THE SYNTHESES OF DEOXYRIBONUCLEIC ACID AND HISTONE IN THE ONION ROOT MERISTEM

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ABSTRACT

A comparison of the times necessary to incorporate tritium-labeled lysine and arginine into histones and tritium-labeled thymidine into DNA indicates that the periods of DNA and histone synthesis prior to division closely coincide. (The comparison was made by determining the times necessary, after pulse labeling, for cells with marked chromosomes to enter and then leave the division stages.) An additional period of chromosomal protein synthesis, of short duration, occurs late in interphase. Most of the chromosomal proteins appear either to be synthesized in the nucleus or to migrate there shortly after synthesis. Much of this protein is conserved from one division to the next. Studies of the effects of puromycin and fluorodeoxyuridine on the syntheses of DNA and histone suggest that continuation of DNA synthesis is dependent on a concurrent protein synthesis. Histone synthesis, on the other hand, can proceed at a normal rate under conditions in which DNA synthesis is inhibited.

INTRODUCTION

Twelve years ago Bloch and Godman (1955) and Alfert (1955) demonstrated parallel increases in staining of DNA with the Feulgen reaction and of histone with alkaline fast green during interphase leading to division. The former work dealt with mammalian cells, the latter with plant. The conclusion was drawn that the parallel increases in staining reflect simultaneous syntheses of these two chromosomal components, at least at the level of the nucleus. Similar results were obtained by others using other systems (McLeish, 1959; Woodard et al., 1960). Gall (1959) found parallel increases in DNA and histone staining in the regeneration band of the *Euplotes* macronucleus, thereby localizing the level of synchrony to a subnuclear region in this complex macronucleus.

Whereas in 1955 the idea of coupled syntheses of DNA and histone prior to division seemed reasonable, the later works of Kornberg's school on DNA synthesis (Lehman et al., 1958; Bessman et al., 1958) and the works of Zamecnik, Hoagland, and others on protein synthesis (Hoagland, 1960) gave good reason to expect the two substances to be synthesized independently. Earlier findings of higher rates of incorporation of glycine into histones than into DNA (Daly et al., 1952) and findings of a low but nevertheless appreciable incorporation into histones of nonproliferating

tissues (Brunish and Luck, 1952) did indicate the existence of a histone synthesis independent of DNA synthesis and cell proliferation (see also Chalkley and Maurer, 1966). In the past few years a number of investigators have also made observations indicating histone synthesis independent of DNA synthesis during the cell cycle. Evans et al. (1962) reported an earlier incorporation into an acid-soluble nuclear protein than into DNA during initiation of rat liver regeneration. Umaña et al. (1962, 1964) found that the ratios of histone to DNA are higher for isolated liver nuclei than for chromosomes and that the values are higher for nondividing cells than for dividing cells. They concluded that histones are synthesized in greater amounts than are needed to combine with DNA, and that this synthesis can occur in the absence of DNA synthesis. They proposed that simultaneous increases in DNA and histone staining, such as noted above, reflect the inability of the fast green staining technique to detect histone not combined with DNA. Thus, they interpreted parallel staining to mean only that DNA synthesis is followed closely by a complexing of DNA with histone, the complexing process rendering the histone detectable (Umaña *et al.*, 1962). Lindner and Kutkam (1962), on the other hand, reported increases in *staining* of histones relative to DNA in dividing cells, compared with nondividing cells, and similarly interpreted this increase to indicate the presence of more histone than that combined with DNA. Bloch and Brack (1964) reported that the histones synthesized in the spermatid of grasshopper during conversion of the sperm nucleo-protein appeared to be synthesized in the cytoplasm and then appeared to move into the nucleus. This histone synthesis not only occurs outside the nucleus but, as in spermiogenesis in other organisms, takes place several weeks after DNA synthesis. These cytoplasmic proteins, incidentally, do not stain with the alkaline fast green method. They are too dilute or perhaps, as would be predicted by Umaña, they are lost during the staining procedure.

Because of possible misinterpretation of the results with staining methods, an attempt was made to reinvestigate with radioautographic methods the problem of timing DNA and histone synthesis. While these results with radioautography may be subject to criticism on other grounds, they are free, at least, from the criticisms which had been leveled at the staining procedures.

The rationale of the present experiment is as follows. If the end of histone synthesis occurs at a different time (say earlier) than the end of the period for DNA synthesis, it will take a different length of time (more time) for labeled amino acids than for labeled thymidine to appear in mitotic chromosomes (see Fig. 1). The hypothetical period of histone synthesis is designated the "S_h" period, and the DNA synthetic period the "S_d" period (after Howard and Pelc, 1953). The "$G\text{-}2_h$" and "$G\text{-}2_d$" (between S and division) periods can be obtained by determining the times necessary, after initial labeling, to find label in mitotic chromosomes. Similarly, the beginning of the S_h and S_d periods can be obtained by determining the lengths of time necessary for unlabeled divisions to appear again *after* the labeled divisions following pulse administration of isotope.

For example, according to Fig. 1 DNA synthesis ends 10 hr and histone synthesis 12 hr before mitosis. DNA synthesis begins about 18 hr and histone synthesis 24 hr before mitosis. Figure 1 shows the results expected if histone synthesis occurs over the entire cell cycle. For simplicity, the mitotic period is considered insignificant in these examples.

This treatment would be applicable as described only if the protein of the condensed chromosome were entirely histone and if the chromosomes could be freed of surrounding protoplasm. Unfortunately, these conditions are not met. Such methods as treatment with hypotonic solutions and acetic acid or acetic alcohol, which permit isolation of the spindle and metaphase chromosomes (Prescott and Bender, 1961) and which effect sufficient softening to permit the high degree of flattening convenient for radioautographic study of chromosomes, also result in the removal of some or most histones (Cave, 1966). The basic approach, as described above, was used with modifications in an attempt to detect the synthesis of histone-like chromosomal proteins in the midst of other proteins. The results show an approximate coincidence of the S_d and S_h periods.

Evidence was also obtained to show that the bulk of the histones either are synthesized in or immediately migrate to the nucleus, that the replication of DNA is dependent upon the concurrent synthesis of protein, and that histone synthesis can proceed in the absence of DNA synthesis.

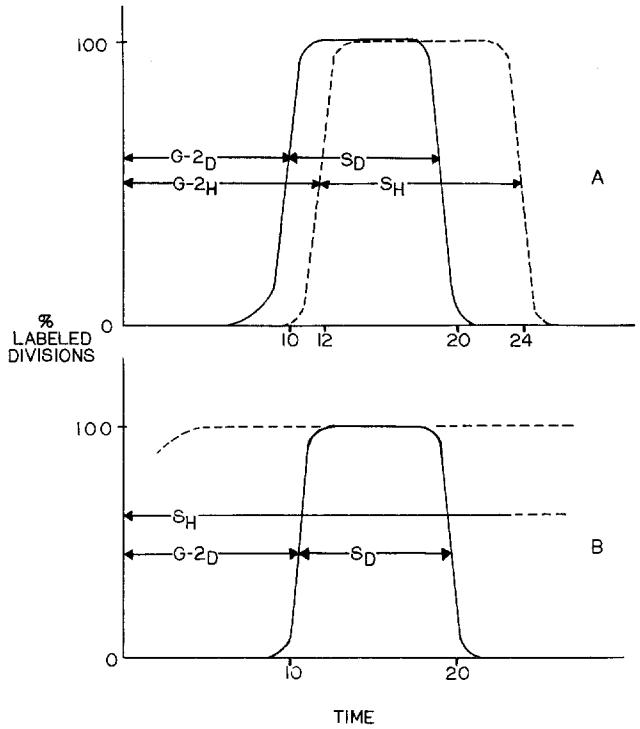

FIGURE 1 Hypothetical curves illustrating the method of determination of the times of DNA and histone synthesis during the cell cycle. Abscissa, time; ordinate, frequency of labeled division figures. Solid lines, thymidine labeling; broken lines, amino acid labeling. See text for further explanation.

