tase system are lost by the method of fixation and embedding used since there is no question that such treatment considerably reduces the intensity of the reaction(10,11). Studies using more efficient methods of tissue preparation such as freezing-drying are continuing. Other components of the phosphatase system are also being studied, and the reversible inactivation by EDTA of 5-nucleotidase has already been demonstrated by histochemical means.

Summary. A method of controlled inactivation of alkaline phosphatase in tissue sections by removal of the activating metallic component through chelation is described. The enzyme activity of alcohol-fixed, paraffin-embedded rabbit kidney can be completely inhibited by EDTA and subsequently reactivated by a variety of metallic cations in 0.1 molar concentration, including Mg^{++}, Co^{++}, Zn^{++}, Ca^{++}, Sr^{++} and Ba^{++}. Ni^{++} is a weak reactivator. The localization of enzyme activity in the tissue is the same regardless of the reactivating ion. Hg^{++}, Be^{++}, and Cu^{++} are constant strong inhibitors. Mn^{++}, Pb^{++} and Cd^{++} fail to reverse EDTA inhibition but will not themselves inhibit the active enzyme in the concentrations used. Preliminary experiments indicate, however, that the alkaline phosphatase of guinea pig and monkey kidney is inhibited by Pb^{++} and Cd^{++} in much lower concentrations.

1. Green, M. M., Gladner, J. A., Cunningham, L. W., Jr., and Neurath, H., *J. Am. Chem. Soc.*, 1952, v74, 2122.

2. Gross, M., *Science*, 1953, v118, 218.

3. Swanson, M. A., *J. Biol. Chem.*, 1952, v194, 685.

4. Meyer, K., and Rapport, M. M., *J. Biol. Chem.*, 1951, v188, 485.

5. Roche, J., Chap. 11: *Phosphatases, in The Enzymes*, edited by Sumner, J. B., and Myrbach, K., Academic Press, New York, 1950.

6. Lehninger, A. L., *Physiol. Rev.*, 1950, v30, 393.

7. *Sequestrene, Tech. Bull.* Alrose Chemical Co., Providence, R. I., 1952.

8. Schwarzenbach, G., Biedermann, W., and Bangerter, F., *Helv. Chim. Acta*, 1946, v29, 811.

9. Schwarzenbach, G., and Biedermann, W., *ibid.*, 1948, v31, 678.

10. Gomori, G., *Microscopic histochemistry. Principles and practice.* Univ. of Chicago Press, Chicago, 1952.

11. Pearse, A. G. E., *Histochemistry, theoretical and applied.* Little Brown and Co., Boston, 1953.

12. Neumann, H., *Biochim. et biophys. Acta*, 1949, v3, 117.

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Effect of Colchicine on Synthesis of Desoxyribonucleic Acid in Tissue Cultured Rat Fibroblasts.* (20641)

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Early observations on the effect of colchicine on growing tissue (Dustin(1)) suggested that this substance exerts a stimulatory effect on the process of cell division. In later analyses it was shown by Ludford(2) and others that the increase in mitotic figures was the result of an inhibition of mitosis at metaphase. Ludford postulated an inhibitory effect on the mitotic spindle, which has recently been convincingly illustrated by the work of Inoue(3) on the birefringence of the spindles of normal and colchicine-treated sea urchin eggs. The suggestion of a specific effect led to experiments which demonstrated that at low col-

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chicine concentrations sufficient to cause mitotic inhibition, other metabolic processes which are not directly concerned with the mechanics of cell division can proceed. For example, Zeuthen(4) reported that although cleavage of echinoderm eggs is inhibited during colchicine treatment, the rhythmic behavior of oxygen uptake and nuclear refractivity which normally occur during cleavage, also occur during colchicine blocking. Skipper *et al.*(5) showed a continuous incorporation of C^{14} into nucleic acids of tumor cells during colchicine treatment. The production of polypoid cells with colchicine (Blakeslee and Avery(6)) gives evidence that this substance does not directly interfere with the synthesis of chromosomal material. This author has observed a few meristematic cells from the buds of Crepis, whose normal diploid complement is 6, with more than 100 chromosomes after a few days of colchicine treatment. The presence of cells with higher than the tetraploid number can only be due to continued synthesis during treatment.

