sured from the rate of incorporation of ^{3}H-thymidine, was retarded in the cells of these colonies, further confirmation of their particular biological features. Cells of macrocolonies weighing over 5 mg possessed enzyme activity and also a rate of DNA synthesis close to those in control areas of the mucosa. Colonies of this type constituted the overwhelming majority, and it is therefore likely that when the functional characteristics of the mucosa as a whole are studied, their indices may mask the functional indices of the small number of highly differentiated cells of "small" colonies (under 5 mg). The existence of functional heterogeneity of the epitheliocytes of the mucosa evidently has definite biological significance, for it endows it with greater resistance to the action of unfavorable factors. For example, in radiation injury to the intestine, cells with lower mitotic activity will be more radioresistant and will ensure the more rapid recovery of the mucosa after irradiation. The cause of the "decrease" in proliferative activity of the epitheliocytes of the mucosa during prolonged fractional irradiation of the abdominal region may perhaps be the greater survival rate of the slowly proliferating cells of "small" macrocolonies [3].

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METABOLISM OF ERYTHROCYTIC CHALONE IN VITRO

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It was shown previously that the chalone activity of erythrocyte extracts is associated with protein fraction 1 [2].

In the investigation described below the metabolism of this fraction was studied during culture of bone marrow cells.

EXPERIMENTAL METHOD

Fraction 1 was isolated from erythrocyte extracts and concentrated to 11 mg/ml by the method described in [2]. The isolated protein, corresponding to a single peak on the densitogram, was conjugated with fluorescein isothiocyanate (FITC, from Serva, West Germany), using 0.5 M carbonate buffer, pH 9.5, for this purpose [3]. The labeled protein was separated from the unbound fluorochrome on a column (1.2 × 30 cm) packed with Sephadex G-25 and reconcentrated to the original volume. The labeled protein was added to bone marrow cells isolated from the femors of mice, pipeted in Hanks' solution, and centrifuged for 15-20 min at 1000 rpm. The sedimented cells were suspended in Hanks' solution and poured in equal numbers into centrifuge tubes. Bone marrow from 20 noninbred albino mice weighing 18-20 g was used. A mixture of cells from five animals corresponded to each experimental point.

The following series of experiments were carried out with the cell suspensions thus obtained.

Series I. Labeled protein was added to the myelokaryocytes at the rate of 0.05 ml to 2 ml of suspension. After 1 min the cells were shaken, washed 3 times with cold Hanks' solution to remove unbound protein, and

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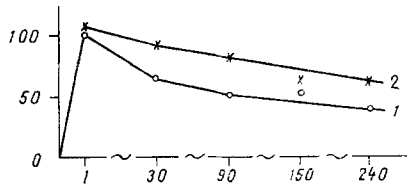

Fig. 1. Intensity of fluorescence of bone marrow cells after incubation for various times. Abscissa, incubation time (in min), ordinate, intensity of fluorescence of myelokaryocytes (in %); fluorescence of bone marrow cells after incubation with labeled protein for 1 min taken as 100%. 1) Results of experiments of series I, 2) results of experiments of series II.

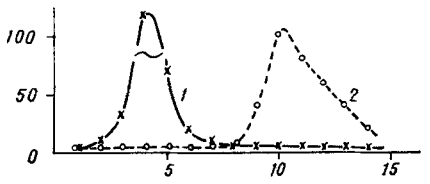

Fig. 2

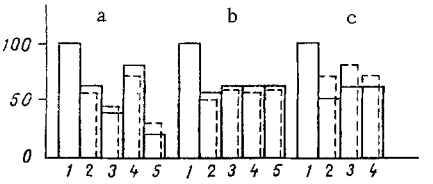

Fig. 3

Fig. 2. Intensity of fluorescence in different samples of labeled protein (1) and supernatant of cells after incubation for 4 h (2) after their separation on Sephadex column. Abscissa, nos. of samples collected; ordinate, intensity of fluorescence (in conventional units based on deflection of microammeter pointer). Elution with physiological saline at the rate of 15 ml/h, Sephadex G-25.

Fig. 3. Intensity of fluorescence of myelokaryocytes after treatment of cells with trypsin and exposure to EP and EC. Ordinates, intensity of fluorescence (in %; fluorescence of cells after incubation with labeled protein for 1 min taken as 100%). a: 1) control, 2, 3) fluorescence of cells and supernatant respectively after their incubation with labeled protein for 1 min and treatment with trypsin, 4, 5) the same, but after treatment with trypsin after incubation of labeled cells for 30 min; b: 1) control, 2, 3) fluorescence of cells after preliminary exposure to EP or EC respectively, 4, 5) fluorescence after repeated exposure to erythrocytic chalone after incubation for 1-2 h; c: 1) control, 2, 3, 4) fluorescence of cells after preliminary treatment with trypsin, before and after incubation for 1-2 h respectively. Results of experiments of series II shown by broken lines.

the fluorescence of the cell suspension was measured before and after incubation of the cells in Hanks' solution for 30 min and 1.5, 2.5, and 4 h. The supernatant obtained by centrifugation of the myelokaryocytes after incubation for 4 h was separated on a column packed with Sephadex G-25 and the fluorescence of the samples was measured. Fluorescence of samples of labeled protein after elution on the column under the same conditions was recorded for comparison.

