IMMUNODIFFUSION ANALYSIS OF PROTEINS SYNTHESIZED IN A SINGLE COLONY OF SPLENIC HEMATOPOIETIC CELLS

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A method of immunodiffusion analysis of proteins produced by a single splenic colony is described.

Immunodiffusion analysis [2, 3] occupies a foremost place in the analytical chemistry of proteins. This method requires no special manipulations to purify the individual proteins, which would be impossible in practice with small objects, and it enables the individual proteins to be analyzed in a mixture containing commensurate concentrations of other proteins.

In the investigation now described an attempt was made to use this method of analysis to investigate single splenic colonies, because the contents of the colonies are a refined material (erythroid or myeloid branches of the hematopoietic system) [1, 5]. The volume of the colonies is very small, and the "useful" volume of the contents on the 10th day after transplantation does not exceed 0.005 cm^3. For this reason the sampling and analysis of these contents present considerable technical difficulties.

To study protein synthesis in vitro the cells of the colonies had to be incubated with C^{14}-amino acids, and for this purpose a drop of the contents of the colonies had to be applied to the cellophane membrane of a Steiner–Anker glass cell. The Steiner cell was modified to enable this to be done.

Modification of the Steiner–Anker Cell

Usually a Steiner cell with covered top compartment [4] was replaced by a modification in which a ring with two flanges was placed on top (Fig. 1, 1). A cellophane membrane is drawn tight over the bottom flange and this is placed on the lower compartment containing medium No. 199, while the upper flange is left uncovered during work, but is covered by a glass plate during incubation only.

Taking Material from a Column

Two methods were devised for taking material from the colonies: 1) aspiration with a micropipet; 2) expression of the contents directly on to the cellophane membrane of the Steiner cell.

1. Aspiration of Material by a Micropipet. The first attempts to aspirate material from a colony with a simple micropipet 100 μ in diameter by puncturing the colony were unsuccessful. Observations with a stereoscopic microscope showed that the edges of the puncture were firmly pressed against the pipet. In this way a vacuum was formed inside the pierced colony, there was no pressure drop between the inside of the pipet and the space inside the colony, the contents of the colony could not be drawn into the pipet. To overcome these difficulties, a double-barreled pipet was made, the second bore of which communicated with the atmosphere. As a result, at the moment of aspiration, when a negative pressure was formed in the first bore, the contents of the colony were forced up into the first bore by the pressure difference.

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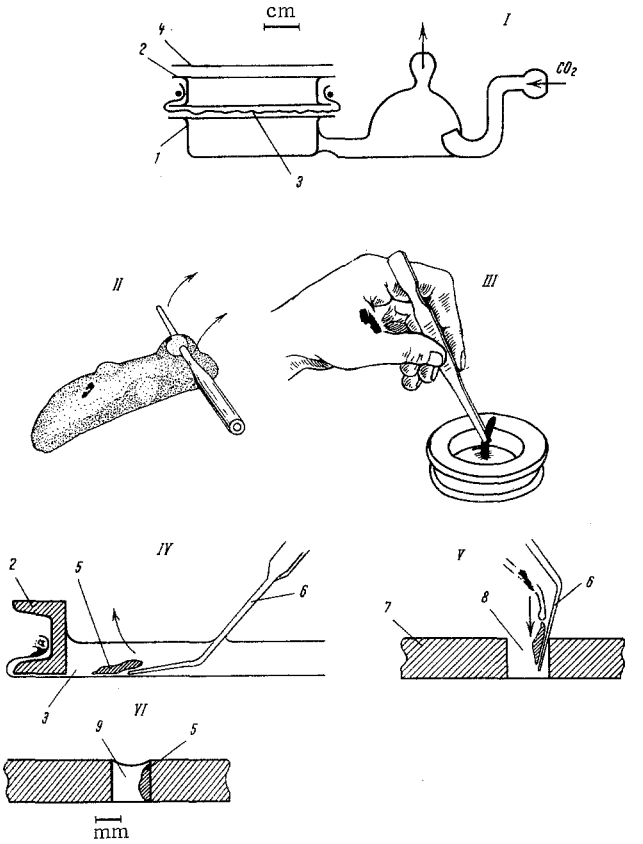

Fig. 1. Diagram showing the preparatory operations. I) Modified Steiner–Anker cell; II) technique of puncturing colony; III) application of material to cellophane; IV) removal of solidified agar drop containing test material from cellophane; V) introduction of solidified drop into well; VI) pouring further agar into the well to join the drop to the whole layer of agar; 1) bottom compartment of cell; 2) top compartment; 3) cellophane membrane; 4) lid of cell; 5) solidified drop with material; 6) spatula; 7) main layer of agar; 8) well in agar layer; 9) well filled with agar.

The manufacture of the double-barreled pipet presents some difficulties and it can be done only if the operations are carried out in a definite order: 1) two capillary tubes with an internal diameter of 1.5 mm and walls 1 mm thick are bent to a right angle, each tube separately; 2) the tubes are soldered together by their short arms and their long arms are kept at an angle of 20-30°; 3) an additional piece of glass tube is soldered to the end of the double-barreled arm of the pipet thus formed and it is drawn out into a double-barreled tip. Pipets whose external tip diameter is 200-300 μ were chosen; 4) one of the arms is broken off: this usually takes place spontaneously, and always at the bend.

