USE OF A COLORIMETRIC TECHNIQUE FOR RHODAMINE 6G FOR FREE FATTY ACID ASSAY IN BIOLOGICAL MEMBRANES

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An advantage of the method of determining the concentration of free fatty acids (FFA) by means of the cationic dye rhodamine 6G, suggested previously [2, 4], over other colorimetric methods used for this purpose [1, 3] is its high sensitivity, so that a small volume of biological material (about 1 mg protein per sample) can be used. At the same time, the method is extremely sensitive to cleanliness of the glassware and to the presence of impurities in the extract at the stages of evaporation and assay.

It was accordingly decided to use silica gel to adsorb phospholipids [5] and other components from the extract, and plastic vessels for evaporation. The method was used by the writers to determine the FFA level in membranes of erythrocytes, mitochondria, and the apical part of enterocytes. From 0.5 to 3 mg biomembrane protein in 0.1-1.0 ml of aqueous medium per sample was used in duplicate tests. FFA were extracted twice with pentane or with the low boiling point fraction of petroleum benzine, 5 ml at a time, for 10 min with vigorous shaking on a "Vortex" apparatus (from Searle, The Netherlands). The pooled extract was transferred to glass test tubes or flasks with a capacity of 20 ml. To remove phospholipids, 200-300 mg of activated silica-gel was added. The sample was centrifuged for 5 min at 1000-3000g, after which the extract was collected and transferred to polyethylene tubes. The pentane extract was evaporated (to dryness) in air, whereas the petroleum benzine extract was evaporated in vacuo. Next 1 ml of 0.005% rhodamine 6G in benzene was added as described in [2]. The solution of rhodamine 6G (0.005%) was made up as follows: 5 mg of dye was dissolved in 5 ml of 0.2 M phosphate buffer, pH 11.0-12.0, extracted in darkness with 100 ml benzene with vigorous shaking on a magnetic mixer for 3-5 min. The solution of the dye in benzene was transferred to a dark glass vessel and used for work for a period of several months. Optical density was measured on a Beckman-25 spectrophotometer (Austria) against 0.005% rhodamine 6G.

The differential absorption spectrum of the rhodamine 6G–FFA complex (6:1) have characteristic maxima of absorption at wavelengths of 515 and 550 nm. The error of the method did not exceed 10-20% in the case of measurement at 515 nm. Using a small quantity of material, the differential absorption spectrum was recorded in the region 480-600 nm. Stearic acid was used to plot calibration curves (Fig. 1). The results of determination of the FFA level in the different biological membranes (in nmole/mg protein) were as follows: in mitochondria 10.8 ± 3.4 (11 samples), in light mitochondria 7.8 ± 1.1 (11 samples), in human erythrocytes taken from inhabitants of Moscow and Magadan 10.6 ± 1.3 (58 samples) and 27.6 ± 2.3 (124 samples) respectively, and in enterocytes of the brush border 12.4 ± 1.7 (15 samples).

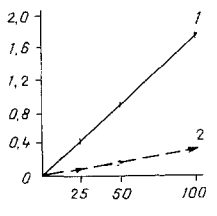

Fig. 1. Calibration curves for stearic acid with rhodamine 6G. Abscissa, optical density; ordinate, FFA concentration (in nmole/ml): 1) at 515 nm, 2) at 550 nm.

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DETECTION AND ISOLATION OF ANTIBODY-FORMING CLONES BY LOCAL CYTOLYSIS IN GEL

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To select clones of hybridomas producing monoclonal antibodies (MCA) of required specificity, many different methods have been suggested. However, all, as a rule, are based on testing culture fluid taken from wells in plastic plates, in which the hybridomas are grown. Cultures from positive wells are cloned in semisolid medium [4] or by the finite dilutions method [5], after which screening was repeated. Combining two stages – screening and cloning – into one would evidently result in considerable economies of time, medium, and plastic ware. Köhler and Milstein [6] suggested a method of local hemolysis in gel for detecting hybridoma clones forming antibodies against sheep's red blood cells. We have extended the scope of this method by conducting the reaction in a monolayer by the method suggested previously [2].

A variant of the method whereby nonerythrocytic targets can be used was described previously.

Hybridoma of strain NATF9.9 (F9), obtained by the writers previously and synthesizing IgM MCA against differential antigen Lyt-3.2 of mouse T lymphocytes [1] was used. This allele of the antigen (2nd) is represented on 80% of thymocytes and on some peripheral T cells of CBA and other lines of mice, but is absent in C58 and AKR mice. Line F9 was cultured in vitro for 2 months before the beginning of the experiment.

Before testing, the hybridoma cells and thymocytes were washed 3 times in medium 199 to remove protein, including MCA contained in the culture medium, by centrifugation at 100g for 5 min, then resuspended in medium without serum and kept until required in the experiment in an ice bath. The viability of the cells was estimated by determining incorporation of trypan blue in a Goryaev chamber. It was not less than 95%. Testing and subsequent culture were carried out in plastic Petri dishes 30 mm in diameter (Flow Laboratories, England). The dishes were washed to remove free PLL, and seeded with a suspension of hybridoma cells (10^3 per dish). After about 10 min, the suspension of thymocytes was added to the dishes. After adhesion of the cells the supernatant was drawn off and the mixed monolayer of hybridoma cells and thymocytes was covered with 0.5 ml of 0.3% agarose (from Calbiochem, USA) on medium RPMI-1640 (Flow Laboratories) with 20% fetal calf serum (N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR). After the agarose had solidified, a layer about 5 mm thick was formed. In the other variant, the target cells were added together with agarose. The dishes were incubated in a CO_2 incubator for 1, 2, or 3 h. Next, rabbit complement (from Cedarlane, Canada), diluted in Hanks' solution 1:15, was layered above the agarose and the dishes were incubated for a further 1 h. Zones of cell lysis around hybridoma cells producing MCA were detected by adding a 0.01% solution of the fluorescent dye ethidium bromide (from Serva, West Germany) in a dose of 10 μl to the dish 15 min berore the end of incubation [3]. The liquid above the agarose was drawn off and the dishes ex-

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