MATERIALS AND METHODS

The standard procedure used in the radioautographic experiments follows. The bottoms of onion bulbs (*Allium cepa*) were scraped and immersed in water. The bulbs were a globe-shaped yellow variety obtained locally. Each bulb was supported on the rim of a 150 ml beaker. The onions were grown in the dark at 15–17°C, or at room temperature, as indicated. After several days most of the roots ranged from 1 to 3 cm in length. Pairs of selected onions were transferred to paired 10-ml beakers, one onion to a beaker. To one beaker of a pair was added 10 μc each of tritium-labeled arginine and lysine (specific activity of arginine, 1.05 c/mmole; specific activity of lysine, 0.2 c/mmole; both obtained from Nichem Inc., Bethesda, Md.). To the other beaker of a pair was added 10 μc of tritium-labeled thymidine (specific activity 1.9 c/mmole) (Schwarz Bio Research Inc., Orangeburg, N. Y.). The onions were maintained in the label for ½ hr, then were put into a solution containing unlabeled precursors in concentrations 100 times that of the labeled material for the second ½ hr, and finally were transferred back to distilled water. Individual roots were measured at the beginning of the experiment and before sampling, to insure that the roots remained healthy and growing. Samples, usually consisting of single roots, were taken at designated intervals. Single roots were used because the roots of individual bulbs did not grow in sufficient numbers to supply more than one root at each time during the experiment. Usually, replicate experiments rather than replicate samples were relied upon to provide a measure of reproducibility.

The roots were fixed in neutral, buffered formalin for at least 3 hr, then rinsed and stained in bulk with the Feulgen procedure. 25 min hydrolysis in 1 N trichloracetic acid at 60°C preceded staining with Schiff's reagent. Trichloroacetic acid was substituted for HCl to insure retension of the histones. The roots were then dehydrated and embedded in an Araldite-Epon mixture (Mollenhauer, 1964) and 1 μ sections were cut on a Porter-Blum Ultramicrotome with a glass knife. Plastic embedding was employed because its high density afforded a means for equalizing the different intrinsic densities and self-absorption of the various areas within the cell. It also permitted more precise localization of sites of radioactivity (Bloch and Brack, 1964). The sections were applied to albuminized slides and coated with Kodak AR-10 stripping film in the manner of Taylor and McMaster (1954). The slides were exposed for varying periods, from 2 wk to 2 yr as indicated for the amino acid experiments, and for 3–6 months for the thymidine experiments. Fig. 2 shows a typical section. The relatively few grains over the vacuoles and between the cells

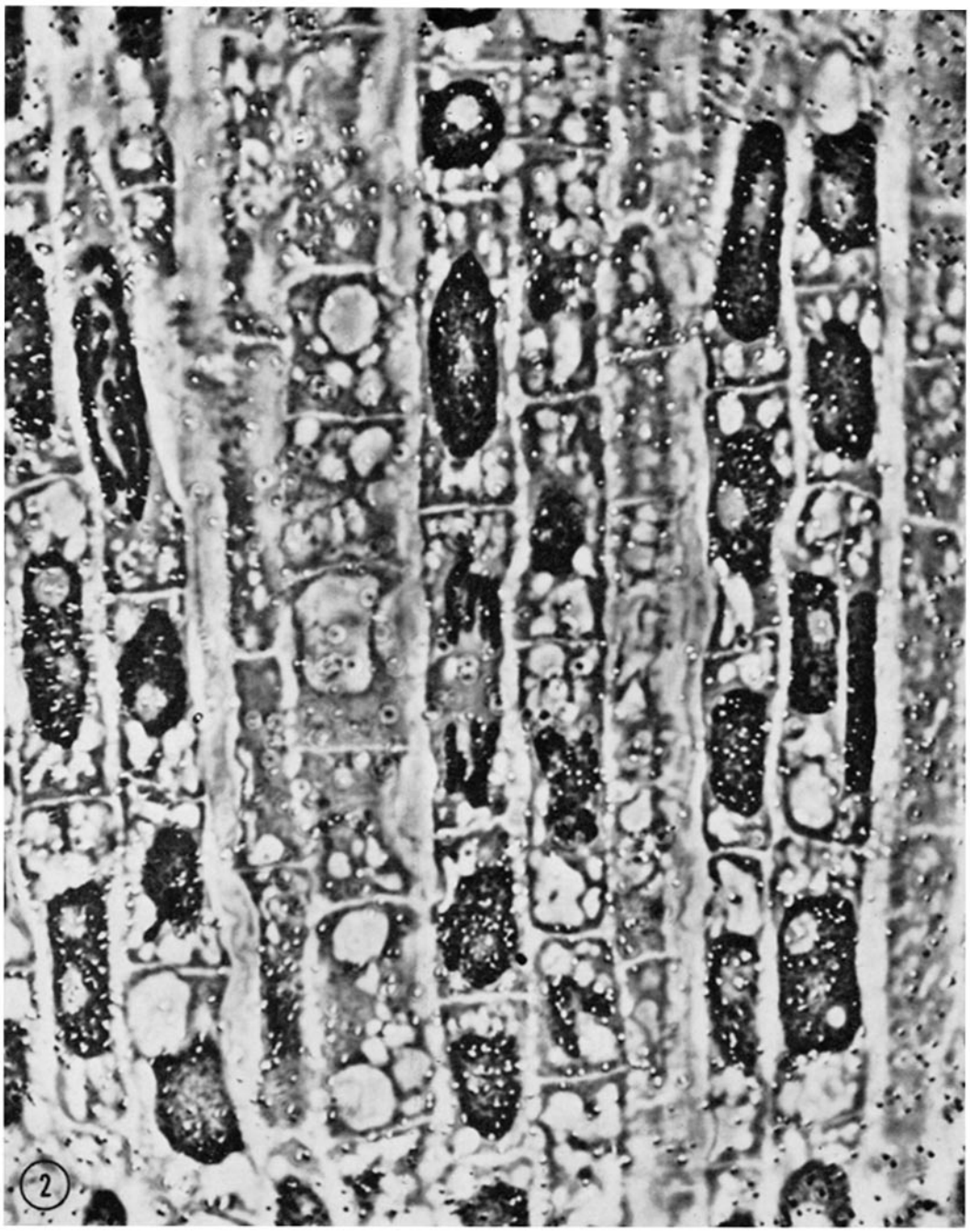

FIGURE 2 1-μ thick section of arginine-^{3}H and lysine-^{3}H labeled, Araldite-embedded, Feulgen-stained onion root tip with overlying emulsion. Dark field phase photomicrograph. Focus is on the upper level of of the section rather than on the silver grains. The latter are seen as white specks. The quality of localization is indicated by the dearth of grains in areas that do not overlie protoplasm. × 1850.

attest to the precision of localization of the silver grains.

