POLYPHOSPHATE ACCUMULATION AND UTILIZATION AS RELATED TO SYNCHRONIZED CELL DIVISION OF *CORYNEBACTERIUM DIPHTHERIAE*[1]

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In previous studies, Sall *et al.* (1956) employing resting cells of *Corynebacterium diphtheriae*, and Mudd *et al.* (1956, 1958) employing resting cells of Mycobacteria, demonstrated that malate as substrate enhances the accumulation of polyphosphate (metaphosphate) in the form of metachromatic granules and glucose promotes the withdrawal of polyphosphate. Similarly, Mudd *et al.* (1958), employing P^{32}, showed that, in growing cultures of *Mycobacterium thamnopheos*, malate as substrate or azaserine as inhibitor favors the deposition of polyphosphate, whereas glucose as substrate or dinitrophenol as inhibitor promotes the utilization of polyphosphate to form ribonucleic acid.

Various applications of synchronized cell division to metabolic and cytological studies have recently been reviewed by Campbell (1957). In the present study, utilizing synchronously dividing cells of *C. diphtheriae*, a physiological deposition and utilization of polyphosphate has been demonstrated in relation to the cycle of cell division.

MATERIALS AND METHODS

Corynebacterium diphtheriae mitis A 679 was used throughout. Stock cultures were kept under oil at 4 C on Morton-Engley (1945) yeast extract tryptone agar enriched with blood serum. Subcultures were prepared from the stock cultures by inoculating tubes of Morton-Engley blood serum broth and incubating for 18 hr at 37 C in a roller-tube aerator.

Synchronized cell division was obtained in the following manner: 18 hr cultures were harvested, washed 3 times with refrigerated saline solution and suspended in refrigerated saline. The cell suspension was placed in an ice bath for 45 min. The suspension was then poured into 250 ml of Morton-Engley blood serum broth at 37 C and aerated. At 10 min intervals, 30 ml aliquots were removed by means of a 50-ml syringe equipped with a 3-way stopcock. One ml was removed for total cell count and estimation of index of metachromasy. The remaining 29 ml of cell suspension were analyzed for the various phosphorus fractions.

Total cell counts were obtained in the usual manner employing a Petroff-Hauser counting chamber. One-quarter ml of cell suspension was diluted with aqueous solution of 1.5 per cent crystal violet, and mixed vigorously in a small test tube. A portion of the diluted cell suspension was removed with a capillary pipette and was used to charge the counting chamber. Ten large squares were counted, 5 diagonally and 5 scattered.

Index of metachromasy was determined by the procedure described by Sall *et al.* (1956). The index of metachromasy is the sum of the products obtained by multiplying the estimated diameters ($4+ = 1\ \mu$, $3+ = \frac{3}{4}\ \mu$, $2+ = \frac{1}{2}\ \mu$, $1+ = \frac{1}{4}\ \mu$) of the granules by the percentage of cells showing such granules.

Phosphorus partitioning was performed in a manner previously described (Sall *et al.*, 1956). The acid soluble fraction was determined *in toto*. The following acid insoluble fractions were pooled and determined: phospholipid, organic, inorganic, and 7 min phosphorus.The ultramicro method of Berenblum and Chain, as modified by Martin and Doty (1956) for the determination of phosphorus was employed. Ogur and Rosen's (1950) method for nucleic acid estimations was used as an assay confirmatory to the estimation of nucleic acids by fractionation of phosphorus.

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RESULTS

Approximately 80 per cent of the cells divide synchronously after an incubation period of about 20 min. A similar division again took place after about 40 min (tables 1 and 3, and figures 1*a* and 8*a*). The various phosphorus fractions are expressed as phosphorus or nucleic acid per bacterial cell in tables 1, 2, and 3 and as phosphorus per volume of culture in figures 1*a* and 8*a*.

