The suggested method is superior to those already known in its simplicity of performance and in its informativeness, for the quantity of SEF is expressed in intestinal units/ml. The results can accordingly be subjected to statistical analysis. Furthermore, unlike in other known methods, in the one described above, the effect of general anesthesia and operative trauma on the animal is ruled out. A particularly important feature is that small quantities of test material (not more than 0.5 ml) are required for determination of SEF. With all these advantages, the above method can be widely used for clinical-physiological investigations.

LITERATURE CITED

1. Yu. Ivanov, Kardiologiya, No. 8, 138 (1975).
2. L. J. Barton, L. H. Lackner, F. C. Rector, et al., Kidney Int., 1, 19 (1972).
3. B. M. Brenner, S. L. Troy, and T. M. Daugharty, J. Clin. Invest., 50, 1596 (1971).
4. J. H. Cort, T. Dousa, V. Pliska, et al., Am. J. Physiol., 215, 921 (1968).
5. J. M. Diamond, J. Gen. Physiol., 48, 15 (1964).
6. J. M. Diamond and J. M. Tormey, Nature, 210, 817 (1966).
7. J. Heller, Physiol. Bohemoslov., 10, 167 (1961).

A DOUBLE LABELING METHOD IN ELECTRON-MICROSCOPIC AUTORADIOGRAPHY

A. A. Pal'tsyn and E. G. Kolokol'chikova

UDC 57.087.347

A method of investigation of synthesis of two different substances (RNA and protein) in the same cell is described. Two specific precursors, uridine and proline, each labeled with tritium, were injected into animals. Differentiation of RNA and protein labeling was based on the fact that the biosynthesis of these macromolecules is located in different parts of the cell. Comparison of the results with those of two control experiments in which only one precursor was used showed that the suggested method is suitable for a simultaneous study of RNA and protein synthesis in wound fibroblasts.

KEY WORDS: *autoradiography; wound; RNA metabolism; protein metabolism.*

The double labeling method is used in light-microscopic autoradiography to investigate the metabolism of two different substances in the same preparation or metabolism of the same substance, usually thymidine, but injected on two occasions separated by a definite time interval, so that the fate of the labeled substance can be studied after being present in the body for different periods. Double labeling has widened the scope of autoradiography, for the method can be used to study problems beyond the reach of biochemistry and of ordinary autoradiography. To identify cells labeled at the first and second injections of thymidine, the labeled amino acid is given in sharply different doses, and the density of the tracks is then studied [4, 7]. When two precursors are injected they are labeled with different isotopes, usually ^{3}H and ^{14}C. Radioactivity of ^{3}H and ^{14}C is identified in sections coated with a double [2, 3] or a thick single [6] layer of emulsion. These methods of differentiating the two labeled substances are unsuitable for electron-microscopic autoradiography and, for that reason, no method of double labeling suitable for use with it has hitherto been described.

The object of this investigation was to determine the possibility of studying DNA and protein synthesis in granulation tissue cells by injecting two precursors. The basis for this investigation was the following arguments. RNA synthesis takes place in the cell nucleus; the cytoplasm, except the mitochondria, does not produce RNA, at least in quantities sufficient to be detected by methods of investigation used. Protein, however, is synthesized

Department of Pathological Anatomy, A. V. Vishnevskii Institute of Surgery, Academy of Medical Sciences of the USSR, Moscow.

(Presented by Academician of the Academy of Medical Sciences of the USSR M. I. Kuzin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 86, No. 11, pp. 626-629, November, 1978. Original article submitted January 16, 1978.

0007-4888/78/8611-1550$07.50

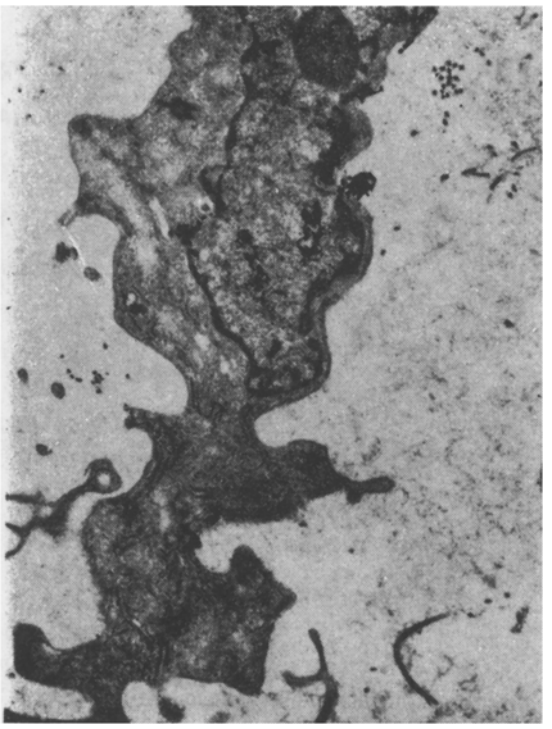

Fig. 1. Electron-microscopic autoradiograph of fibroblast fixed 1 h after injection of uridine-5-^{3}H (control group 1). Grains of silver reflecting RNA synthesis located above nucleus (21,000×).

