INCORPORATION OF LEUCINE-H^3 INTO SUBNUCLEAR COMPONENTS OF ISOLATED PEA NUCLEI.

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We have developed methods for the isolation in relative pure condition of nuclei from pea embryos and seedlings (Rho et al. 1961) and have shown that such nuclei incorporate labeled amino acids into protein in vitro (Birnstiel et al., 1961a). By elaboration of the methods of Johnston et al., (1959), we have been able to fractionate nuclei into various distinct subcomponents (Birnstiel et al., 1961b), namely: nucleoli, chromatin, ribosomal fraction and nuclear supernatant, each characterized by appearance under the microscope, sedimentation range and chemical composition. We now report the findings of a study in which the above methods have been applied to the investigation of the site of amino acid incorporation within the nucleus. We are particularly interested in differentiating between the behavior of the nucleolus and that of the remaining subnuclear components.

Methods: Nuclei were obtained from 4-day-old pea stems or from embryonic axes of 36-hour-old pea seedlings, by differential centrifugation of a roller-mill-produced homogenate as reported earlier (Rho et al., 1961). The nuclei were then incubated with 50 or 100 μC/ml of D, L-leucine-4, 5-H^3 (3.57 mC/μM) in the presence of a mixture of the other 19 amino acids, and an ATP regenerating system, under the conditions described in the legend of figure 2. The incubated nuclei were next further purified by centrifugation in concentrated sucrose, disrupted by grinding, and fractionated by differential centrifugation as described in the legend of figure 1 (see also Birnstiel et al., 1961b). In this procedure each fraction was examined

under the microscope to determine the presence or absence as well as the abundance of nuclei and of nucleoli. In addition, each fraction was examined analytically for content of DNA, RNA and protein. Well defined fractions containing principally nucleoli, chromatin, etc. were reproducibly obtained as well as intermediate fractions which were physical mixtures of the subnuclear components.

The individual pellets were exhaustively washed by an appropriate and rigorous method in order to remove all tritium activity other than that incorporated into protein. In this procedure, the pellets were washed successively, and in presence of a large excess of unlabeled leucine, with cold TCA, NaOH (1 M at 37°C), hot TCA, and alcohol. Radioactivity was determined with an automatic TRICARB Model No. 314-DC of Packard Instrument Co. and with an ethanol-toluene medium containing 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP).

Results: The data of figure 1 concern the distribution of incorporated label among the subnuclear components of pea embryo nuclei previously incubated in tritiated leucine for 90 sec. at 37°C. The principle incorporation during this short incubation period, with respect to both total activity and specific activity, is by the nucleoli, (Fractions 4 and 5 of figure 1). These two fractions are composed of intact nucleoli, in contrast to fraction 3 which is a mixture of nuclei and nucleoli. The incorporation by other fractions decreases with decreasing abundance of nucleoli and with increasing content of DNA-rich chromatin. Thus the DNA-rich chromatin and the protein-rich nuclear supernatant incorporate but little labeled leucine into protein during a brief incubation period.

The data of figure 2 concern a pulse experiment in which, after 5 minutes of incubation in labeled leucine, a large (1000 fold) excess of unlabeled leucine was added to the nuclear preparation, obtained in this case from tips of young pea stems. The nuclei were incubated for an additional 7, 17, or 40 minutes in unlabeled leucine before being subjected to fractionation. It is clear from the data of figure 2 that during the first