Preliminary experiments have shown that the individual nuclei of growing cultures which have been treated with colchicine for approximately one day, invariably contain higher amounts of desoxyribonucleic acid (DNA) than untreated controls. The following experiments were designed to trace the course of DNA synthesis during colchicine treatment.

Materials and methods. Fibroblast cultures obtained from explants of the subcutaneous fascia of a 5-day-old rat were grown on Maximow slides for about 7 days. This tissue affords a source of fibroblasts in which the division rate is relatively steady, and which are relatively genetically pure with regard to tissue type (Murray *et al.*(7)). The cultures were treated with colchicine in the feeding solution at a concentration of 10^{-5} molar. After treatment they were fixed at various intervals along with controls in neutralized 50% formalin, followed by washing for 3 hours in running tap water, and 15 minutes in 80% ethanol to remove excess formaldehyde. They were then stained according to the Feulgen procedure using a hydrolysis time of 15 minutes. Fifty to 60 randomly selected interphase nuclei, distributed among 3 explants, were measured from each culture, totaling approximately 400. One culture was used for each treatment, and 2 for the controls. The intensity of stain was determined by absorption measurements using a microspectrophotometer of the type described by Pollister and Ris(8), as modified by Moses(9). The slides were mounted in oil with a refractive index of 1.548, approximately that of the stained cells, to minimize light scattering. A wave-length of 565 $m\mu$ was used, which lies at the absorption peak of the stain. Relative amounts of DNA in the individual nuclei were calculated by multiplying the extinction by the nuclear area. The latter was approximated by direct measurement of the 2 diameters of the flattened elliptical nuclei with an ocular micrometer. The mitotic indices are the fractions of cells found in mitosis and were determined by counting up to 1000 nuclei in randomly selected fields. Fewer nuclei, approximately 500, were counted in the cultures showing very high indices.

Results. The upper curve in Fig. 1 shows a typical distribution curve of the amounts of DNA in the individual nuclei of a young growing culture. The first peak, which predominates, consists of nuclei containing the normal diploid amount. The nuclei in the second peak have doubled their DNA, presumably during interphase (Swift(10)), and are about to undergo division. This curve is in accord with the results obtained by Frazer and Davidson(11) on the distribution of DNA in cultured chick fibroblasts. That the cells in the second peak are not true tetraploids is made evident by the curves obtained from older cultures which are no longer growing. In such cultures the second peaks are almost non-existent. Henceforth Swift's(12) designation: Class 2, 4, 8, . . . pertaining to nuclei with diploid, tetraploid, octoploid, . . . amounts of DNA will be used in describing the various classes. The lower curves show the shifting of the class distributions with time in colchicine. After 10 hours of treatment, the ratio of class 4 to class 2 nuclei increases, and some class 8 nuclei appear. At 21 hours the class 4 predominate and class 8

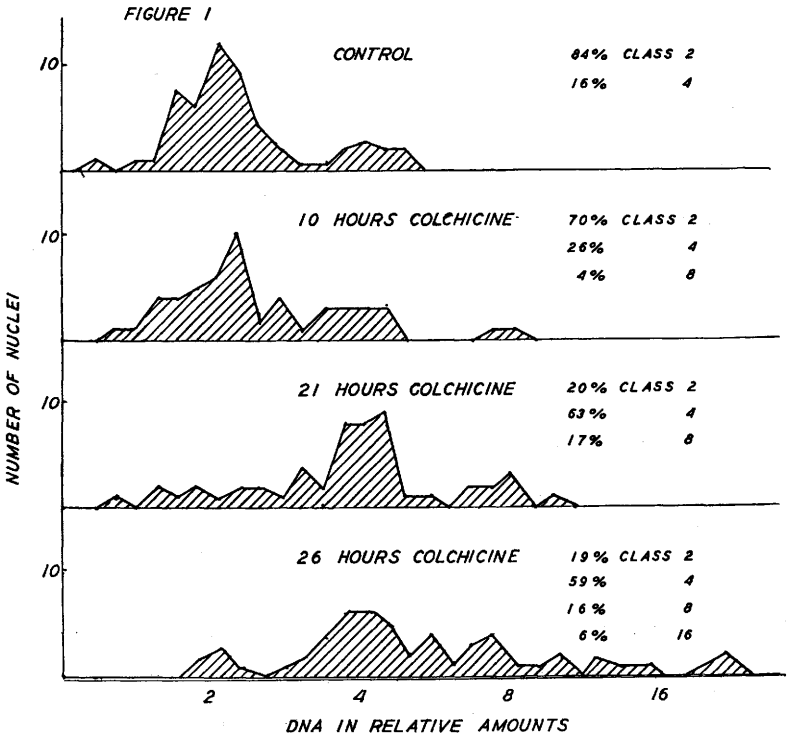

FIG. 1. Effect of time of colchicine treatment on frequency distributions of DNA per nucleus.

