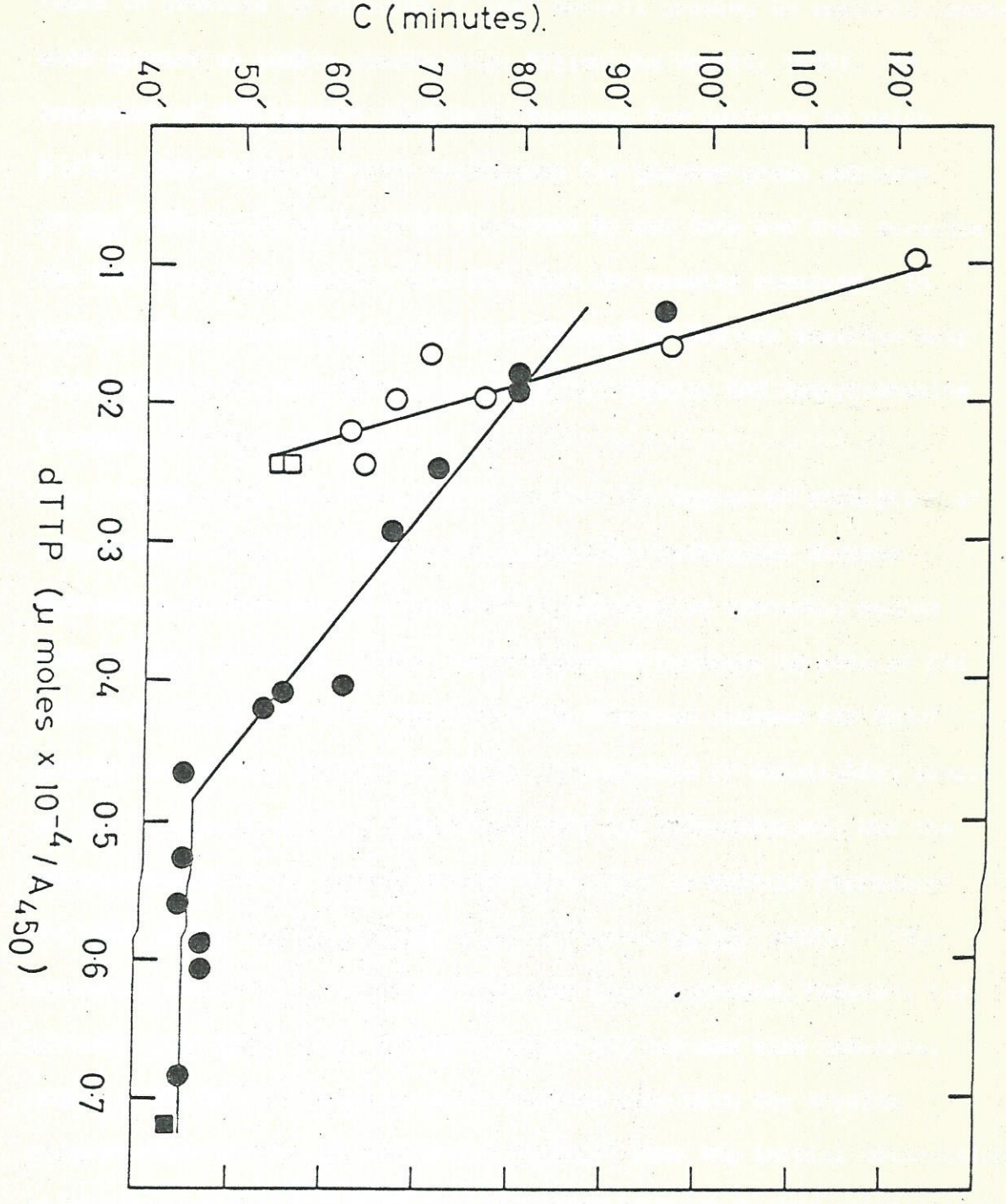

FIG. 22. C against dTTP concentration in P162 and P162-8 (open symbols) and in P178 (closed symbols). □ and ■, when cultures were supplied with 200 μg/ml deoxyguanosine. Values of dTTP concentrations were taken from Beacham et al (1971). C values were derived from the lines of Fig. 21.

found in practice in cultures of thy⁻ mutants growing in synthetic media with glucose as carbon source (e.g. Billen and Hewitt, 1966). An increment of 39% is only predicted, however, for cultures in which $C = \tau$. This frequently made assumption for glucose grown cultures of thy⁻ mutants is shown to be incorrect by our data and this accounts for the increments greater than 39% that are usually observed. In both strains used in this study an increment of 39% was observed only when the culture was grown on a mixture of thymine and deoxyguanosine (section (9)).