Series II. Myelokaryocytes were labeled with fraction 1 for 1 min, washed to remove unbound protein, and treated for 30 min with cold (4°C) 0.25% trypsin solution made up in Hanks' solution without Ca ions. Similar experiments were set up with labeled cells, which were incubated for 30 min in Hanks' solution at 37°C and treated with trypsin. The myelokaryocytes were sedimented by centrifugation, washed to remove trypsin, and the fluorescence of the cell suspension and supernatant was measured.

Series III. Cold 0.25% trypsin solution was added to the myelokaryocytes for 30 min, after which the cells were washed free from trypsin, diluted in Hanks' solution, and half was poured into each of two centrifuge tubes. The intensity of fluorescence of the cells treated with trypsin was measured after the end of trypsin treatment, and after incubation for 1 and 2 h at 37°C. For this purpose, labeled protein was added at appropriate intervals to the cell suspension for 1 min, after which the cells were washed to remove the unbound label and the cell suspension was subjected to spectrofluorometry.

Series IV. Rat serum enriched with erythropoietin (EP) as a result of acute blood loss, or extracts of rat erythrocytes containing chalones (EC) were added to the myelokaryocytes at the rate of 0.5 ml to 2 ml of the cell suspension for 1 min, the cells were washed by centrifugation to remove unbound EP or EC, and then suspended in Hanks' solution. Labeled protein was then added to the suspension of myelokaryocytes for 1 min immediately after their suspension in Hanks' solution or after incubation of the cells for 1-2 h at 37°C. After removal of unbound labeled protein from the cells their fluorescence was measured on a double monochromatic spectrofluorometer at excitation wavelength 435 nm and fluorescence wavelength 522 nm. Unlabeled myelokaryocytes did not give fluorescence in Hanks' solution at these parameters.

EXPERIMENTAL RESULTS

It will be clear from Fig. 1 that labeled protein binds in vitro with bone marrow cells as early as after 1 min, evidently because of the presence of specific receptors on their surface. With lengthening of the incubation periods, degradation of the absorbed protein fraction 1 and its transfer into the incubation medium were observed. Comparison of peaks of fluorescence of labeled protein and supernatant of cells incubated for 4 h showed that labeled protein bound with the cells is degraded to low-molecular-weight compounds (Fig. 2).

Degradation of the labeled protein by myelokaryocytes took place intracellularly and not extracellularly. This is shown by the following observations. Treatment of the labeled cells with trypsin removed part of the label from the surface of the cells and transferred it into the supernatant (Fig. 3a). If the labeled cells were incubated for 30 min and then treated with trypsin, a much smaller fraction of label was found in the supernatant and more of it inside the cells. These facts suggest that the test erythrocytic protein, after binding with the outer receptors of the membranes for a short time, penetrates inside the cell, where it is degraded and eliminated from the cells into the surrounding medium in the form of protein fragments.

It will be clear from Fig. 3b that the receptors for EP and EC are evidently the same, for otherwise a decrease in the intensity of fluorescence of the cells would be observed only after addition of EC. Despite the fact that after incubation of the myelokaryocytes for 30 min protein had entered the cells, the liberated receptors were not ready to bind fresh batches of protein after incubation of the cells for 1-2 h. This may be due to partial degradation of the receptors together with the assimilated protein molecules and to the impossibility of their resynthesis de novo within the specified time. The results are confirmed by those of another series of experiments (Fig. 3c). If myelokaryocytes are treated with trypsin they lose their ability to bind with labeled protein, evidently because of destruction of the receptor sites. Incubation of such cells for 1-2 h did not lead to any significant recovery in the number of receptors on the surface of the myelokaryocytes. Evidently this period of time is insufficient for their resynthesis or restoration in vitro.

The results are in agreement with those of investigations by other workers [4] who studied the metabolism of epidermal growth factor in cell culture. On the basis of a comparison of these results it can be postulated that the metabolism of growth stimulators [4] and also of inhibitors of proliferation, such as chalones, shares a number of common general principles. According to data in the literature [4], degradation of epidermal growth factor is an energy-dependent process and is suppressed by protease inhibitors. The ability of cells to respond a second time to the action of epidermal factor is not exhibited until after 10-12 h. During that period the injured receptors are evidently resynthesized. The present writer also showed previously that restoration of mitotic activity of bone marrow cells in vivo is observed 10 h after administration of chalones [1], and evidently after that moment the cells are capable of responding a second time both in vitro and in vivo.

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