In one experiment, roots were fixed in Carnoy's (3:1) ethanol:acetic acid for several hours, carried through alcohols to water, hydrolyzed for 12 min in trichloroacetic acid, stained, and then, after sulfite and water rinses, treated with a mixture of 1% cellulase and 5% pectinase (Nutritional Biochemicals Corporation, Cleveland, O.) in distilled water for 1 hr at room temperature. The roots were then rinsed, quickly teased apart in 45% acetic acid, and squashed between slide and cover slip. As indicated below, use of trichloroacetic acid during hydrolysis prevents loss of most of the histone during subsequent treatment. The slides were frozen (cover slip down) on dry ice and the cover slips chipped off. They were then air-dried and stored until application of emulsion. Fig. 3 shows such a squashed preparation.

Label was assayed in several ways. In the case of thymidine, percentages of labeled cells were determined by scoring the mitoses as labeled or unlabeled.

In the amino acid experiments, the value for the number of grains per chromosome length was determined by counting the grains in individual *sets* of chromosomes as seen in the sections. Grains whose centers lay within the boundaries of the individual chromosomes within a set were counted, and this number was divided by the total length of the chromosome pieces of the set. The value of grains/length at a given time is the average value of at least 10 sets in a sample. The sets were selected by random scanning of the slide. In the experiment shown in Fig. 7 (the squashed preparations), the grain counts represent, at each time, the average number of grains overlying the flattened chromosomes of 10 division figures. Only chromosome sets that were present in their entirety were considered. In the experiments shown in Figs. 6 and 17, an independent but subjective estimate of labeling was made by exposing the sections for longer times, 6 wk in the first case and 2 yr in the second. In the former case, mitotic figures were considered "labeled" when they appeared heavily labeled compared to the cytoplasm, and "unlabeled" when they did not stand out against the surrounding cytoplasm. The results of the subjective estimate and of the determination of the grains per length were similar. In the experiment in which the sections were exposed for 2 yr (Fig. 17), areas were photographed after development, for purposes of relocation, and the number of visible mitoses was counted within these designated areas. Many division figures were unrecognizable as such because they lay hidden under the silver grains, but they were revealed after removal of the silver by immersion of the slide for ½ hr at room temperature in a solution containing equal parts of 5% cupric sulfate and 5% sodium chloride. The value of "percent hidden divisions" represents that proportion of divisions that were so heavily labeled that they were rendered invisible until their overlying grains were removed.

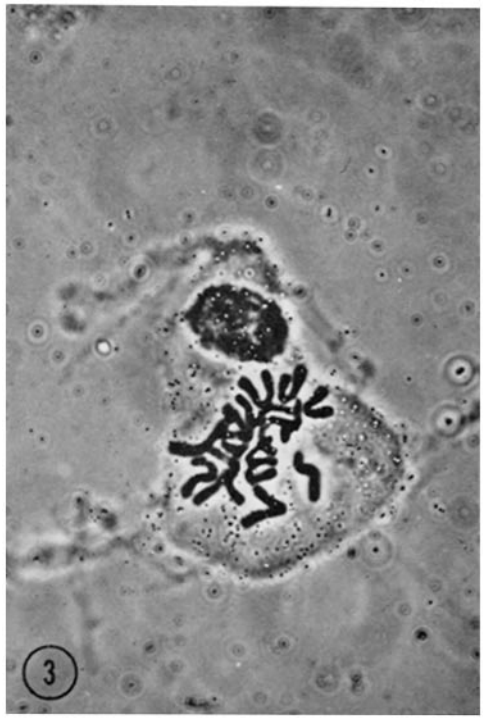

FIGURE 3 Phase photomicrograph of a squashed preparation such as was used to obtain the data in Fig. 7. The few grains over the chromosome set appear as white specks. × 950.

Grain counts per unit area were obtained in a similar manner for interphase nuclei, nucleoli, and cytoplasm. The areas were determined from planimetric measurements of tracings on photomicrographs. The volumes of the cytoplasm, nuclei, and nucleoli were obtained from Chalkley counts (Chalkley, 1943). The relative volumes of interphase nuclei and mitotic chromosomes were obtained by determining the relative lengths of segments of a straight line in an optical reticule overlying interphase nuclei and mitotic chromosomes, and then by dividing these values by the mitotic index. The values are expressed in arbitrary units, with that of the nucleus equal to unity. The lengths of lines projected on a class of objects in a section is proportional to the volume of the section occupied by the objects. The volumes of different classes of objects may be compared by dividing the lengths of the projected lines by the relative frequency with which the objects occur in the section.

Incorporation of isotope into whole roots was

determined in the following manner. Onions were grown as before. Isotope was administered. Roots, at least two per sample, were collected and fixed in neutral buffered formalin. Tips 3 mm long were cut, transferred to 70% ethanol, and then to 1 N trichloroacetic acid in which they were heated at 60°C for 25 min. They were then transferred to 70% ethanol, absolute ethanol, and scintillation medium [0.4% 2,5 diphenyloxazole (PPO) and 0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) dissolved in reagent grade toluene]. The counts per minute were determined in a Packard Model 314E Scintillation Counter.

Histones for incorporation studies were prepared as follows. The roots of five isotope-treated onions (180 roots) were collected. The end 2 mm of the root tips were excised, treated with a solution containing 1% cellulase and 5% pectinase for several hours at room temperature, and homogenized in 15 ml of ice-cold 0.25 M sucrose for 10 min in a Potter-Elvejhem glass homogenizer. The chilled homogenate was pooled with a similar one obtained from 300 unlabeled root tips, to increase the bulk of material. The combined homogenate containing cells, nuclei, and fibers was centrifuged for 10 min at 800 *g* in an International refrigerated centrifuge. The pellet was washed with cold sucrose, and the centrifugation and washing were repeated twice. The pellet was suspended in 7 ml of distilled water, and the suspension was frozen and thawed three times to break the nuclei. The suspension was rehomogenized, cold HCl was added to bring the concentration to 0.2 N, and the mixture was homogenized a third time. After 24 hr the mixture was dialyzed against water and concentrated by dialyzing against carbowax, and the solution was lyophilized.

The lyophilized protein was assayed for radioactivity with the scintillation counter, and for total nitrogen with a microphotometric modification of the Kjeldahl method (Ballentine, 1957). Samples on filter paper were applied to starch gels and electrophoresis was carried out in a manner following that of Neelin and Connell (1959) with some modifications. The gel was prepared in a 0.2 M NaCl solution brought to pH 3.9 with formic acid. 40 ma were applied to the gel, whose cross-sectional area was 2.75 cm^2. At the end of a run of 24 hr, the gels were sliced into equal 2-mm sections which were dehydrated with alcohols and impregnated with scintillation fluid. Counts were determined as in the case of the root tips.

Other chemicals used were labeled deoxycytidine (specific activity 2.4 c/mmole; Schwarz Bio Research Inc.), puromycin dihydrochloride (Nutritional Biochemicals Corporation), and 5-fluorodeoxyuridine (FUDR) (Hoffman-La Roche, Nutley, N. J.).