chiefly in the granular endoplasmic reticulum and on the ribosomes of the cytoplasm, although a certain amount of protein is also formed in the cell nucleus. Consequently, if the cell is fixed soon after the single injection of tritiated precursors of RNA and protein, labeling of the extra mitochondrial part of the cytoplasm must reflect protein synthesis only, and labeling of the nucleus predominantly RNA synthesis, although a certain number of grains of silver above the nucleus may be accounted for by intranuclear protein synthesis. With an increase in the pulse, i.e., in the interval of time between injection of the precursor and fixation of the cell, the likelihood of clear differentiation between RNA label (above the nucleus) and protein label (above the cytoplasm), becomes theoretically smaller and smaller. The migration of RNA, synthesized in the nucleus, into the cytoplasm and migration of protein from cytoplasm into the nucleus [5] lead to mixing of the protein and RNA labels in the two parts of the cell and it becomes impossible to distinguish them.

EXPERIMENTAL METHOD

A wound of skin and muscle with a tissue defect measuring 3 × 3 × 3 mm was inflicted on four mice, and seven days later a mixture of precursors was injected subcutaneously into the animals: uridine-5-^{3}H in a dose of 4 μCi/g (specific activity 22.5 Ci/mmole) and proline-3,4,5-^{3}H in a dose of 20 μCi/g (specific activity 115 mCi/mmole). In the two control groups, animals with identical wounds received either uridine-5-^{3}H only or proline-^{3}H only, in the same doses. Tissue taken from the wound 1 h after injection of the precursors was fixed with 2.5% glutaraldehyde solution in phosphate buffer, pH 7.4. The tissue was then washed for 24 h with phosphate buffer, postfixed with 1% OsO_4 solution, and embedded in Epon. To begin with an autoradiographic investigation was made of semithin (1-2 μ) sections stained with toluidine blue and azure. An area containing granulation tissue cells was chosen under the light microscope, and a pyramid was cut out from this area for ultrathin section cutting. Electron-microscopic autoradiographs were prepared by the method described by Sarkisov et al. [1].

EXPERIMENTAL RESULTS

In the animals of the control group receiving uridine only, mainly the nuclei of the granulation tissue cells were labeled. Highest activity of RNA synthesis was observed in

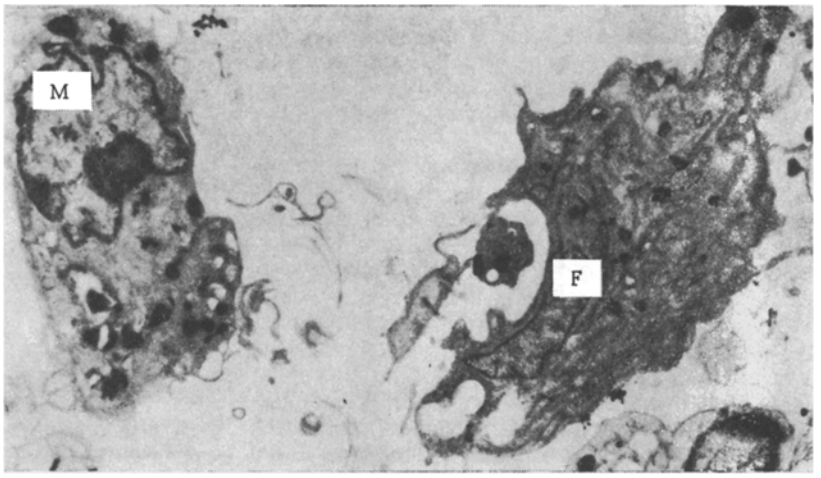

Fig. 2. Electron-microscopic autoradiograph of fibroblast (F) and macrophage (M) fixed 1 h after injection of proline-^{3}H (control group 2). Many grains of silver indicating active protein synthesis concentrated above cytoplasm of fibroblast; nucleus of fibroblast not labeled; a few grains of silver above cytoplasm of macrophage reflect weak incorporation of proline-^{3}H into it (11,000×).

macrophages and fibroblasts (Fig. 1). Single grains of silver were found above the cytoplasm of these cells. After injection of proline only into the animals a high concentration of silver grains was observed only above the cytoplasm of the fibroblasts. Single grains of silver were found above some fibroblast nuclei, but many nuclei were not lableed at all (Fig. 2). As regards macrophages, the control tests with uridine showed very intensive RNA synthesis in them. Since the only function of RNA so far known is its participation in protein synthesis, it can be concluded from the uridine uptake that macrophages produce large quantities of protein. However, these cells incorporated little proline. This can be explained on the grounds that proline is used mainly for the synthesis of collagen, and other proteins contain only small amounts of it. This is confirmed by the weak proline labeling of fibroblast nuclei, where noncollagen proteins are synthesized. Consequently, the autoradiographic data indicate the relatively high specificity of proline as a collagen precursor.

