ISOLATION OF A THYMUS HORMONE, LSH

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Early work by Bonskov and Sladovic (1940) and Roberts and White (1949) with injections of cell-free thymic extracts showed that the thymus contains a specific agent capable of causing lymphocytosis. Metcalf (1956-59) described a "lymphocytosis stimulating factor" (LSF) which was saline extractable from human or mouse thymus. When injected intracerebrally into newborn mice, this material caused a lymphocytosis which reached a maximum at six days. This factor also maintained lymphocyte levels in thymectomized mice. Further confirmation of the presence of this agent(s) was obtained when Levey et al. (1963), Osoba and Miller (1963) and Osoba (1965) were able to prevent lymphocytopenia and other pathologic effects of neonatal thymectomy by enclosing thymus tissue in cell impermeable diffusion chambers and implanting the chambers into thymectomized animals. Using a modification of the Metcalf assay we have chemically fractionated thymus to isolate one compound which increases the lymphocyte/polymorph ratio. This hormone, designated LSH has characteristics of a histone with a molecular weight of about 17,000.

METHODS

Activity of isolated fractions and other test materials was assayed by intraperitoneal injection of neonatal Swiss Webster mice with 0.01 to 0.02 ml of test material 6-12 hours following birth. Six days after injection, blood was obtained from the tail, differential white cell counts were made using Wright's stain and the lymphocyte/polymorph ratio was calculated.

Fresh bovine thymus from animals 12 to 18 months old was freed of excessive fat and connective tissue, weighed and cut into small segments. Unless other wise noted all further steps in the procedure were carried out in the cold room at 0° to 5° C, all water used was distilled and

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deionized, and all centrifugations were 16,300 g for 20 minutes. The thymic segments were placed in a Waring blender and emulsified at medium speed for five minutes in 0.85% saline (1:12 W/V). After standing 30 minutes, the emulsion was centrifuged, and the cellular debris was discarded. Crystalline ammonium sulfate was added to the saline extract until a concentration of 20% (W/V) was reached. This mixture was stirred slowly for one hour and centrifuged. The precipitate was taken up in enough water to give a volume equal to 1/2 of that volume of saline originally used to extract the thymus. This suspension was allowed to stand for six hours with slow stirring, and then centrifuged. The precipitate was discarded. Three parts (W/V) of absolute methanol were then added to the supernatant. The mixture was stirred for 15 minutes, centrifuged and the supernatant discarded. The precipitate was dissolved in water equal to 1/4 the volume of saline originally used to extract the thymus and slowly stirred for 6 hours. The suspension was centrifuged and the supernatant was dialyzed with slow stirring for 15 hours against 50 volumes of water. The fractionation scheme and the activity of the fractions is presented in Figure 1. The activity of the lyophilized residue showed a 500 fold concentration over the fresh thymus.

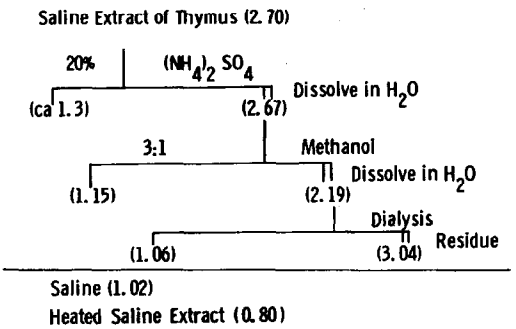

Figure 1. Fractionation scheme for thymus. The activity of the preparations is indicated by the number in parentheses. This number is the average ratio for the differential counts of 5 mice (Ratio = lymphocytes/polymorphs). Ratios greater than 1.5 indicate activity.

Further purification was achieved using polyacrylamide disc electrophoresis. The gel formula of Reisfield, Lewis, and Williams (1962) was modified by the addition of more tetramethylenediamine and a reduction in potassium hydroxide and acetic acid ion concentrations to facilitate rapid cold room polymerization and reduce ohmic heating during electro-

phoresis. The lyophilized sample was dissolved in enough water to give a protein concentration of 300γ/0.1 ml. This was then mixed 1:1 with upper gel reagent and 0.2 ml applied to each of 16 tubes. The sixteen tubes were electrophoresed for threehours at 48 milliamperes. Following electrophoresis the leading ion-trailing ion fronts on all columns were marked with a dissecting needle and 15 columns were immediately frozen on dry ice. One gel column was placed in staining solution at room temperature for one hour after which it was destained. The stained protein bands and the perforations were used as reference points to cut the 15 thawed, unstained columns into segments A, B, C, D, and E (Figure 2). Similar segments from the 15 tubes were pooled and expressed through the orifice of a 2 ml syringe and mixed in 50 ml water. The proteins were ex-