RESULTS

An assessment of the lag in the incorporation of precursors into the DNA and proteins of the root tips and of the time needed for labeled precursor, once incorporated, to be diluted out by carrier is shown in Fig. 4. Labeled material was added at zero time and unlabeled at ½ hr. Any lag in in-

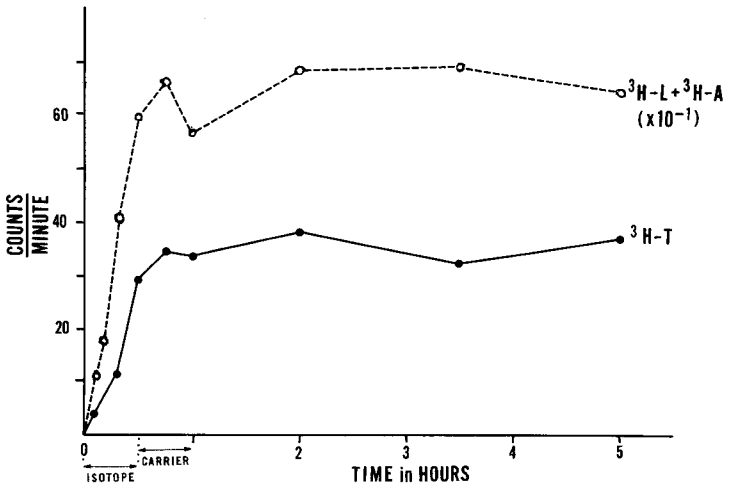

FIGURE 4 Incorporation of labeled amino acids (dotted line) and thymidine (solid line) into root tips. Each point represents an average of counts on three roots. The doses were as follows: ½ hr in 1 μc per ml of each of lysine-^{3}H and arginine-^{3}H followed by ½ hr in 0.086 mg/ml lysine and 0.018 mg/ml arginine for the amino acid experiment; and 5 μc/ml thymidine-^{3}H followed by 0.12 mg/ml cold thymidine for the thymidine experiment.

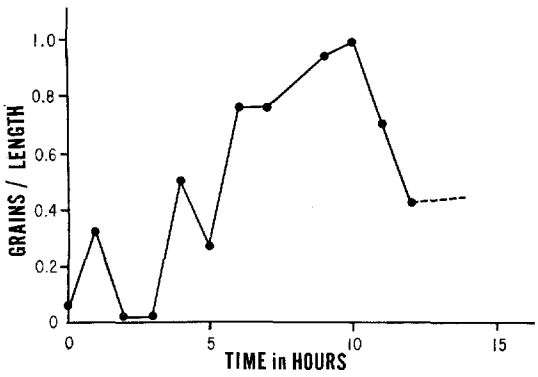

FIGURE 5 Incorporation of lysine-^{3}H and arginine-^{3}H into mitotic figures. Each point represents the average values for 20 mitotic figures taken from a root. Each time is represented by a single root. Doses were 1 μc/ml of each of lysine-^{3}H and arginine-^{3}H for ½ hr followed by 0.075 mg/ml cold lysine and 0.0175 mg/ml cold arginine for 1½ hr. The incorporation is expressed as grains per chromosome length, the length given in arbitrary units. The onion was grown at 17°C.

corporation or any residue of undilutable label is minute compared to the times and amounts of label concerned in this study.

Figs. 5–7 show the times required for thymidine and amino acid labels to appear and then disappear from cells going through division. In all of these experiments paired onions were used, one for thymidine labeling, the other for amino acid labeling. The data given in Fig. 5 are for amino acid incorporation only; the thymidine incorporation, for unexplained reasons, was too low to provide useful counts. The increase in the number of grains overlying the chromosomes after a lag of from 5 to 9 hr is fairly typical and reflects a lag in the appearance in these chromosomes of at least a fraction of the label that ultimately is incorporated into the protein of the mitotic chromosomes.

Fig. 6 shows the results obtained in an experiment in which each of the paired onions successfully incorporated label. 7 months' exposure of the radioautographic film was needed for the thymidine experiment; higher doses were not used because of the reports of Wimber and Quastler (1963) on the effects of tritiated thymidine in delaying the G-2 period.

2 wk exposure proved adequate for determination of amino acid incorporation. The data from this amino acid experiment were obtained in two ways. In the first, the value for grains per unit length of chromosomes was determined, as in the previous experiment. In the second, a subjective estimate of labeling was assessed by assigning the designation "labeled" to division figures with many grains compared to the surrounding cytoplasm and the designation "unlabeled" to division figures with relatively few grains. The slides used for this scoring were exposed for approximately 6 wk. The results are expressed, as in the thymidine experiments, in terms of fraction of labeled divisions which are literally divisions whose grain density is higher than that of the cytoplasm. The shape of the curve follows quite closely that obtained by the objective scoring of grains per unit length of chromosome. The proximity of the absolute values of grains/length and fraction of divisions is coincidental.

Fig. 7 represents an attempt to increase confidence in the assignment of label to the chromosomal protein. Squashes made from Carnoy fixed material were used. The resulting dispersion of cytoplasm, the flatness of the mitotic figures, and the preservation of entire sets of chromosomes (Fig. 3) were advantages gained at the risk of losing some histone. Preliminary experiments indicated that much of the material staining with alkaline fast green, i.e. histone, was retained in these preparations, provided the hydrolysis prior to Feulgen staining was done with trichloroacetic acid rather than with HCl.

With the exception of the peak during the G-2 period, the pattern of incorporation of amino acids into protein is again similar to that of thymidine into DNA. It is concluded from these experiments that there are two periods during interphase in which much, although not all, of the protein of the mitotic chromosome is synthesized. The first and major period corresponds fairly closely to that of the DNA synthesis. That this protein includes the histone is assured by the fact that 75% of the label is lost during hydrochloric acid hydrolysis of roots fixed in Carnoy's medium. The number of grains per micron of chromosome length is 0.40 $\pm$ 0.03 after standard fixation and staining, and 0.09 $\pm$ 0.02 after HCl hydrolysis following Carnoy fixation.

The approximate coincidence of the S_h and S_d periods suggests that at any given time a population of cells within the meristem may be found synthesizing this chromosomal protein.

Labeling experiments with tritiated thymidine show that the population of cells in the S period within the meristem of a growing root comprises

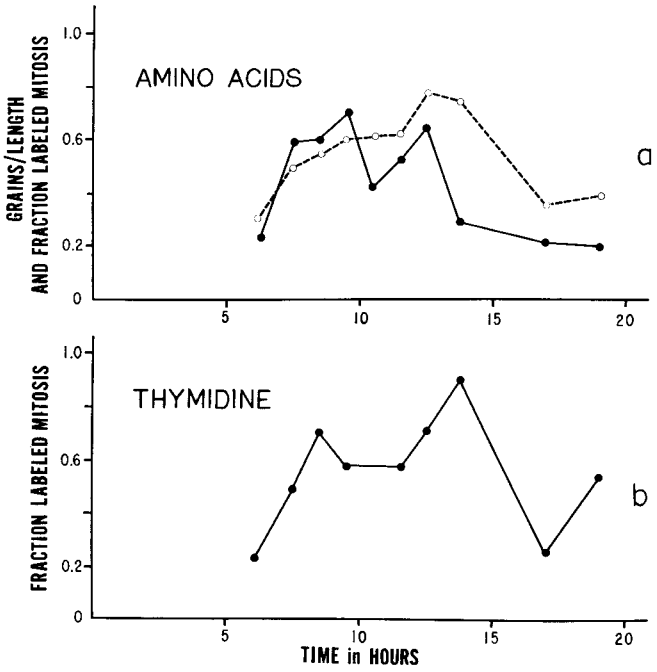

FIGURE 6 Comparison of times of labeling of protein (*a*) and DNA (*b*) of mitotic chromosomes. The dosage with the amino acids is as given for the experiment in Fig. 3, with the exception that the roots in *a* were put into carrier for only ½ hr. The dose in B was 1.0 μc/ml of thymidine-^{3}H for ½ hr, followed by 0.012 mg/ml cold thymidine for ½ hr.