2. Taking Material from the Colony by Expression. Experience of work with colonies showed that the other method is better. On the 10th day after transplantation the colony is pierced with a glass needle through the side, holding the needle parallel to the spleen surface. After puncture, an upward jerk is made with the needle. The dome of the colony is torn. After pressure from the sides with ophthalmic forceps, a droplet of fluid is expressed from the colony. The contents of erythroid colonies are usually fluid, while those of myeloid colonies are crumbly or porridge-like in consistency. This drop is applied, in turn, to the cellophane membrane of the Steiner–Anker cell, into which mineral oil is immediately poured, and to a drop of isologous serum or, better still, blood of an irradiated mouse (2-3 mm^3) on a slide, to make films for cytological investigation, blood or serum being taken as medium. The material applied to cellophane may become macerated because of the hygroscopic properties of the cellophane, and to prevent this the

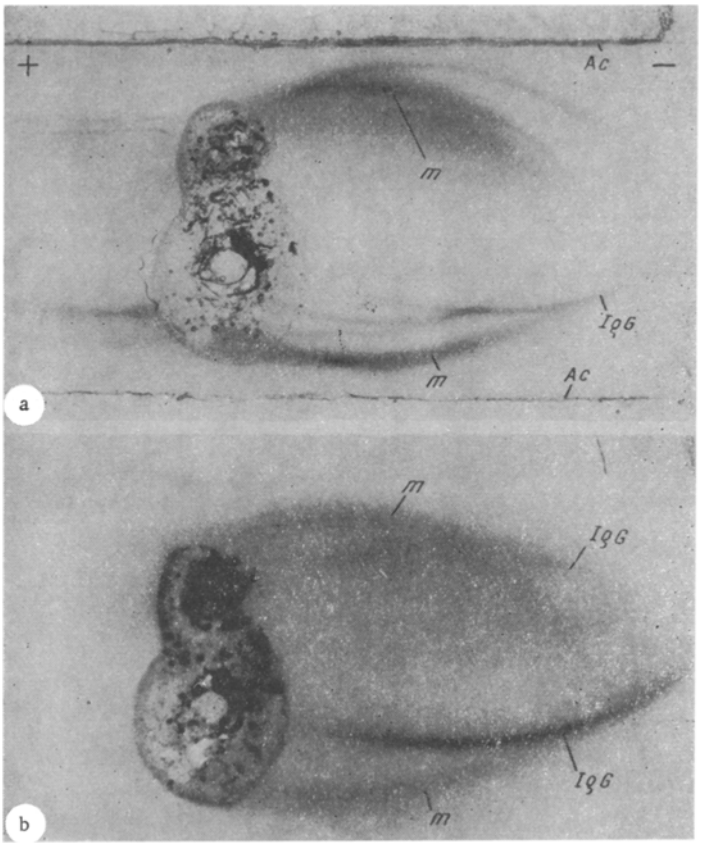

Fig. 2. Immunoelectrophoresis of contents of a single splenic colony (a) and autoradiograph of the same preparation (b). IgG) Immunoglobulin IgG; m) transferrin; Ac) gutter with antiserum. Line IgG is clearly seen on the autoradiograph (b) because of intensive synthesis, but it is not visible everywhere on the stained specimen (a).

cellophane is first moistened with medium No. 199. Material from the colonies on the 8th day after transplantation cannot in general be expressed, for it is compact, and it must be excised with a microscalpel by the method of Curry and Trentin [1]. After the operations described above, the upper compartment of the cell is connected to the lower, which is filled with medium No. 199 with the addition of C^{14}-glycine (1μCi/ml medium). Incubation proceeds for 24 h, and the material is then kept in a frozen state until the cytologist's report on the cell composition of the films made from individual colonies is received. Material is then taken for immunoelectrophoresis.

Taking Material for Immunoelectrophoresis

When the drop applied to the cellophane begins to be aspirated from beneath the layer of mineral oil, usually there is a considerable loss of material, and often none can be aspirated at all. To withdraw the material more completely, the top compartment of the Steiner cell is placed on a hot-plate. About 1 mm^3 of melted agar is introduced by a micropipet, connected to a micrometer mechanism, into the drop. To prevent the agar from solidifying, mineral oil is first drawn up into the pipet and the pipet itself is warmed. After introduction of agar into the drop, the system is cooled. The mixture of agar with the cell contents of the colony, solidified into a mass, is taken off with a pointed spatula (the mass weighs about 1-1.5 mg), rinsed with petroleum ether to remove the mineral oil, introduced into ordinary wells in the main layer of agar, and electrophoresis is then carried out. Further agar is poured into the well to connect the solidified mass with the rest of the layer of agar.

Parameters of Electrophoresis

A 1% agar gel in veronal buffer, pH 8.6, 0.05 M, is used. Agar (17 ml) is poured on to a glass plate measuring 9×12 cm. The duration of electrophoresis is 35 min and the potential gradient 5 V/cm. All the remaining technical details are as for oridnary electrophoresis. After immunoelectrophoresis the agar is dried and stained with amido black, exposed with photographic plates (for the isoorthochromatic plates with a sensitivity of 90 State Standard Units the exposure is 3-6 months), and developed in a peak developer such as D-76. A typical autoradiographic picture as described by Hochwald and Thorbecke [2] is obtained: very clear arcs corresponding to components which are synthesized by that tissue in vitro, and absolutely empty where there are arcs (even intense ones) corresponding to proteins present as impurities (Fig. 2). It is difficult to wash out the serum proteins, and in the present case it was impossible. In this way, immunodiffusion analysis of splenic colonies weighing not more than 1 mg, with a content of individual proteins in the sample of about 10 μg, has been carried out for the first time.

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