Another puzzling phenomenon has been the degree of stimulation of DNA synthesis induced by a period of one mass doubling of thymine starvation in cultures of strain 555-7, growing on synthetic medium with glucose as carbon source. The predicted increase in rate of DNA synthesis in a culture in which $C = \tau$ (as has been assumed for 555-7 growing on glucose synthetic medium) is threefold if reinitiation occurs at all chromosome origins (RSF = 3.0; and see Introduction), but the observed increase in rate is invariably closer to twofold (Pritchard and Lark, 1964; Donachie et al, 1968; Pritchard et al, 1969). This increase in rate is in good agreement with that predicted, however, for cultures in which C = 60 minutes (section (6) and see also Donachie, 1969). This RSF was shown in this study to vary with the thymine concentration present in the growth medium. Thus the initial observation of Pritchard and Lark (1964) which led them to suggest that reinitiation of replication after a period of thymine starvation might take place on only one of the two arms of a replicating chromosome is seen now to be consistent with reinitiation on both arms.

The higher values of RSF recorded by Pritchard et al (1969) after a period of one mass doubling of inhibition of DNA synthesis by nalidixic acid (NAL) might be due to varying the concentration of thymine used in

different experiments and after the treatment (which changes the replication velocity). In order to exclude a possible effect of NAL on the regulation of DNA synthesis other than that induced by thymine starvation, a series of RSF experiments should be repeated (as in section (6)) using inhibition by NAL instead of thymine starvation.

Estimations of C from $\bar{G}/\bar{M}$ depends on the validity of the hypothesis (Donachie, 1968; Pritchard, 1968; Pritchard et al, 1969) that initiation of cycles of replication occurs at a defined mass/chromosome origin ratio. The quantitative agreement between estimates of C obtained by this method and those obtained by other methods provides further evidence supporting this hypothesis. Since the replication time of the chromosome was shown to vary over a wide range without affecting significantly the doubling time of the culture it seems that the only event in the cycle of DNA replication which is coupled to the growth rate is initiation.

There is apparently no mechanism in the bacterial cell which adjusts the DNA/mass ratio to a constant value for any given growth rate (Maaløe, 1961; Copeland, 1969) since this can be varied at will without any correlated change in growth rate. It is also apparent that the attainment of a specific DNA/mass ratio is not a pre-requisite for cell division (Donachie et al, 1968) since cells can still divide exponentially in cultures with significantly different DNA/mass ratios.

The fact that the replication time can be varied over such a wide range without varying the growth rate introduces a serious problem facing the bacterial cell. A culture of P178 growing on media containing both deoxyguanosine and thymine has a chromosome replication time of about 44 minutes and a doubling time of about 45 minutes. There will

consequently be on average about one replication fork per chromosome and two doses of genes near the chromosome origin for every one near its terminus. When a culture of the same strain is grown in the presence of 0.25 μg/ml thymine without deoxyguanosine the generation time is around 40 minutes but the replication time is increased to around 120 minutes. Under these circumstances the average number of forks per chromosome will be about 7 and the relative dosage for genes near the origin will rise to 8. The fact that such a large change in relative gene dosage can be accommodated without reduction in the growth rate suggests that the cell is well buffered against changes in gene dosage, presumably as a result of its capacity to regulate the output of gene products. Below a sharply defined thymine concentration there is a reduction in growth rate. It may be that at this point the buffering capacity against changes in relative gene dosage is exhausted. Below this critical concentration the relative number of colony forming units to particles also falls, indicating an increased proportion of non-viable cells. In both strains these phenomena occur when the estimated value of C reaches about 130 minutes. The cause of loss of viablity is not clear: possibly there is a maximum number of replication forks per chromosome, or a minimum DNA/mass ratio (*this decreases as C increased*), or a *maximum DNA/cell ratio (this increases as C increases) compatible with normal growth and cell* division. Alternatively, there may be an impairment in replication or damage to DNA at the replication fork. This was observed in conditions of thymine starvation of thy$^-$ strains of several species of bacteria (e.g. Ramareddy and Reiter, 1970).