The values in the amino acid experiment are expressed as grains/length (solid curve) as in Fig. 5 and also as percentage of labeled mitoses (broken curve) as assessed subjectively by determining frequency of mitoses showing heavier labeling than the cytoplasm.

up to 30% of the total population. The roots used for the data presented in Fig. 8 were exposed continuously to thymidine-^{3}H during the course of the experiment. Samples were taken at intervals and were stained in bulk with the Feulgen reaction. Squashes were made and were radioautographed. The frequencies of labeled interphases were determined for the different lengths of time. The extrapolation of the regression curve of frequency versus time to zero time provides an estimate of the frequency of cells in the S period at zero time. This frequency varies with rate of growth. The three sets of data represented in Fig. 8 show the results obtained from three different onions in different experiments. The 30% value is high, and probably constitutes an approximate upper limit to the frequency of cells within the S period. A more typical value is around 15%.

Figs. 9–11 show the distribution curves and probits of the values of grains/area over the cytoplasm, nucleolus, and nucleus of interphase cells taken from a 1-hr sample shown in Fig. 5. The value for grains/area provides a measure of concentration of label. As indicated by the cytoplasmic concentrations, the cells appear to belong to a single population whose distribution is skewed to the right. The nucleoli constitute a single population with a normal distribution. The nuclei, on the other hand, present two populations as indicated by the discontinuous change of slope on the probit diagram. The change occurring at a probit of about 6.2 corresponds to a cummulative percentage of approximately 90%. Apparently 10% of the nuclei belong to a different population whose mean grain density is about twice that of the majority of the population. It is thought that these nuclei with their higher rate of incorporation belong primarily to the S_h population.

Table I shows the results of an attempt to compare the amounts of protein synthesized in the S period with the amounts that appear later in the mitotic chromosomes by expressing the grain counts in terms of grains overlying the entire nonnucleolar nucleus, nucleolus, cytoplasm, and set of mitotic chromosomes. It is seen that the amount of radioactive material in the mitotic chromosomes represents about 80% of that of the average interphase nucleus and is roughly equal to the difference between the amounts of label in the major and minor populations of nuclei represented in Fig. 11. This equivalence might be expected if the increment of protein synthesized during interphase accounts for most of the protein of the mitotic chromosomes.

In view of the different mechanisms involved in the syntheses of nuclei acid and proteins, it was

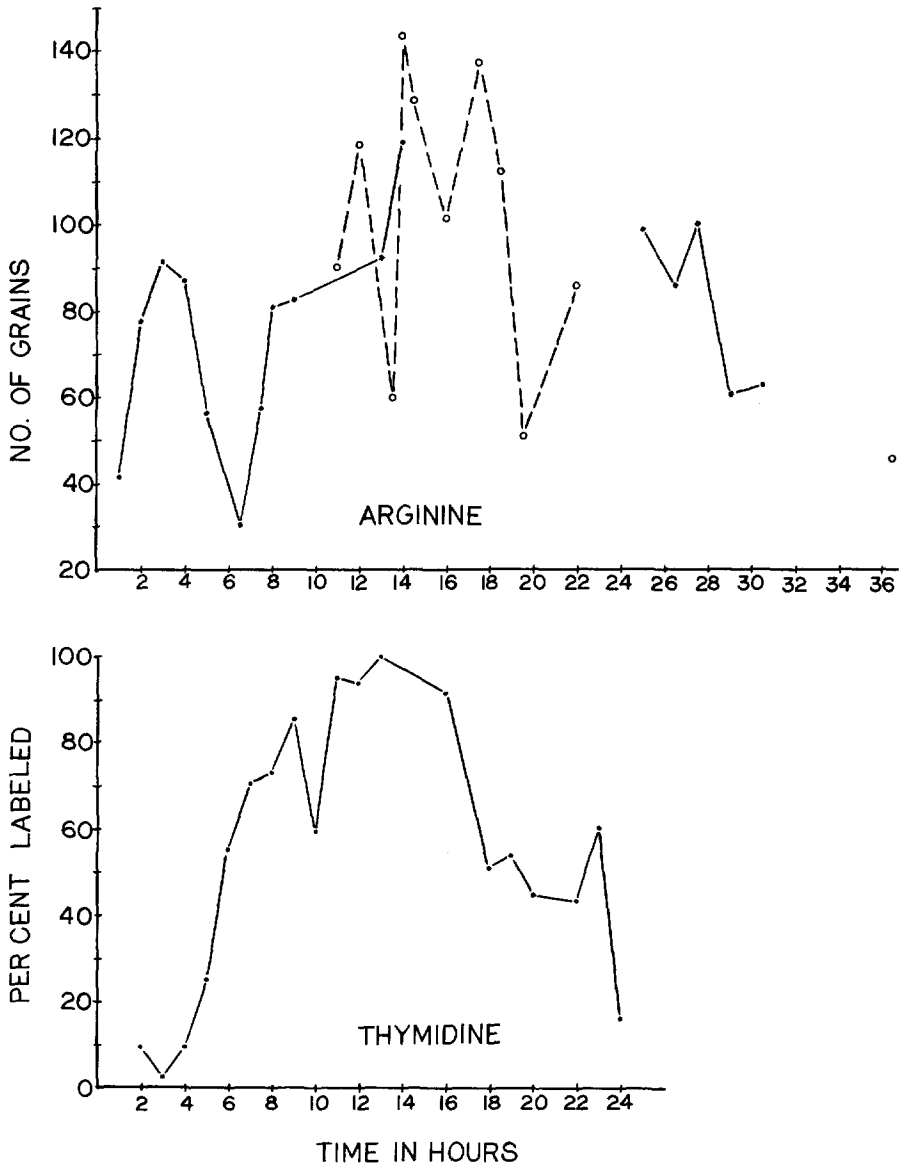

FIGURE 7 Similar to Fig. 4, except that in this experiment the onions A (dots) and B (open circles), given only arginine, were grown at 25°C, and the preparations are squashes of Carnoy-fixed material. Onion C was given thymidine. Each sample represents two to three roots. Only late prophases and metaphases were considered. The data on onions A and B were normalized by multiplying the value for onion B by the factor 1.7. This was made necessary by the lower over-all incorporation by onion B. The factor was obtained from the ratio of numbers of grains in samples taken from onions A and B in the region of overlap, at 11–14 hr.

considered possible that the parallel syntheses of the DNA and histone reflect coordinated control of the two processes rather than independent syntheses, as had once been considered likely. Attempts were made to determine whether the two syntheses could be dissociated from each other by finding out whether specific inhibitors of protein synthesis have any immediate effect on DNA synthesis or whether inhibitors of DNA synthesis have an effect on histone synthesis. Figs. 12 and 13 show

the effects of puromycin, an inhibitor of protein synthesis (Hultin, 1961; Morris et al., 1962), on the incorporation of precursor into protein and DNA. The inhibitory effect on DNA synthesis at various levels of concentration of puromycin is seen to parallel the inhibition of protein synthesis.

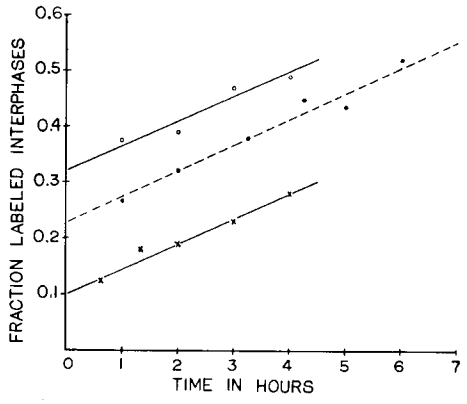

FIGURE 8 Rate of increase of labeled cells with time during continuous labeling with thymidine-^{3}H. The intercept shows the frequency of cells in the S_d period at any given time. The three curves were obtained from three different experiments.

Of importance is the fact that the effect of puromycin at these concentrations is immediate for thymidine incorporation as well as for amino acid incorporation.