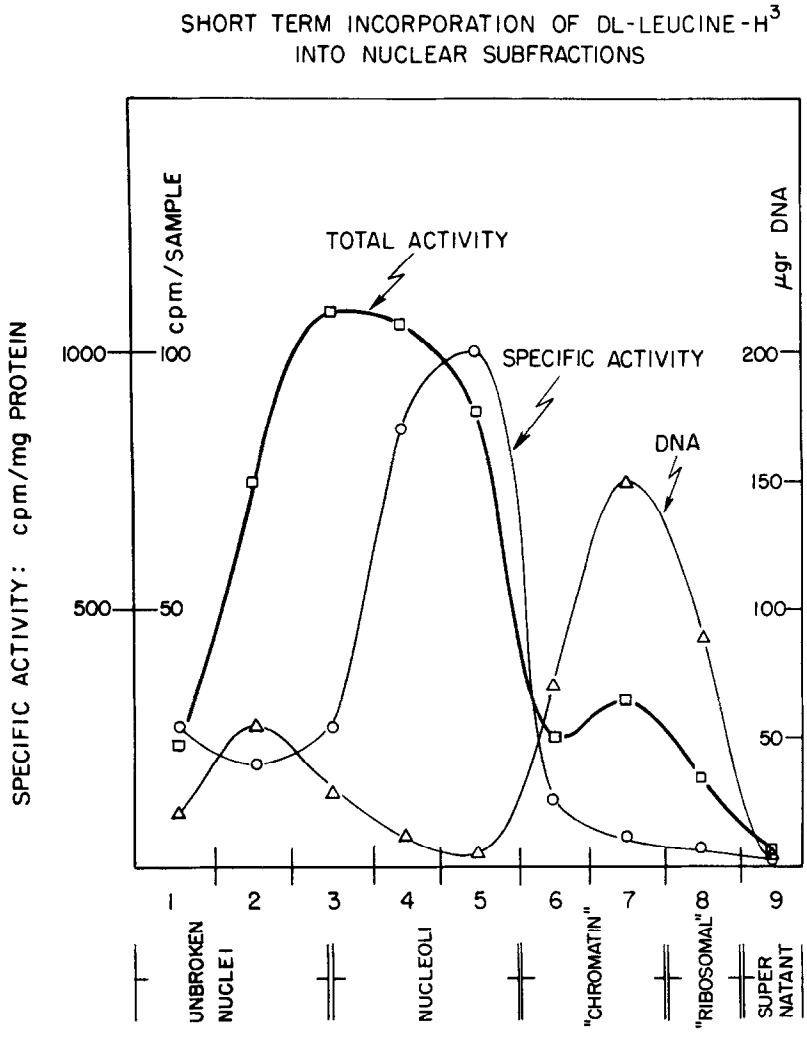

Fig. 1

Incubation for 90 sec at 37°C with 100 μC/ml of DL-leucine-4,5-H^3 under optimal conditions as specified in Fig. 2. The nuclei were purified in a sucrose gradient and the density of the recovered nuclear suspension adjusted to 1.310 gr/cm^3. The nuclei were ground in the presence of 0.5 mM sodium citrate (pH 7.2) in a Omnimix (40 V for 2½ min) and the suspension (1.260 gr/cm^3) was centrifuged differentially (Spinco rotor 25) for 20 min at 1,935 g, 4,340 g, 14,500 g, 30,900 g, 48,200 g and for 2 hrs at 72,000 g (Fraction 1-6). After 5 fold dilution with a solution of 0.01 M KCl, 0.0005 M $CaCl_2$ and 0.002 M tris (pH 7.2) the centrifugation was continued (Spinco rotor 30) for 25 min at 34,850 g and 6 hrs at 78,410 g (Fraction 7 and 8), yielding a supernatant fraction 9.

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7 minutes after addition of the unlabeled leucine the rate of increase of the specific activity of the protein of the nucleoli diminishes, whereas that of the chromatin fraction remains high. Over the longer periods, 17