Deoxyribonucleic acid phosphorus values per cell (tables 1 and 3) were approximately double

TABLE 1

Relationship of phosphorus fractions to cyclic cell division; cells initially metachromatic

Time	Total Cell Count	I.M.*	Total P	Poly P	DNA P	RNA P	A.S. P	A.Ins. P
min	× 10^{-9}							
0	1.54	119	6.0	2.3	.13	0.84	1.3	0.78
10	1.61	63	6.7	1.6	.12	1.7	1.6	1.2
20	1.65	113	8.7	2.2	.24	1.8	1.7	1.8
30	3.18	9	5.2	0.31	.16	1.3	1.0	1.4
40	3.24	62	6.9	1.7	.31	1.6	1.2	2.1
50	6.09	14	4.2	0.20	.20	1.4	0.66	1.6
60	6.80	80	5.4	1.2	.29	1.5	0.65	1.7

All phosphorus fractions are expressed as μg × 10^9 P per cell.

Total cell counts are expressed as cells × 10^{-9} per 29 ml of culture.

* I.M. = Index of metachromasy; Poly P is the acid insoluble polyphosphate P; DNA and RNA = deoxyribonucleic and ribonucleic acid, respectively; A.S. is a pool of the total cold trichloracetic acid-soluble phosphorus fractions; A.Ins. is a pool of the trichloracetic acid-insoluble fractions consisting of phospholipid, inorganic, organic, and 7 min phosphorus.

TABLE 2

Ultraviolet absorption determination of the nucleic acid fractions

Time	Total Cell Count	I.M.*	DNA	RNA
min	× 10^9		μg	μg
0	1.20	10	3.1	20
10	1.32	4	3.0	32
20	1.55	42	5.4	41
30	2.76	6	3.8	31
40	3.09	66	6.6	42
50	6.45	8	3.8	27
60	7.50	74	5.7	35

The nucleic acid fractions are expressed as μg × 10^9 per cell.

Total cell counts are expressed as cells × 10^{-9} per 29 ml of culture.

* I.M. = Index of metachromasy; DNA = deoxyribonucleic acid; RNA = ribonucleic acid.

TABLE 3

Relationship of index of metachromasy, nucleic acids and polyphosphate to cyclic cell division; cells initially nonmetachromatic

Time	Total Cell Count	I.M.*	Poly P	DNA P	RNA P
min	× 10^{-9}				
0	1.92	6	0.20	.05	0.78
10	2.05	3	0.44	.10	1.3
20	2.12	40	1.2	.24	1.6
30	3.71	0	0.27	.16	1.2
40	3.86	81	0.91	.26	1.8
50	6.82	0	0.26	.16	1.4
60	7.75	63	0.77	.27	1.4

The nucleic acid and polyphosphate fractions are expressed as μg × 10^9 P per cell.

Total cell counts are expressed as cells × 10^{-9} per 29 ml of culture.

* I.M. = Index of metachromasy; Poly P is the acid insoluble polyphosphate P; DNA and RNA = deoxyribonucleic and ribonucleic acid, respectively.