In contrast to the clear quantitative conclusions which can be drawn from the main part of this study about the replication velocity of the bacterial chromosome, no meaningful conclusion can be drawn concerning the molecular mechanisms controlling the process of cell division. Although correlation was found between C and "D" (Fig. 18), it is far from clear whether "D" is the real D defined by Cooper and Helmstetter (1968). It is tempting to think so since this correlation ("D" = 0.83C - 16) seems to be too near to the relation D = 0.5C, proved by Helmstetter *et al* (1968) in slow growing bacteria, to be a matter of chance.

However, whether a true correlation between C and D exists or not, there are indications in the literature that D is longer in some *thy*$^-$ strains of *E.coli*. Thus, Vielmetter *et al* (1968) estimating the segregation of randomly distributed mutations, conclude that in 15T$^-$ derivatives segregation is consistently shifted toward later times. This indicates a longer D in these strains than in the K12 strains (*thy*$^+$) they used.

Kubitschek (1969) observed that the fraction of doublets (constricted cells that seem to be in the final stages of their division) is higher in a culture of *E.coli* 15T$^-$ (THU$^-$) than in other *thy*$^+$ strains of *E.coli* growing at a similar rate. Qualitatively I confirmed that a high proportion of the cells in the cultures described in section (1) (Results) were doublets. This proportion increased with time and after many generations I could see cells which contained more than one constriction, some of them being asymmetrically distributed. One of the possible factors contributing to this high fraction of constricted cells is an increased D.

The fact that cell division in cultures of E.coli B/r treated with NAL (to inhibit DNA synthesis) continues for about 20 minutes (Helmstetter and Pierucci, 1968; Clark, 1968a,b) seems to me to be a weak proof for the argument that completion of a cycle of chromosome replication is a sufficient condition for cell division. It is certainly not so in thy⁻ strains (as shown here and in other publications, e.g. Donachie, 1969) as well as in lon⁻ strains of E.coli (E.g. Green et al, 1969). If cell division in slow growing cultures of E.coli B/r treated with NAL is shown to continue for "D" minutes (where D > 20 minutes) it will be necssary to assume a different control of cell division in thy⁻ strains than in thy⁺ ones. However, since even in their data (Helmstetter and Pierucci, 1968) cell division continued for 15-20 minutes, where D = 24 minutes, I tend to believe that this similarity is fortuitous, and that completion alone is not a sufficient condition for cell division.

APPENDIX 1

Average Number of Forks per Chromosome (F) in Steady-State Exponentially Growing Cultures of Bacteria

Assuming Cooper and Helmstetter's model (1968) and a volume (mass) controlled initiation model (Donachie, 1968; Pritchard et al, 1969), the following relationship between τ, C, and D are possible (and see Fig. 23):

(a) $C = \tau - D$ (Helmstetter et al, 1968)

(b) $\tau - D < C < \tau$

(c) $\tau < C < 2\tau$

(d) $2\tau < C < 3\tau$

. . . . (n) $n\tau < C < (n+1)\tau$

Fig. 23 describes the patterns of the chromosomes in a series of cultures during the cell's cycle for each of these cases. Assuming also that the age distribution of chromosomeal forks is the same as the age distribution of cells in a s.s.e.g. culture (Sueoka and Yoshikawa, 1965) and is described by the Function (Powell, 1956) (1)

$$f(x) = (\ln 2).2^{1-x}$$

where $0 \leqslant x \leqslant 1$ is the age of a cell (fork), in fractions of generation, then it is possible to calculate F for each of the above conditions:

(a)

$$F = \frac{\int_0^{C/\tau} f(x)dx}{\int_0^{C/\tau} f(x)dx + 2\int_{C/\tau}^{1} f(x)dx} = \frac{2 - 2^{1-C/\tau}}{2 - 2^{1-C/\tau} + 2.2^{1-C/\tau} - 2} =$$

$$2^{C/\tau} - 1$$

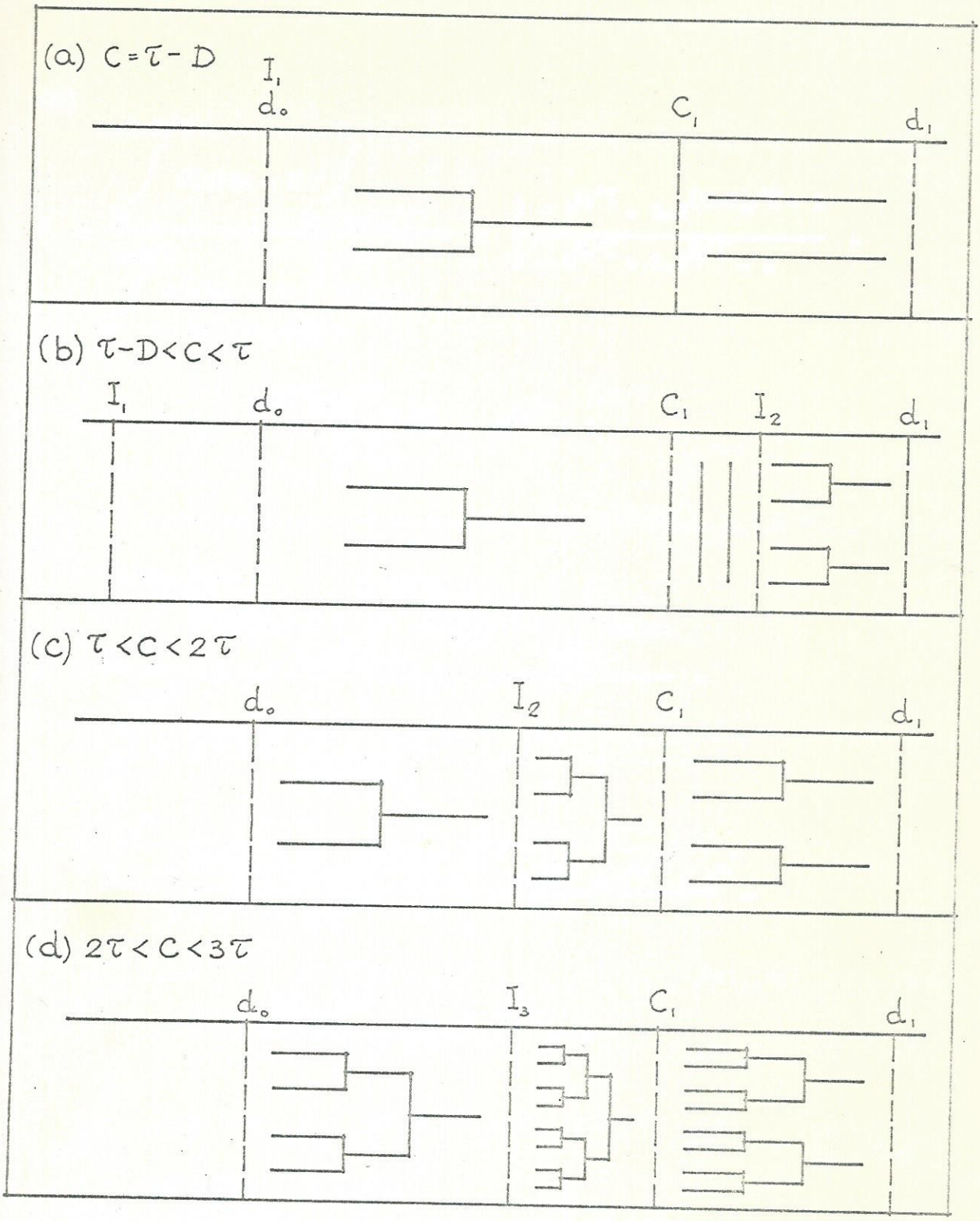

FIG. 23 Chromosome patterns during the cell cycle in cultures with varying C/τ ratios and a constant D/τ ratio. The cases where $n\tau - D < C < n\tau$ ($n = 1, 2, ..$) are not included, but they give basically similar patterns, and the same solutions for the relevant parameters (Appendices 1, 2 and 3).