are numerous. At 26 hours even some class 16 nuclei are found. The boundaries of the various classes were established by multiplying the values obtained for the boundary between the first 2 peaks by the appropriate integers. The minimum between peaks 2 and 4 became very obvious after constructing the distribution curve for the pooled nuclei of all the cultures. After 21 hours, a progressively increasing number of multinucleate cells makes any attempt to determine relative numbers of nuclei in the various classes difficult. Most of these apparently belong to the higher classes, although the errors involved in adding quantitatively determined values of the micronuclei are sufficiently great to preclude their assignment to any definite class. These cells amount to approximately 1% at 17 hours, 5% at 21 hours, and 30% at 26 hours. In determining the class distributions the multinucleate cells were eliminated, resulting in a slight underestimation of the degree of polyploidy as time of treatment increased.

In accord with previous investigations, the mitotic index was found to rise irregularly during the first 24 hours of treatment, after which it declined in the same manner. Continuous observation of several fields during the first 24 hours showed that cells in "colchicine mitosis" revert to the resting state during this period.

Fig. 2 shows the changing mitotic index with time, and also the changing "degree of polyploidy." (The use of the latter term does not presume to distinguish between true polyploids and nuclei of lower classes with increased DNA.) The degree of polyploidy was calculated by assuming the mitotic nuclei to be distributed among classes 4, 8, and 16 in the same ratio as the interphase nuclei, and by multiplying the adjusted (to include mitotic nuclei) percentages in the various classes by their class value. Thus degree of polyploidy, D, equals:

$$\frac{I}{N_T - N_2}(4N_4 + 8N_8 + \ldots) + \frac{1-I}{N_T}(2N_2 + 4N_4 + \ldots)$$

where I is the mitotic index, N_X the number of nuclei in class x, and N_T the total number

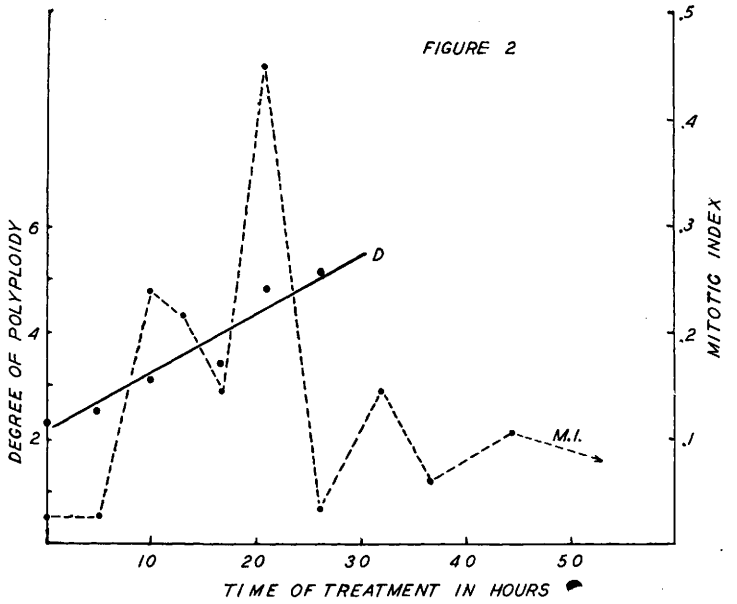

FIG. 2. Effect of time of colchicine treatment on mitotic index and "degree of polyploidy."

of nuclei. D would be 2 in a normal non-growing culture, between 2 and 4 in a growing culture, and 8 in a hypothetical homogeneous octoploid population. The assumption that the proportions among the higher classes is the same in the mitotic nuclei as in the resting is an approximation which is sufficient for the purpose to which it is to be put. It can be seen from Fig. 2 that the degree of polyploidy approximately doubles during the first 18 hours. The coefficient of correlation between D and time during this period, was calculated to be .966. The probability that such a coefficient would be obtained where no correlation exists, is less than 1%. Assuming that during this period the number of cell divisions of any kind is small, the total number of cells therefore remaining the same, the increase in D will be proportional to the increase in the DNA of the culture. Hence DNA approximately doubles during this period. This is at variance with the results of Davison *et al.*(13) who have reported a slight decrease of total DNA phosphorus after colchicine treatment, using chemical extraction technics on colchicined cultures.