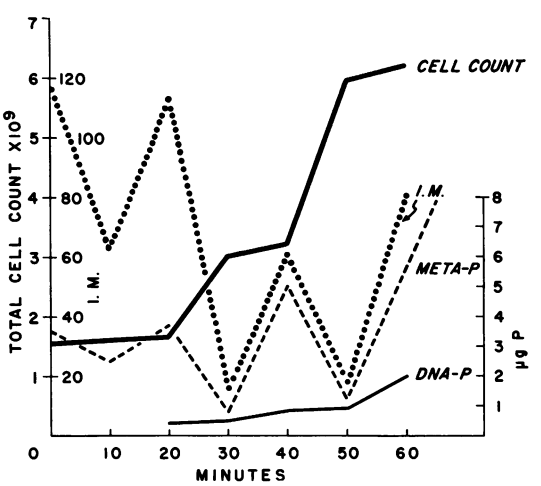
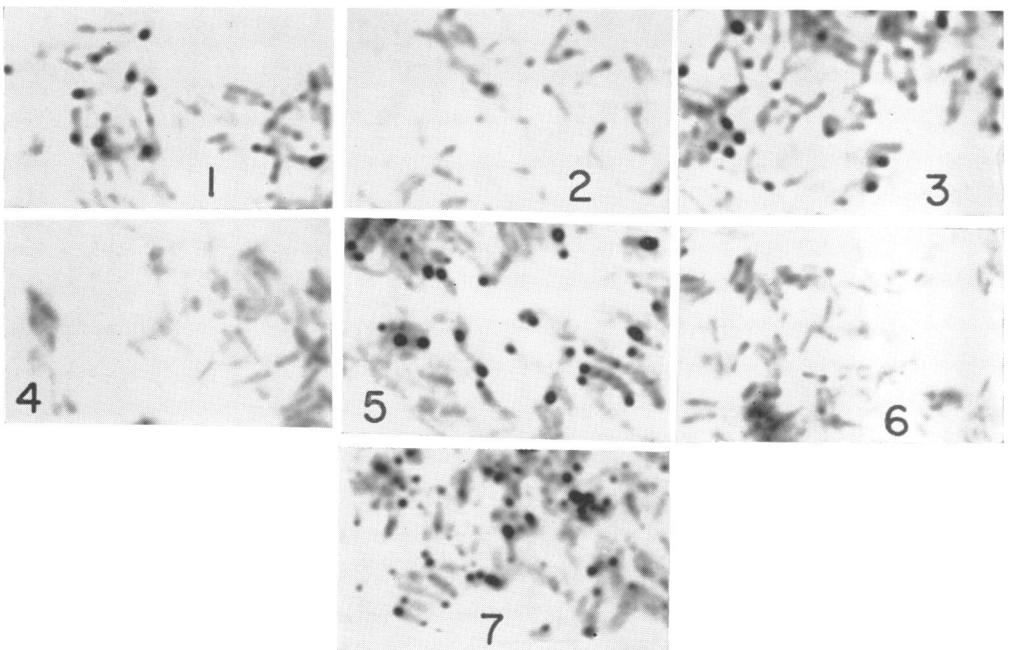


Figure 1a. Synchronized cell division of *Corynebacterium diphtheriae*. The culture had progressed through 2 cycles. Cells initially metachromatic. All P values plotted as μg P per 29 ml of culture. I.M. = Index of metachromasy; Meta-P = Polyphosphate (metaphosphate); DNA-P = Deoxyribonucleic acid phosphorus.



Figures 1–7. Light micrographs of Neisser stained cells which correspond to table 1 and the curve in figure 1*a*. Magnification 1250 ×.

Figure 1, 0 time cells. Note the many darkly stained metachromatic granules; index of metachromasy = 119.

Figure 2, 10 min cells. Note the fewer metachromatic granules; index of metachromasy = 63.

Figure 3, 20 min cells. Note the increase in the number and size of the granules; index of metachromasy = 113.

Figure 4, 30 min cells. Note the absence of the granules; index of metachromasy = 9.

Figure 5, 40 min cells. Note the increase in the number of granules; index of metachromasy = 62.

Figure 6, 50 min cells. Note the marked decrease in the number and size of the granules; index of metachromasy = 14.

Figure 7, 60 min cells. Note the increase in the number and size of the granules; index of metachromasy = 80.

their preceding values at 20 and 40 min and increased by 45 to 70 per cent at 60 min. Ultraviolet spectrophotometric analysis of the nucleic acids (table 2) indicated similar step-wise increases in deoxyribonucleic acid. These data are in agreement with data from cells of higher forms, which approximately double their chromatin content prior to cell division. Total, ribonucleic acid, acid soluble, and acid insoluble phosphorus per cell showed a less clear relationship to the cycle of cell division.