This specificity could be seen particularly clearly when autoradiographs of experimental animals receiving both precursors were examined. A fibroblast and macrophage are shown side by side in Fig. 3. The distribution of the grains of silver in these cells is so different that it would seem that different substances had been introduced into them; in the fibroblast the label was concentrated almost entirely in the cytoplasm, but in the macrophage in the nucleus. The difference is explained on the grounds that these two cells, in accordance with the specific character of their metabolism at the time of investigation, "extracted" different substances from the mixture of precursors. The macrophage incorporated mainly uridine, for the proteins synthesized by it contained little proline; a fibroblast with intensively developed granular endoplasmic reticulum, present in the section, evidently was in a stage of active collagen production and was labeled chiefly with proline. Besides the type of localization of label described in the fibroblast, after injection of a mixture of precursors many cells were seen in which the label was more uniformly distributed and in which many grains of silver were present both above the nucleus and above the cytoplasm.

After simultaneous injection of RNA and protein precursors the distribution of label in cells such as fibroblasts thus differed significantly from pictures observed after injection of each precursor separately. To determine the statistical significance of these differences, the ratio between the densities of label above the nucleus and above the cytoplasm in the experimental group was compared with each of the controls. For this purpose, the number of grains above the nucleus and cytoplasm of the cell was counted on the negatives and the area of cross section of the nucleus and cytoplasm measured in conventional units (the product of the length and width in millimeters). The density of label was determined by dividing the number of grains of silver above the given part of the cell by its area of cross section.

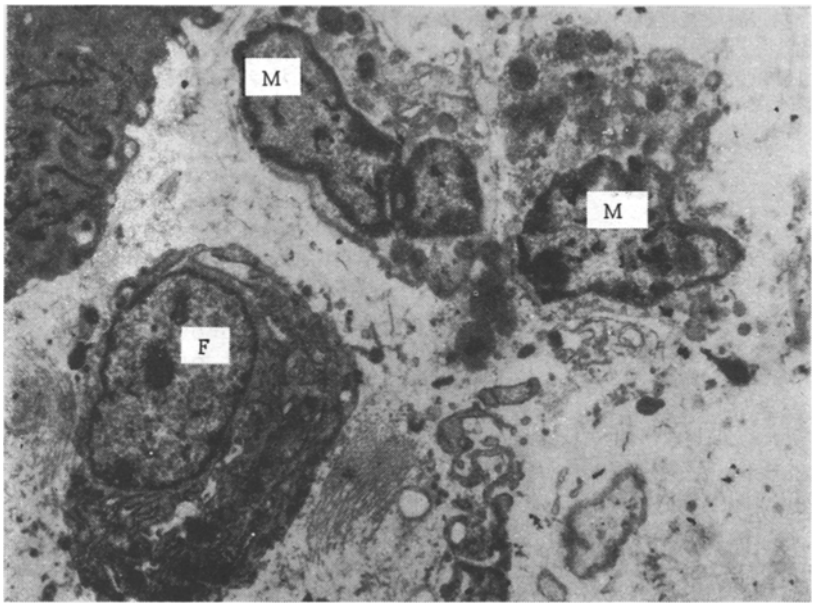

Fig. 3. Electron-microscopic autoradiograph of fibroblast (F) and macrophages (M) fixed 1 h after injection of mixture of uridine-5-^{3}H and proline-^{3}H (experimental group). Label in fibroblast contained mainly in cytoplasm, but in macrophages mainly in nucleus (12,000×).

The following assumptions were made for the statistical analysis. The autoradiographs obtained by double labeling are suitable for simultaneous study of synthesis of two substances only if they differ significantly from each group of control sections. The comparison was therefore made under definite conditions, for 10 randomly chosen cells (fibroblasts) were taken for analysis from the experimental group and each of the control groups. The ratio between densities of nucleus and cytoplasm in the control with proline was between 0 and 1, in the control with uridine between 8.5 and infinity, and in the experimental group between 0.57 and 5. Comparison by means of Wilcoxon's test showed that the difference between the experimental results and the results of the two controls was statistically significant ($P < 0.01$). Consequently, under the conditions suggested it is possible to investigate the synthesis of RNA and protein (collagen) simultaneously in fibroblasts. There is no doubt that the principle of the suggested method, based on the difference in the intracellular localization of biosynthetic processes, can also be used for the simultaneous study of metabolism of other substances and in other types of cells.

LITERATURE CITED

1. D. S. Sarkisov, A. A. Pal'tsyn, and B. V. Vtyurin, Adaptive Reorganization of Biorhythms [in Russian], Moscow (1975).
2. R. Baserga, J. Cell Biol., 12, 633 (1962).
3. E. O. Field, K. B. Dawson, and J. E. Gibbs, Stain Technol., 40, 295 (1965).
4. P. P. Galand, P. Mainguet, M. Arguello, et al., J. Nucl. Med., 9, 37 (1968).
5. L. Goldstein, in: The Cell Nucleus, (ed. by H. Busch), Vol. 1, New York (1974), p. 387.
6. S. W. Perdue, R. F. Kimball, and A. W. Hsie, Exp. Cell Res., 107, 47 (1977).
7. G. Willems, P. Galand, and J. Chrétien, Lab. Invest., 23, 635 (1970).