Figs. 14 and 15 show the effects of 5-fluorouracil deoxyriboside, an inhibitor of DNA synthesis (Cohen et al., 1958), on deoxycytidine incorporation into DNA and arginine incorporation into protein. 10^{-4} M FUDR, which shows almost complete inhibition of DNA synthesis, has no comparable effect on general protein synthesis.

An assay of the effect of FUDR on histone synthesis was made by extracting histones from the roots of onions which had been exposed first to 10^{-4} M FUDR for 1 hr and then to 10μc/ml of each of arginine-^{3}H and lysine-^{3}H during a second hour. Another set of onions, without prior treatment with FUDR, was similarly exposed to the isotope. Equal numbers of roots were taken from the treated set and control set. These were rinsed; carrier was added to dilute out the labeled material; and the roots were macerated along with the roots from unlabeled onions. The nuclei were isolated and the histones were extracted. Fig. 16 shows a comparison of the radioactivities of the

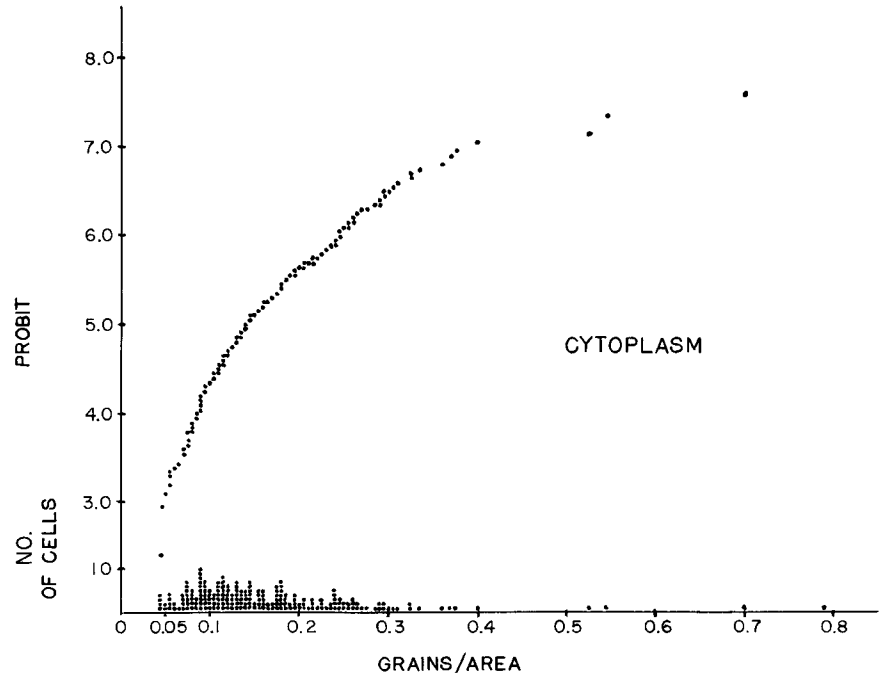

FIGURE 9 Distribution of frequencies and probits of concentration of grains per unit area over the cytoplasm of interphase cells 1 hr after administration of lysine-^{3}H and arginine-^{3}H (1 μc/ml of each).

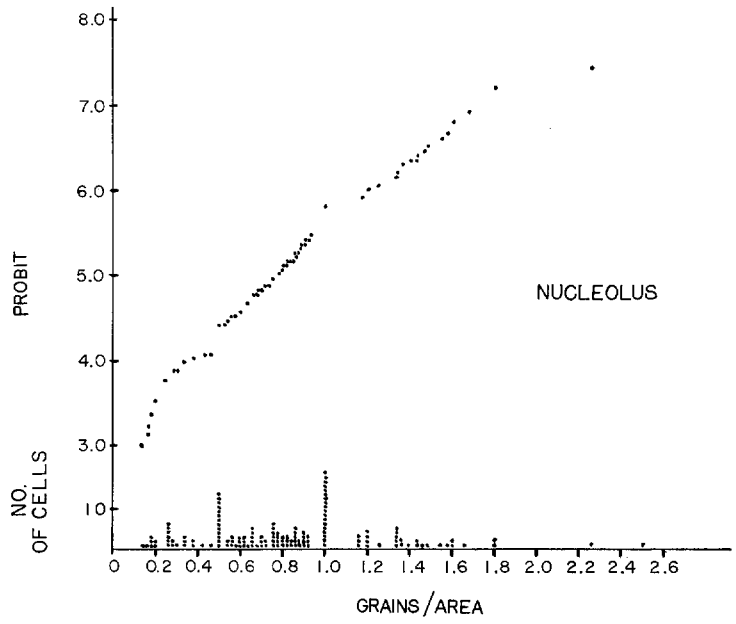

FIGURE 10 Frequency distribution and probits of concentration of grains per unit area overlying the nucleoli of the interphase cells of Fig. 9.

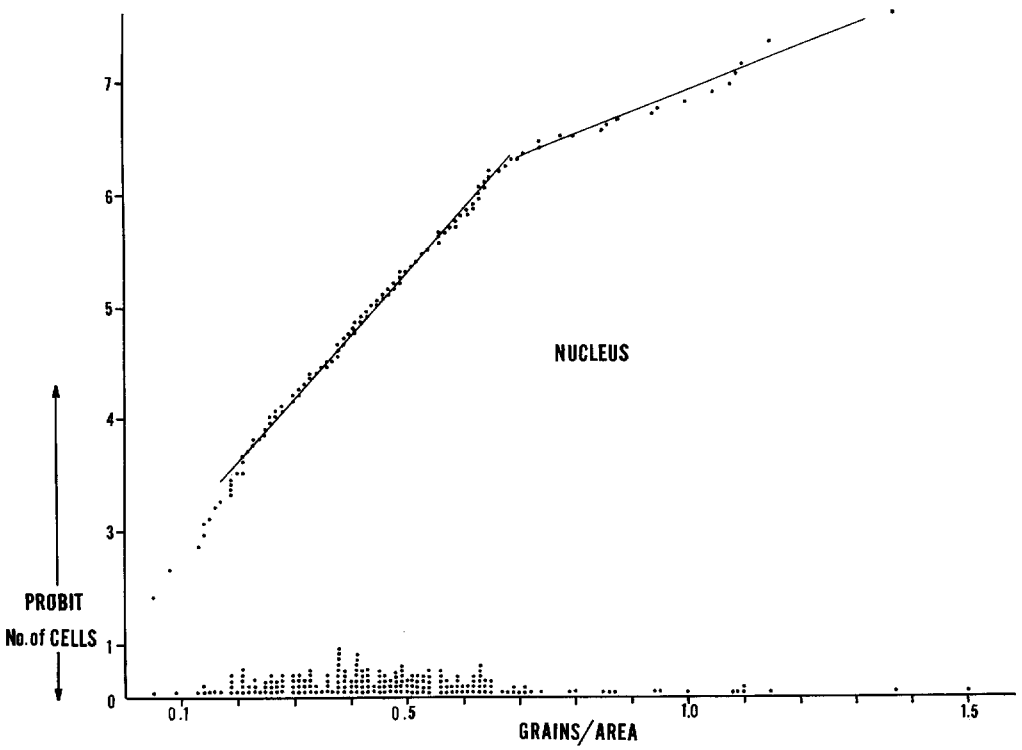

FIGURE 11 Frequency distribution and probits of concentration of grains per unit area overlying the nuclei of the interphase cells of Fig. 9. The break occurs at a probit of 6.3, corresponding to a cumulative percentage of 90.