(b)

$$F = \frac{\int_0^{1-D/\tau} f(x)dx + 2\cdot\int_{2-(C+D)/\tau}^{1}}{\int_0^{1-D/\tau} f(x)dx + 2\cdot\int_{1-D/\tau}^{1} f(x)dx} = \frac{2 - 2^{D/\tau} + 2.2^{(C+D)/\tau} - 1}{2 - 2^{D/\tau} + 2.2^{D/\tau} - 2} =$$

$$\frac{2^{D/\tau}\,(2^{C/\tau} - 1)}{2^{D/\tau}} = 2^{C/\tau} - 1$$

(c)

$$F = \frac{\int_0^{2-(C+D)/\tau} f(x)dx + 3\cdot\int_{2-(C+D)/\tau}^{1-D/\tau} f(x)dx + 2\cdot\int_{1-D/\tau}^{1} f(x)dx}{\int_0^{1-D/\tau} f(x)dx + 2\int_{1-D/\tau}^{1} f(x)dx} =$$

$$\frac{2 - 2^{(C+D)/\tau-1} + 3.2^{(C+D)/\tau-1} - 3.2^{D/\tau} + 2^{D/\tau} - 2}{2 - 2^{D/\tau} + 2.2^{D/\tau} - 2} =$$

$$\frac{2^{D/\tau}(2^{C/\tau} - 1)}{2^{D/\tau}} = 2^{C/\tau} - 1$$

(d)

$$F = \frac{3.\int_0^{3-(C+D)/\tau} f(x)dx + 7.\int_{3-(C+D)/\tau}^{1-D/\tau} f(x)dx + 6\cdot\int_{1-D/\tau}^{1} f(x)dx}{\int_0^{1-D/\tau} f(x)dx + 2\cdot\int_{1-D/\tau}^{1} f(x)dx} =$$

$$\frac{3.2 - 3.2^{(C+D)/\tau-2} + 7.2^{(C+D)/\tau-2} - 7.2^{D/\tau} + 6.2^{D/\tau} - 6}{2 - 2^{D/\tau} + 2.2^{D/\tau} - 2} =$$

$$\frac{2^{D/\tau} \cdot (2^{C/\tau} - 1)}{2^{D/\tau}} = 2^{C/\tau} - 1$$

and for the general form:

$$(n)\quad F = \frac{(2^n-1) \cdot \int_0^{(n+1)-(C+D)/\tau} f(x)dx + (2^{n+1}-1) \cdot \int_{(n+1)-(C+D)/\tau}^{1-D/\tau} f(x)dx + (2^{n+1}-2) \cdot \int_{1-D/\tau}^{1} f(x)dx}{\int_0^{1-D/\tau} f(x)dx + 2 \cdot \int_{1-D/\tau}^{1} f(x)dx} =$$

$$\frac{(2^n-1).\ 2 - (2^n-1).\ 2^{(C+D)/\tau-n} + (2^{n+1}-1)\ 2^{(C+D)/\tau-n}}{2 - 2^{D/\tau} + 2.2^{D/\tau} - 2} +$$

$$+ \frac{-(2^{n+1} - 1) - 2^{D/\tau} + (2^{n+1} - 2)\ 2^{D/\tau} - (2^{n+1} - 2)}{2 - 2^{D/\tau} + 2.2^{D/\tau} - 2} =$$

$$\frac{1}{2^{D/\tau}} [2^{n+1} - 2 + 2^{(C+D)/\tau-n}(2^{n+1} - \underline{1} - 2^n + \underline{1}) + 2^{D/\tau}(\underline{2}^{n+1} - 2 - \underline{2}^{n+1}) +$$

$$+ 2 - 2^{n+1}] =$$

$$= \frac{1}{2^{D/\tau}} [2^{(C+D)/\tau-n} \cdot 2^n - 2^{D/\tau}] = 2^{-D/\tau}[2^{(C+D)/\tau} - 2^{D/\tau}] = \underline{\underline{2^{C/\tau} - 1}}$$

Therefore this equation

$$F = 2^{C/\tau} - 1 \qquad (5)$$

is valid for any τ and C, and F is independent of D.