The acid insoluble polyphosphate (metaphosphate) phosphorus values per cell increased concomitantly with deoxyribonucleic acid phosphorus, i.e., at 20, 40, and 60 min, and decreased markedly during the times of increasing cell numbers, i. e., at 30 and 50 min. Because of the minute quantities of acid soluble polyphosphate accurate estimations of this fraction were not feasible. Fields of cells stained for polyphosphate by the technique of Neisser (Sall *et al.*, 1956) were prepared at intervals corresponding to the preparations for microchemical analysis. The indices of metachromasy fluctuated in parallel with polyphosphate P (tables 1 and 3); these values were strikingly high at 20, 40, and 60 min, but low at 30 and 50 min. The rather dramatic accumulations of polyphosphate granules at 20, 40, and 60 min are shown in figures 1 to 14.

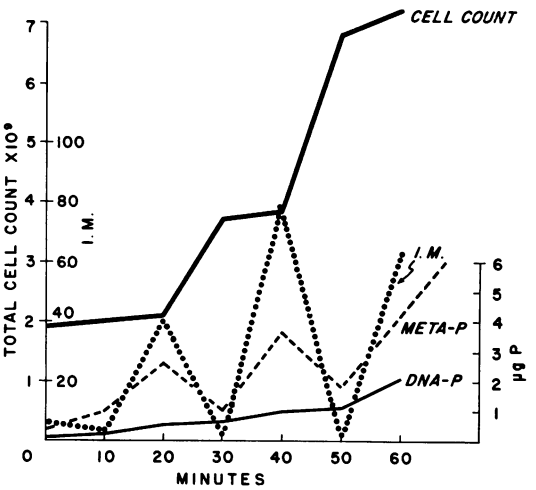
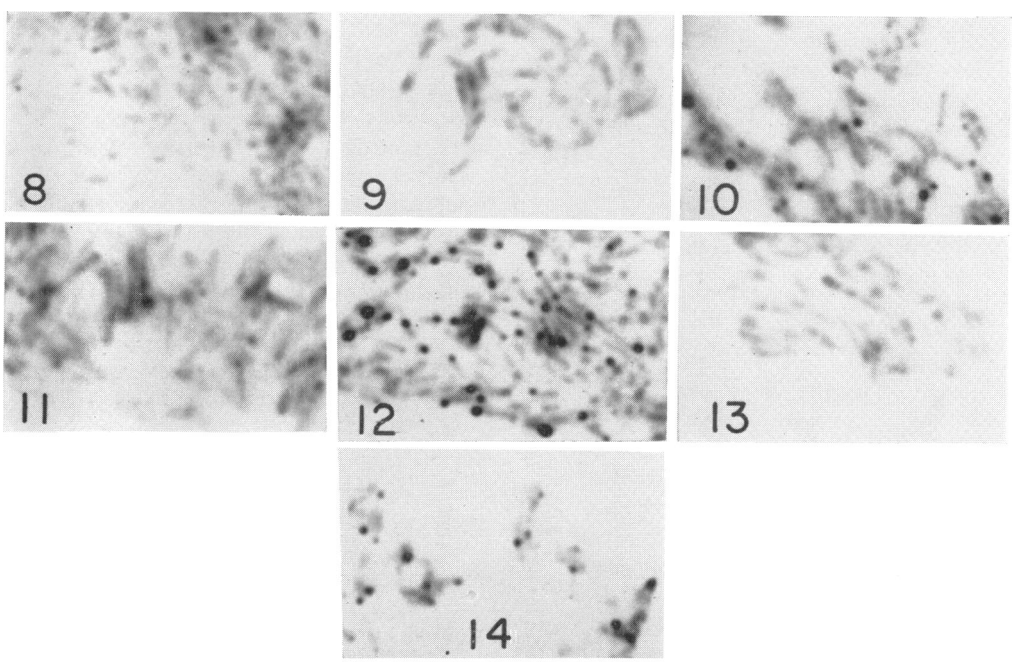


Figure 8a. Synchronized cell division of *Corynebacterium diphtheriae*. The culture had progressed through 2 cycles. Cells initially non-metachromatic. All P values plotted as μg P per 29 ml of culture. I.M. = Index of metachromasy; Meta-P = Polyphosphate (metaphosphate); DNA-P = Deoxyribonucleic acid phosphorus.