TABLE I

Relative Concentrations, Volumes, and Numbers of Grains for Different Cellular Compartments

	Relative volume	Relative concentration of grains	Relative No. of grains
Nucleus, 1 hr	1.00	1.00	1.00
Nucleolus, 1 hr	.111	1.77	.20
Cytoplasm, 1hr	9.15	.35	3.20
Nucleus, 13 hr	1.00		
Chromosomes, 13 hr	.58	1.39	.81

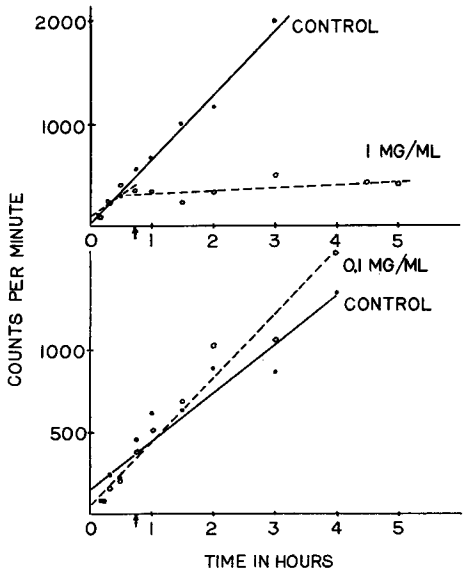

FIGURE 12 Effect of puromycin on incorporation of labeled arginine by onion root tips. Puromycin was added at the designated concentration at 45 min (arrow) after addition of isotope. The concentration of isotope was 2.5 μc/ml. Concentrations higher than 1 mg/ml of puromycin showed complete inhibition. Concentrations lower than 0.1 mg/ml showed no inhibition.

electrophoresis profiles of these histones. The relative incorporations of amino acids into the histones of the treated and untreated roots are 0.18 and 0.23 cpm/mg protein, respectively. There is little if any immediate effect of FUDR on histone synthesis under conditions in which DNA synthesis is blocked almost completely.

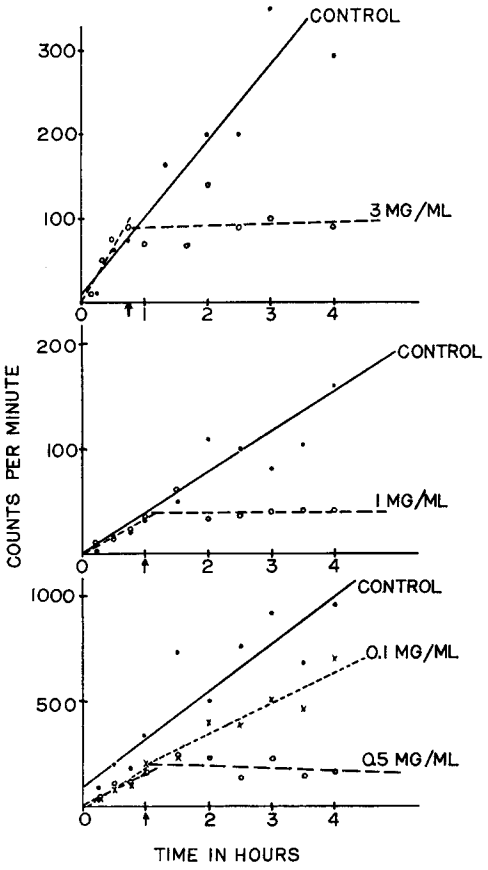

FIGURE 13 Effect of different concentrations of puromycin on incorporation of labeled thymidine by onion root tips. Puromycin was added at the designated concentrations, at the time shown by the arrow, after prior addition of isotope. The doses of thymidine were 2.5, 1.0, and 5.0 μc/ml, respectively, in the three figures.

DISCUSSION

The Concurrence of DNA and Histone Syntheses

Comparison of the times needed for the appearance and then disappearance of label from the chromosomes of cells going through mitosis, after pulse labeling with amino acids and thymidine, reveals that the S_d and S_h periods are concurrent over most of their lengths. The procedures required for distinguishing cytoplasmic labeling and chromosomal labeling while retaining histones precluded the collection of large amounts of data needed for the precise estimation of the times of the

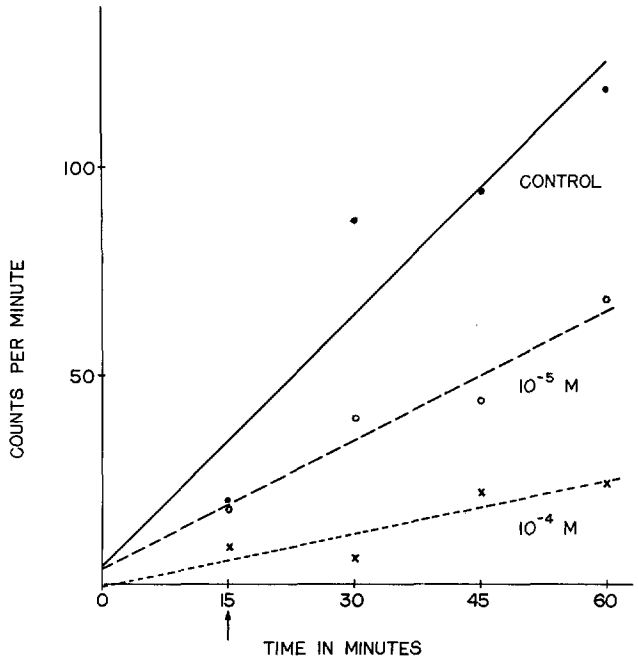

FIGURE 14 Effect of different concentrations of FUDR on the incorporation of deoxycytidine into DNA. The dosage of label was 2 μc/ml. FUDR was added 15 min after administration of the isotope.

different stages of the cycle. Whether small differences exist in the times of initiation and termination of the S_d and S_h periods is not indicated by these experiments. That much of the protein labeled during the S period is in fact histone is indicated by its susceptibility to extraction from the chromosomes by hydrochloric acid following Carnoy fixation.

Other attempts employing labeling techniques to follow the course of DNA and histone syntheses during division in onion and other organisms have yielded a variety of results. Zweidler (1965) has recently reported that histone and DNA syntheses run essentially parallel in the onion root meristem but that the histone synthesis is initiated approximately 1 hr before DNA synthesis, a difference in timing that cannot be resolved by the present experiments. Zweidler based his conclusion on the observation that the frequency of nuclei that label heavily with amino acids is consistently greater than the frequency of nuclei that incorporate thymidine. Some of this difference may be attributable to nuclei in the short period of protein synthesis prior to division. In a biochemical study of incorporation by G-1 and G-2 nuclei obtained from the regenerating rat liver and separated by gradient centrifugation, Niehaus and Barnum (1965) found that amino acids injected shortly

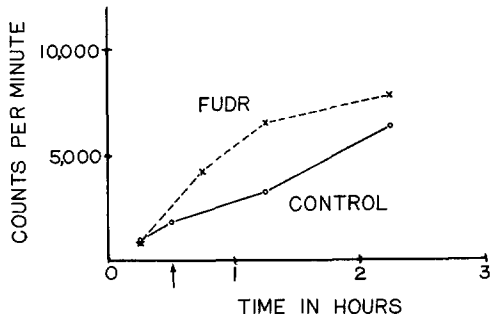

FIGURE 15 Effect of FUDR on incorporation of amino acid into onion root tips. FUDR was added 30 min after administration of 25 μc/ml of arginine. Root tips were contained in 1 ml of solution.

before collection of the nuclei are incorporated into the histones of the G-2 and late S nuclei. This condition would not have been realized had the histones been synthesized prior to the S period as claimed by Evans et al. (1962).