According to Lambert and Hanes(14), the average period between the onset of prophase and the end of telophase in cultured rat fibroblasts is about 35 minutes at 37°C. A similar figure was obtained for chick fibroblasts (Hoffman(15)). The amount of time spent in any one phase of mitosis would be proportional to the number of cells found in that particular stage. Therefore the mitotic index of .027 for the control cultures indicates that the average cell completes the division cycle in about 22 hours. The typical interphase period has been found to be approximately 9 hours (Fell and Hughes(16)) by direct measurement of the length of time between successive mitoses. However, the 22 hours arrived at here includes in the average, cells which are not actively dividing. During this 22-hour period the number of cells, hence total DNA for the control cultures, also approximately doubles. Considering the approximations used, the agreement between these 2 figures, 18 hours for the colchicined cultures and 22 hours for the controls, is as good as could be expected. It can be stated with assurance that the increase in the total DNA over the first 26-hour periods is approximately the same in the colchicine treated cultures as in the controls, and colchicine is not apparently directly concerned with DNA synthesis as such.

After the first day, there appears to be an

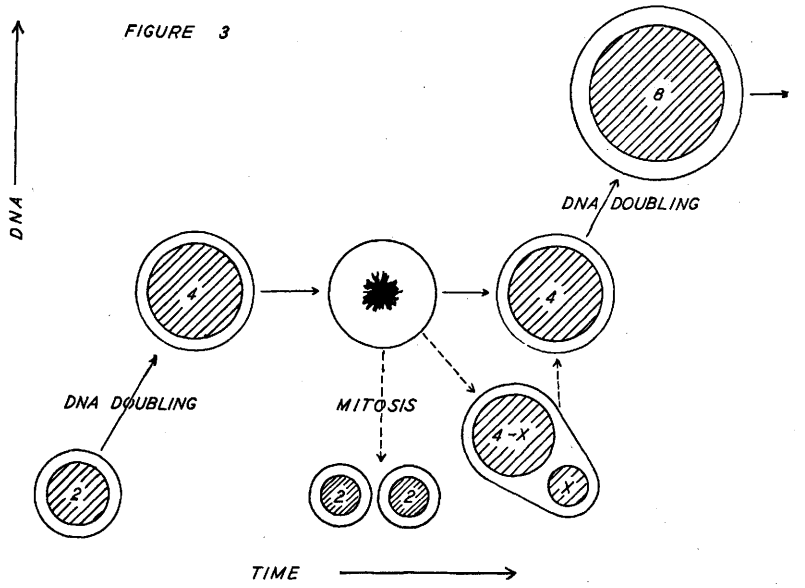

FIG. 3. Possible scheme for production of higher DNA class cells during colchicine treatment.

eventual diminution of activity, as indicated by the decreased mitotic index. Subsequent growth of the cultures is slight as compared by inspection with the controls. Whether this decreased growth is due to a general toxicity of the colchicine, or to production of genetically unbalanced cells is not certain. Interestingly enough, colchicine had no apparent effect on the single peaked distribution curves of non-growing cultures. This is in accord with the long established fact that colchicine in these low concentrations does not drastically disturb the resting cells of adult differentiated tissues.

Fig. 3 illustrates a possible scheme to explain the production of the various class nuclei under the influence of colchicine. The majority of cells probably follow the unbroken line leading through DNA duplication to the metaphase like figure typical of colchicine treated cells. From this point, several pathways might be open. One is through resumption of mitosis, a "colchicine escape," especially after prolonged treatment. Earlier, the most likely pathway is directly back to the interphase, maintaining the class 4 amount of DNA. A more devious route would be through separation of the chromosomes to form a multinucleate cell, thence restitution to the mononucleate class 4 stage. This whole process could be repeated forming the class 8 and 16 nuclei.

Summary. 1. In spite of its action in blocking mitosis, colchicine does not directly inhibit the synthesis of DNA. During the first 26 hours of treatment, DNA synthesis proceeds at the same rate as in untreated controls. 2. The result of colchicine treatment is a progressive increase in the number of cells belonging to the higher DNA classes, up to 26 hours. 3. Prolonged treatment eventually results in decreased growth.

