Short Communications

Antigenicity of Histones

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Since the proposal by Stedman & Stedman (1950) that histones regulate gene activity, attempts have been made to demonstrate chemical and biological differences between histones of various tissues and species. Obviously, antigenic analysis might throw more light on this problem. Although immunological techniques are increasingly used for the characterization of proteins, immunological studies of histones have as yet not given clear-cut results. This appears to be due to the fact that histones, isolated by conventional methods, are either weak antigens or are not antigenic (Black, Ansley & Mandl, 1964; Wilson *et al.* 1966).

In this laboratory Sluyser, Thung & Emmelot (1965) have developed a procedure for isolating histones from various tissues, employing brief heat treatment (5 min. at 80°) of these tissues, before extraction. Heat treatment inactivates the proteolytic enzymes and thus breakdown of the histone is diminished. The histones obtained in this way from rat liver are strongly antigenic when injected into rabbits. In our initial experiments, one rabbit was immunized by intramuscular injections of 10 mg. of lysine-rich histone (fraction 1) in 1 ml. of 0.15 M-NaCl, emulsified with 1 ml. of complete Freund's adjuvant (Freund, 1947) obtained from Difco Laboratories, (Detroit, Mich., U.S.A.). Three intramuscular injections (10 mg. each) were given at 1-week intervals and arterial blood was drawn from the ear 3 weeks after the last injection. In a later experiment even a total of 3 mg. of lysine-rich histone incorporated in adjuvant, of which the first 1 mg. was injected into the footpads, was found to be sufficient to elicit a good antibody response. Another rabbit was immunized, according to the first-mentioned procedure, with lysine-poor rat-liver histone (fraction 2a + 3). However, this antiserum was rather weak, and therefore the schedule was repeated 5 weeks after the last injection and serum of blood drawn about a month after the second course was used for most experiments. Gel double-diffusion tests were performed in 1.5% agar or agarose in buffered saline on slides (5 cm. × 5 cm.);

the wells were either 4 mm. in diameter or, for weak antisera, 9 mm. in diameter and 6 mm. apart edge-to-edge. Immunoelectrophoresis was performed according to the micro method of Scheidegger (1955) and the indirect immuno-fluorescent antibody technique of Weller & Coons (1954). Rat-liver homogenates were prepared in 0.15 M-NaCl in a glass homogenizer with the pestle rotated at 500 rev./min. during 2 min. (3 ml. of 0.15 M-NaCl/5 g. of tissue). Several dilutions in 0.15 M-NaCl were tested for the double-diffusion procedure. For the fluorescent antibody technique, small pieces of rat liver were frozen in liquid N₂; sections 4 μ thick were cut in a cryostat and air-dried or ethanol-fixed. In other tests smears of rat-liver cell suspensions were used. For this purpose rats were decapitated and immediately perfused for 3 min. via the aorta with 0.04 M-sodium citrate-0.15 M-sucrose (adjusted to pH 7 with citric acid). Liver minces were then homogenized very briefly by hand and gross material was removed by filtration through gauze. The minces were washed twice with 0.15 M-sucrose. The sections or smears were incubated for 30 min. at room temperature with the antisera, diluted 1:10 (v/v) in phosphate-buffered saline, pH 7.4. Repeated washings after incubation with serum or fluorescent conjugate were carried out in buffered saline during 30 min. Roboz commercial sheep anti-(rabbit γ-globulin)-isothiocyanate conjugate was used in appropriate dilutions in phosphate-buffered saline (in general 1:10, v/v). Incubation was carried out at room temperature for 30 min.

With antiserum against lysine-rich rat-liver histone, the following results were obtained. In the double-diffusion test one precipitation line was observed with rat-liver histone fraction 1. No precipitation occurred with rat serum or with lysine-poor rat-liver histone (fraction 2a + 3). Rat-liver homogenate gave one precipitation line that was identical with the line obtained with histone fraction 1. Immunoelectrophoretically this line was localized towards the cathode. The mobility was approximately equal to that of the medium portion

of rat-serum γ -globulin. Fluorescent antibody studies revealed that the antiserum against lysine-rich rat-liver histone reacted with the contents of the nuclei of cryostat sections or of cell-suspension smears of rat liver (Plate 1).

Different results were obtained with the antiserum against lysine-poor rat-liver histones (fraction 2a + 3). In the first place this antiserum did not react with lysine-rich rat-liver histone but reacted weakly with one or two rat serum components. After sufficient absorption with rat-serum powder, the antiserum revealed two lines with rat-liver histone fraction 2a + 3, and the same two lines with liver homogenate. Both lines were also observed with separate fraction 2a but only one with fraction 3. This indicated that histone fractions 2a and 3 were immunologically different from each other, and that fraction 2a was contaminated with fraction 3. Immunoelectrophoretically the two lines were localized towards the cathode, where they crossed each other completely.

Our finding that rat-liver homogenates, prepared in 0.15M-NaCl, reacted with antisera against rat-liver histones suggests that part of the histone of rat liver dissociates from DNA after homogenization of the tissue in 0.15M-NaCl. The possibility that

saline-soluble protein contaminating our histone preparations had acted as antigen seems unlikely, since the histones were prepared from rat liver after removal of saline-soluble material (Sluyser *et al.* 1965). Immunoelectrophoresis also showed that the antigen had a basic character and fluorescent antibody studies showed that it was located in rat-liver nuclei.

It can thus be concluded that: (1) rat-liver histones, prepared according to our procedure, are antigenic in the rabbit; (2) rat-liver histone fractions 1, 2a and 3 are immunochemically distinct.

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Isolation of 5-Amino-4-ribitylamino-uracil from a Riboflavineless Mutant of *Aspergillus nidulans*

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Studies to elucidate the mode of the formation of riboflavine in the riboflavine-synthesizing organism *Eremothecium ashbyii* (Goodwin & Treble, 1957) have indicated that a pyrimidine compound, 4,5-diamino-uracil, is a probable precursor in flavinogenesis. This observation has been extended by Katagiri, Takeda & Imai (1959) and Kishi, Asai, Masuda & Kuwada (1959), who showed that the ribityl derivative of 4,5-diamino-uracil is converted by extracts of *E. ashbyii* and *Aerobacter aerogenes* into 6,7-dimethyl-8-ribityl-lumazine, which has been reported to be the immediate precursor of riboflavine (Plaut, 1963).

Our observations on the utilization of the ribityl derivative of 4,5-diamino-uracil by the riboflavineless mutants of *Aspergillus nidulans* (Sadique,

Shanmugasundaram & Shanmugasundaram, 1966) also indicate that it can be a precursor of riboflavine. The identification of 4,5-diamino-uracil in a riboflavineless mutant of *Aspergillus nidulans* was reported by Sadique *et al.* (1966). The present communication deals with the isolation and identification of the ribityl derivative of 4,5-diamino-uracil in growing mycelia of a riboflavineless mutant of *Aspergillus nidulans*.

A riboflavineless mutant designated as wribo₆ was grown in 2l. of the minimal medium, as used by Pontecorvo (1953), in Hoffkin flasks supplemented with riboflavine. After 7 days' growth at 37° the mycelia were harvested. About 100g. wet wt. of mycelia were homogenized with 50ml. of water in a cold room (3–4°) with a Servall Omnimixer. The