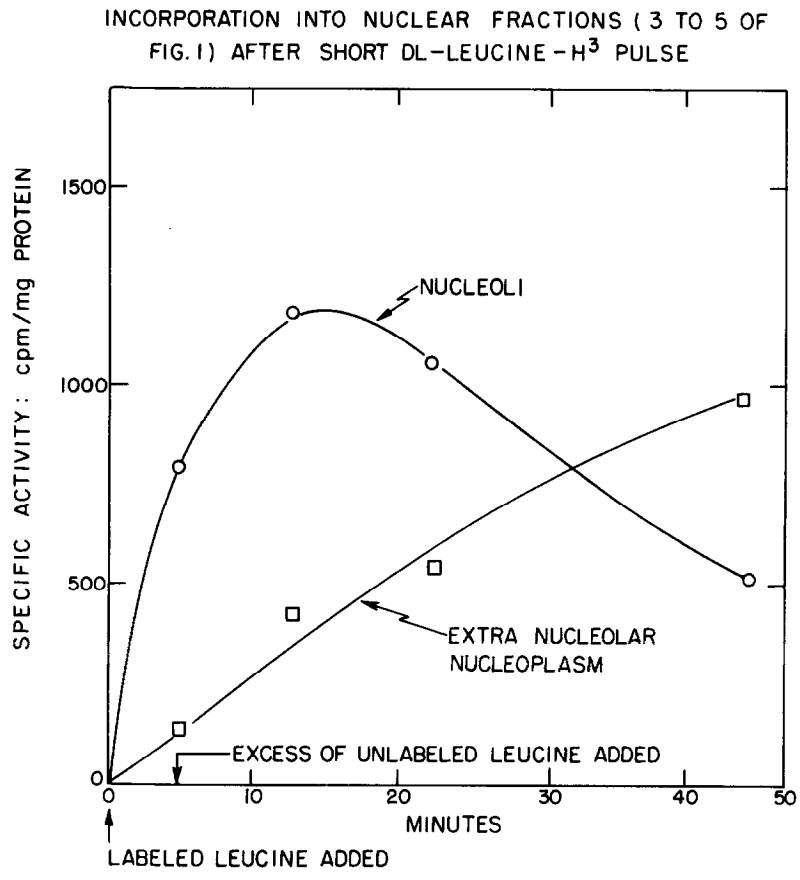

Fig. 2

Incubation under optimal condition: 50μC/ml of DL-leucine-4,5-H^3; 0.003 M $CaCl_2$; 0.2 M sucrose; 0.005 tris; 0.0001 ATP; 0.02 M phosphocreatine, sodium salt; 50 γ/ml creatine kinase; a complete mixture of 19 L-amino acids, 40γ/ml of each except leucine; 50 γ/ml streptomycin; final pH 7.0. Incubation aerobically at 37°C. Fractionation as described in Fig. 1.

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and 40 minutes, the specific activity of the proteins of the nucleoli (in each case microscopically identified) decreases markedly. This is however accompanied by an increase in specific activity of the extranucleolar nucleoplasm; an increase found principally in the DNA-rich chromatin.

Discussion: The washing procedure here used in preparation of samples for counting (Birnstiel et al., 1961a) destroys all sRNA-amino acid complexes and we feel, on this basis as well as on the basis of the observed requirement for the presence of all amino acids, that the leucine incorporated in these experiments represents incorporation into protein or into other

peptide linkage. The results from incubations of short duration such as that of figure 1 and from pulse experiments such as that of figure 2 indicate that the nucleolus is the principal site of amino acid incorporation within the nucleus and further suggest movement of labeled product from nucleolus to the remaining nucleoplasm, principally to chromatin. An exact balance between the amount of activity lost from the nucleolar fractions and the amount gained by the chromatin fraction is not found in the present experiments due to the fact that incorporation into the nucleolar fraction continues for several minutes after addition of unlabeled leucine. This may be in part accounted for by the presence of a considerable pool of sRNA-amino acid complexes, which we know to be present in these nuclei. Although the present results establish the nucleolus as a principal site of nuclear protein synthesis, they do not exclude the possibility of a slower synthesis of protein by the chromatin itself.

The nucleolus as we prepare it from the pea nucleus contains in addition to RNA a significant amount of DNA, (Birnstiel et al., 1961b), apparently as an integral part of its structure. This is in agreement with the work of Peveling (1961) whose electron microscope studies showed that the nuclei of young plant cells contain embedded chromatin material.

The results here presented are in agreement with those of many radioautographic studies, reviewed and summarized recently by Sirlin (1960), which also suggest the nucleolus as a site of nuclear protein synthesis. Our techniques of fractionation and isolation of subnuclear components provide quantitative support of a more biochemical nature for the view that the nucleolus is the principal site of protein synthesis in the nucleus of young cells.

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