1. Dustin, A. P., *Bull. Acad. Roy. Med. Belg.*, 1934, v14, 489.

2. Ludford, R. J., *Arch. Exp. Zellforsch.*, 1936, v18, 411.

3. Inoue, S., *Exp. Cell Research*, 1952, Supp. 2,305.

4. Zeuthen, E., *Publ. Sta. Zool. Napoli*, 1951, 23 Supp., 47.

5. Skipper, H. E., Mitchell, J. J., Bennett, J. L., Newton, M. A., Simpson, L., and Eidson, M., *Cancer Research*, 1951, v11, 145.

6. Blakeslee, A. F., and Avery, A. G., *J. Heredity*, 1937, v28, 393.

7. Murray, M. R., de Lam, H. H., and Chargaff, E., *Exp. Cell Research*, 1951, v2, 165.

8. Pollister, A. W., and Ris, H., *Cold Spring Harbor Symp. Quant. Biol.*, 1947, v12, 147.

9. Moses, M. J., *Exp. Cell Research*, 1952, Supp.

2, 75.
10. Swift, H., *Phys. Zool.*, 1950, v23, 169.
11. Frazer, S. C., and Davidson, J. N., *Exp. Cell Research*, 1953, v4, 316.
12. Swift, H., *Proc. Nat. Acad. Sci.*, (Wash.), 1950, v36, 643.
13. Davidson, J. N., Leslie, I., and Waymouth, C., *Biochem. J.*, 1949, v44, 5.
14. Lambert, R. A., and Hanes, F. M., *Virch. Arch.*, 1913, 211, 89.
15. Hoffman, J. G., *Science*, 1947, v106, 343.
16. Fell, H. B., and Hughes, A. F., *Quart. J. Microscop. Sci.*, 1949, v19, 355.

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Filter Paper Electrophoresis of Serum Proteins of the Domestic Fowl.* (20642)

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(Introduced by C. P. Leblond.)
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Filter paper electrophoresis(1,2) of avian serum proteins has demonstrated (a) the separation of albumin, 2 α-globulins, a β-globulin, and a γ-globulin from the sera of cockerels and immature pullets; and (b) the appearance of a presumptive lipovitellin (PLV) fraction in the sera of laying hens or estrogenized immature pullets(3). It has further been shown(3) that electrophoresis in a veronal buffer made up with 20% methanol results in the separation of a new slow-moving fraction from the sera of estrogenized immature pullets or laying hens. This fraction is not present in comparable electropherograms of sera of immature pullets; it is poorer in lipid than the PLV fraction and rich in phosphorus. For convenience this fraction has been designated presumptive phosphoprotein (PP).

The following additional observations are reported in the present communication: (a) the presence of a third α-globulin fraction on electropherograms of fowl sera; (b) the application of the Feulgen plasmalogen reaction to the electropherograms; (c) the double nature of the PP fraction; and (d) the results of studies using P^{32} labeled inorganic phosphate.

Methods. Electrophoresis was performed as described previously(3) with the exception that the apparatus was kept in a cold room at 6° to 7°C. At this lower temperature it was necessary to raise the potential to 200 volts in order to obtain 8 to 9 ma. with filter paper 17 inches in width and 16 inches in length and run in methanolic buffer. This lower temperature has given closer reproducibility. It is specially recommended if methanolic buffer is being used.

Experimental results. The third α-globulin was first observed when making quantitative determinations of the protein fractions by eluting them from the electropherograms with 1% NaCl and applying Weichselbaum's biuret method(4) to the eluates. The positions of the fractions were established in the first place by rapidly staining a strip of the paper with Brom Phenol Blue. It was subsequently found that this α_3-globulin may be seen well on papers stained for at least 30 minutes in a saturated solution of Naphthalene Black in 60% methanol and then washed in methanol containing 10% glacial acetic acid. It is less readily seen on papers stained with Brom Phenol Blue because it seems that this dyestuff, when associated with α_3-globulin, is relatively easily washed off the papers. However, papers stained with Brom Phenol Blue may subsequently be restained with Naphthalene Black. We have in this way successfully restained older electropherograms on which the original Brom Phenol Blue had faded quite badly. The α_3-globu-

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