Figures 8–14. Light micrographs of Neisser stained cells which correspond to table 3 and the curve in figure 8*a*. Magnification 1250 ×.

Figure 8, 0 time cells. Index of metachromasy = 6.

Figure 9, 10 min cells. Only occasional metachromatic granules; index of metachromasy = 3.

Figure 10, 20 min cells. Note the marked increase in the number of metachromatic granules; index of metachromasy = 40.

Figure 11, 30 min cells. Comparatively few granules; index of metachromasy = 0.

Figure 12, 40 min cells. Note the large increase in size and number of metachromatic granules; index of metachromasy = 81.

Figure 13, 50 min cells. Note the marked decrease in the size and number of the granules; index of metachromasy = 0.

Figure 14, 60 min cells. Note the increase in the number of granules; index of metachromasy = 63.

DISCUSSION

The data presented are representative of the successful experiments, about one-half of the total number that were performed. The principal difficulties were involved in obtaining synchronized cell division. It is of interest to note that the normal generation time of this organism under the same experimental conditions with the exception of cold shock, is 22 min, the same generation time as was observed with synchronously dividing cells. However, a period of 45 min was necessary for the cold shock treatment in order to obtain synchronized cell division. This could be accounted for if portions of the population reached the "particular stage of unbalanced growth" (Campbell, 1957) at different times, resulting in the need for a 45-min shock period for at least 80 per cent of the organisms to attain the unbalanced growth stage.

It is characteristic of bacilli of *C. diphtheriae* to form sharp angles with each other following cell division. This characteristic tended to the formation of clumps, which made the total cell count arduous to obtain. This difficulty was surmounted, to an extent, by vigorously shaking the cell suspension with the crystal violet diluting solution in a small test tube containing several small glass beads.

Winkler (1953) states that during the early stages of the log phase Corynebacteria and Mycobacteria usually have minute metachromatic granules, which are detectable only with the electronmicroscope. Towards the end of the log phase the granules are large enough to be seen in stained preparations with the light microscope, and in the stationary phase the granules increase in size. This is in agreement with Kornberg (1957) and Winder and Denneny (1957) who postulate that during periods of rapid growth, or synthetic processes which require adenosine triphosphate, the utilization of polyphosphate exceeds its formation. This is substantiated by Mudd *et al.* (1958) who demonstrated with Mycobacteria that polyphosphate could be used for nucleic acid synthesis and cell growth.

Recently Kornberg *et al.* (1958) obtained from *E. coli* an enzyme which is capable of converting deoxyguanosine triphosphate to deoxyguanosine and tripolyphosphate. The implications of this reaction further support the possibilities that polyphosphate can be utilized for nucleic acid synthesis and cell growth.

The demonstration that polyphosphate may be stored and utilized in physiological relationship to the cycle of cell division is in agreement with the evidence presented in earlier papers (Sall *et al.*, 1956; Winder and Denneny, 1956; Kornberg *et al.*, 1956; Lieberman, 1956; Mudd *et al.*, 1958) that polyphosphate may serve in microorganisms as phosphagen. Scott and Chu (1958) have shown that during cell division of *E. coli* there is a sharp increase in glucose assimilation. It is possible that phosphate may be utilized in the transport mechanisms involved.

SUMMARY

Polyphosphate is found, by cytochemical and microchemical procedures, to increase and decrease in relation to the cyclic division of synchronized dividing cells. The peaks of polyphosphate accumulation are found immediately before the periods of increasing cell numbers; minimal amounts of polyphosphate are found at the ends of the recurrent periods of cell division. Increments of deoxyribonucleic acid are found to occur between the periods of cell division. Accumulation and disappearance of polyphosphate are, then, physiological events related to cell division; these findings support the interpretation of polyphosphate as phosphagen.

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