Cave (1966), using an approach similar to the present one in a study of human leukocytes, found that the initiation of synthesis of DNA and an increase in incorporation of lysine into chromosomal protein show a fair degree of coincidence. However, the protein studied in this case was essentially nonhistone. The method employed did

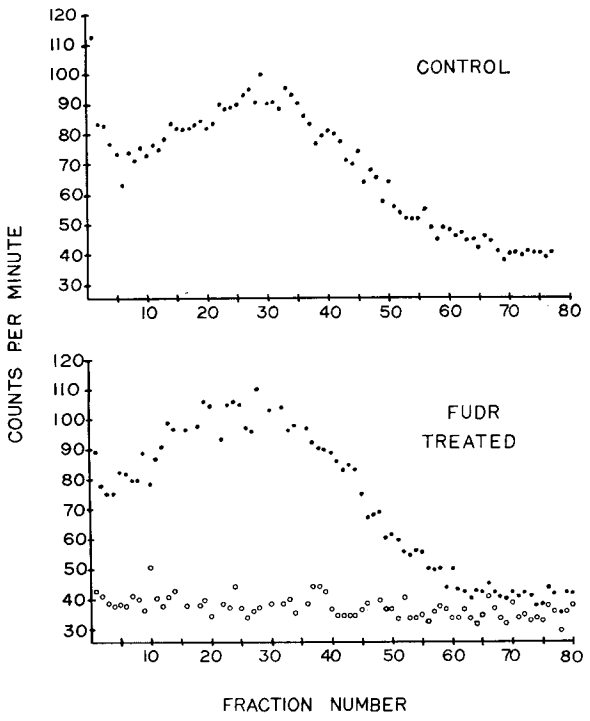

FIGURE 16 Incorporation into histones of FUDR-treated and control root tips. The histone samples were applied to starch gel and run electrophoretically. The number on the abscissa refers to the number of the slice (No. 1 being the slice nearest the point of application of the sample, No. 80 being closest to the cathode) of the starch gel from the origin. Each slice was 2 mm thick. The open circles represent background.

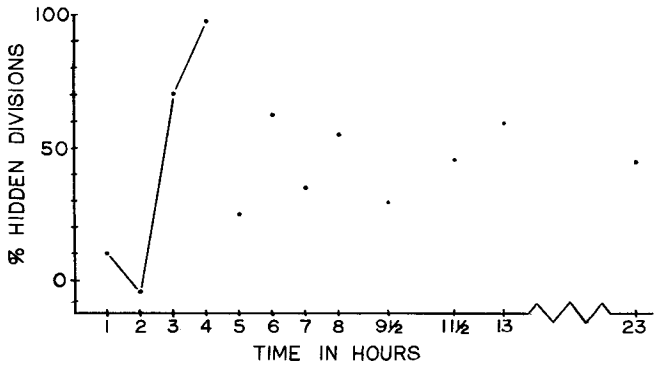

FIGURE 17 Frequency of division figures completely hidden by their grains at different times after labeling with arginine-^{3}H and lysine-^{3}H. The emulsion was exposed for 2 yr.

not preserve the histones. Furthermore, Cave stressed the observations of (*a*) peak incorporation into the protein late in the S period, (*b*) continuing incorporation through the G-2 period, and (*c*) labeling of close to 100% of the mitotic cells at all times. He concluded that the syntheses of DNA and protein do not occur simultaneously.

In the present work, as in that of Cave, the mitotic chromosomes do contain some proteins that are synthesized throughout the entire cell cycle. However, most of the proteins are synthesized during the S period and during a second period of short duration in the G-2 period. The chromosomal protein synthesized during the latter time is of particular interest. There are some indications of differences between this protein and the protein synthesized during the S period. The peak of the G-2 synthesis is more obvious in the squash preparations than in the sections. It is uncertain whether this is indicative of a greater stability toward acid of this late protein, a characteristic of nonhistone protein, or of a greater arginine/lysine ratio. Of interest also is the different distribution of the two proteins among the chromosomes. A series of slides which were duplicates of those represented in Fig. 5 was exposed

for 2 yr. The proportion of division figures whose presence was completely obscured by overlying silver grains was determined. Fig. 17 suggests that the protein synthesized in a given ½ hr during the S period covers the chromosomes nonrandomly, much as the DNA synthesized "asynchronously" (Taylor, 1960) during a portion of the S period can be localized within limited regions of the chromosomes. Because of the heterogeneous distribution of silver grains, the divisions containing protein synthesized during the S period can be recognized more often than not, by the presence of unlabeled chromosomes or arms. However, that protein synthesized during the G-2 period is distributed more homogeneously, resulting in silver grains overlying the whole chromosome complement.

Also of interest is the apparent conservation of the protein between divisions, as suggested by the large number of grains overlying the chromosomes at times corresponding to the second division (Fig. 7). These results are at variance with those of Prescott and Bender (1963) and of Prensky and Smith (1964) but may reflect differences, in these reports, in the proteins retained by the chromosomes. Cave (1966) and Zweidler (1965) both find conservation of chromosomal protein.

Chromosomal Protein: Its Site of Synthesis and Time of Coupling with DNA

The exact site of synthesis of the protein is not certain. The existence, an hour after administration of isotope, of a population of cells with high nuclear labeling indicates that the protein, wherever it is made, is to be found within the nucleus within an hour of its synthesis. If the protein were synthesized in the cytoplasm and then transferred to the nucleus, a separate population of cells showing high cytoplasmic labeling might also be expected. Such is not seen, although the skewness of the cytoplasmic grain distribution might conceal the type of discontinuity that is so apparent in the case of the nucleus.

As discussed above, most of the microspectrophotometric data indicate that histone is coupled with DNA as the latter is synthesized. This implies availability of the protein to the chromosomes as DNA is replicated. The present work is in accord with this idea.

The Dependence of DNA Synthesis on Protein Synthesis

There have been numerous observations of the requirement of protein synthesis for DNA synthesis. However, most of these show the need of protein synthesis for initiation of DNA synthesis (Mueller et al., 1962; Gottlieb et al., 1963) but not for its maintenance once initiated. The present finding of the immediate and parallel inhibition by puromycin of both protein and DNA syntheses suggests that a continued supply of new protein is essential for continued DNA synthesis. That the inhibition may be the result of a general toxicity at these high concentrations of puromycin cannot be discounted. However, the lack of observable effect at lower concentrations indicates that the puromycin is not readily accessible to the cell. It is not considered likely that a nonspecific toxicity would be revealed before the more commonly observed inhibition of protein synthesis, as the concentration of puromycin is increased. The possibility that puromycin directly interferes with DNA synthesis in a manner that is independent of its known effect on dissociation of peptide from ribosomes seems less likely in view of the similar ranges of concentrations over which its effect begins to take place on both DNA and protein syntheses. It seems plausible that DNA synthesis may be tied to protein synthesis in higher organisms in which the chromosome contains both DNA and protein.

The relationship, in this respect, between the syntheses of general chromosomal protein and histone is not known. In special cases DNA synthesis does precede complexing with histone by a substantial margin, and in fact this coupling may not occur at all. We have recently observed that the doubling of DNA in the late replicating X chromosome of the katydid *Rehina spinosa* goes almost to completion before histone doubling begins (Teng et al., unpublished data). In microsporogenesis in *Hippiastrum belladonna*, the occasional DNA doubling that occurs in the vegetative cell is apparently unaccompanied by histone synthesis (Pipkin, personal communication).

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