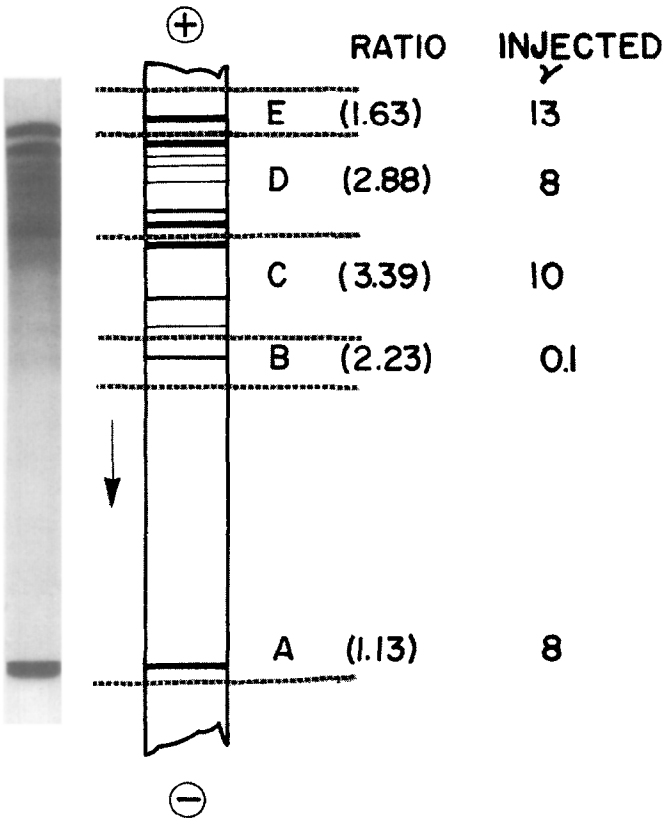

Figure 2. Gel electrophoresis pattern and activity ratios (Ratio = lymphocyte/polymorphs).

extracted from the homogenized gels by allowing the mixtures to stand 8 to 10 hours. The gel-protein mixtures were then centrifuged at 1,800 r.p.m.; the supernatants were dialyzed against 50 volumes of water for 12 to 18 hours and lyophilized.

RESULTS

Bioassay of the fractions obtained (Figure 1) showed LSF to be saline soluble, ammonium sulfate precipitable, methanol precipitable, heat labile and non-dialyzable. This material was found to be 99+% protein by the method of Lowry et al. (1951). Activity of the electrophoresis fractions (Figure 2) resided in fractions B, C, and D. Re-electrophoresis of these fractions confirmed the evidence that a single compound was present in fraction B. Comparison of the amount of protein required for activity in fractions C, D, and E, (10γ, 8γ, 13γ respectively) with the amount in fraction B (0.1γ) makes it probable that fraction B is the most active compound.

TABLE 1

CHARACTERIZATION OF LSH

Source	Thymus (ca. 0.01%, wet basis)
Crystals (from H_2O)	Clear, Birefringent plates
Purity	One band on gel re-electrophoresis
Molecular weight	17,000 $\pm$ 5000
Chemical nature	Basic Protein
Heat stability	Labile (56° C for 30 minutes
Bioassay	Increase lymphocyte/polymorph ratio
Biological Activity, I.P.	0.1 γ/mouse

DISCUSSION

Considering the previous experimentation by Jolly and Hinshaw (1965) LSF originates only in the thymus and is conveyed through the blood to peripheral lymphoid organs where it causes a stimulation in lymphocyte production or proliferation (observed here as an increased lymphocyte/polymorph ratio). Since this statement concerning LSF closely parallels the classical description of a hormone, the pure compound isolated (fraction B) may be called a hormone. Therefore, the designation of lymphocyte stimulating hormone (LSH) shall be used. Its characteristics are summarized in Table I.

The protein character of LSH is supported by the fact that the stain used in electrophoresis, amido black 10 B, is specific for proteins. The fact that LSH is non-dialyzable suggests that it is greater than 10,000 in molecular weight. Preliminary work with Sephadex indicated that LSH was probably between 10 and 50 thousand in molecular weight. This was corroborated by the results with polyacrylamide gels. Using 15% gels, an average pore size of approximately 30 Å was obtained; this gel effectively separates molecules with molecular weights between 10 and 30 thousand. LSH was separated in this system and showed a high mobility, indicating a molecular weight well under 30,000 and/or a highly charged structure. These results suggest that LSH is a basic protein with a molecular weight of 17,000 ± 5,000. Confirmation of this by chemical analysis is presently hampered by the small quantities of LSH isolated using the present polyacrylamide technique. The re-electrophoresis pattern suggested that LSH was one pure protein. Further support of its purity was obtained by lyophilizing fraction B to near dryness and crystallizing it. The microscopic crystals were clear, birefringent, hygroscopic, rectangular plates.

The relationship of LSH to the materials used by Comsa (1965), Ord et al. (1965), Bernardi and Comsa (1965), Goldstein et al. (1966), Pansky et al. (1965) and Basso et al. (1964) is not clear. The preparations of Bernardi and Comas (1965) and Goldstein et al. (1966) are glycoproteins. Glucosamine and carbohydrate were detectable in trace amounts in our methanol precipitate. The preparation of Goldstein, et al. (1966) is a heat stable, dialyzable glycoprotein. In contrast, LSH follows the characteristics of the Metcalf (1965) extracts: heat labile and non-dialyzable. Although the material of Bernardi and Comsa is one chromatographic fraction, we have separated one Sephadex fraction into 10 proteins by gel electrophoresis. Although we have isolated what appears to be the most active compound, the data suggest the existence of several compounds which have LSH activity. The extensive work of Comsa suggests that one compound, presumably LSH, will allow (a) antibody production, (b) survival, (c) increased function of thyroid, gonads and adrenal cortex, (d) increased growth and (e) restoration of lymphatic tissue and lymphocytes in thymectomized guinea pigs.

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