PREPARATION, ASSAY, AND PARTIAL PURIFICATION OF A THYMIC LYMPHOCYTOPOIETIC FACTOR (THYMOSIN)*

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In recent years, investigations involving thymic ablation and replacement by thymic grafts or extracts in several species have suggested a central role for the thymus gland in the maturation, proliferation, and immunological competence of the lymphocyte.[1] Two major types of observations imply that the influence of the thymus is in part endocrine. The first is the *in vivo* effect of thymic extracts on lymphopoiesis, lymphocytosis, and immunological competence;[2–11] the second is the effect of thymus grafts, enclosed in cell-impermeable Millipore diffusion chambers, on thymectomized animals.[12–14]

Previous attempts to isolate and purify "thymic hormones" have been hampered by the lack of a satisfactory, rapid assay method. In two earlier publications, Klein, Goldstein, and White[10, 11] have described the preparation of thymic extracts and an *in vivo* method for testing their biological activity. In this communication, we wish to report the preparation and partial purification from calf thymic tissue of a product which stimulates incorporation of H^3-thymidine into mesenteric lymph node cells. The lymphocytopoietic factor, which we term *thymosin*, is active when administered *in vivo*, as well as when added directly to a lymphocyte suspension incubated *in vitro*. This *in vitro* activity has permitted the development of a new assay procedure for thymosin and has facilitated purification studies.

Materials.—Animals: Used for the *in vitro* and *in vivo* experiments were 29-day-old male Swiss Webster CD1 mice, 8–10-week-old male Sprague-Dawley rats, and 8–10-week-old male New Zealand white rabbits. All animals received food and water *ad libitum* until sacrificed.

Radioactive precursors: H^3-thymidine (3.0 c/mmole) and H^3-deoxycytidine (2.4 c/mmole) were purchased from Schwarz BioResearch, Inc. H^3-uridine (1.8 c/mmole), C^{14}-lysine (222 mc/mmole), C^{14}-leucine (223 mc/mmole), and C^{14}-phenylalanine (333 mc/mmole) were purchased from New England Nuclear.

Chemicals and reagents: Trypsin (2 × crystallized, salt-free), soybean trypsin inhibitor (5 × crystallized), RNase (5 × crystallized), bovine serum albumin, and the nucleosides used were all purchased from Nutritional Biochemicals Corp. Phytohemagglutinin in a powdered form was obtained from the Burroughs Wellcome Co. Pooled calf serum (sterile and filtered) was purchased from the Pentex Corp. Eagle's medium for spinner cultures (MEM, without phosphate and calcium) was purchased in powdered form from General Biochemical Corp. Phosphate was added to the powder during preparation of the medium. Bio-Gel P-10 (50–150 mesh) was purchased from Bio-Rad Laboratories.

All other chemicals used in this study were of analytical or reagent grade and were used without further purification.

Fractionation procedure: Figure 1 presents a diagram of the fractionation procedure. Fresh or frozen calf thymus, obtained from a local abattoir, was cleaned, defatted, and homogenized in 0.15 *M* NaCl (tissue:saline = 1:3) at 0–5°C in a Waring Blendor. All isolation procedures were carried out in the cold. The homogenate was centrifuged at 1200 × *g* in an International refrigerated centrifuge at 0–5°C for 15 min. The supernatant fluid was then centrifuged at 105,000 × *g* in a Spinco model L ultracentrifuge for 1 hr. The supernatant fluid was passed through glass wool to remove floating particulate material. The clear extract (*fraction 1*) was then treated by either of two procedures. In method 1, developed initially, the solution was added slowly with

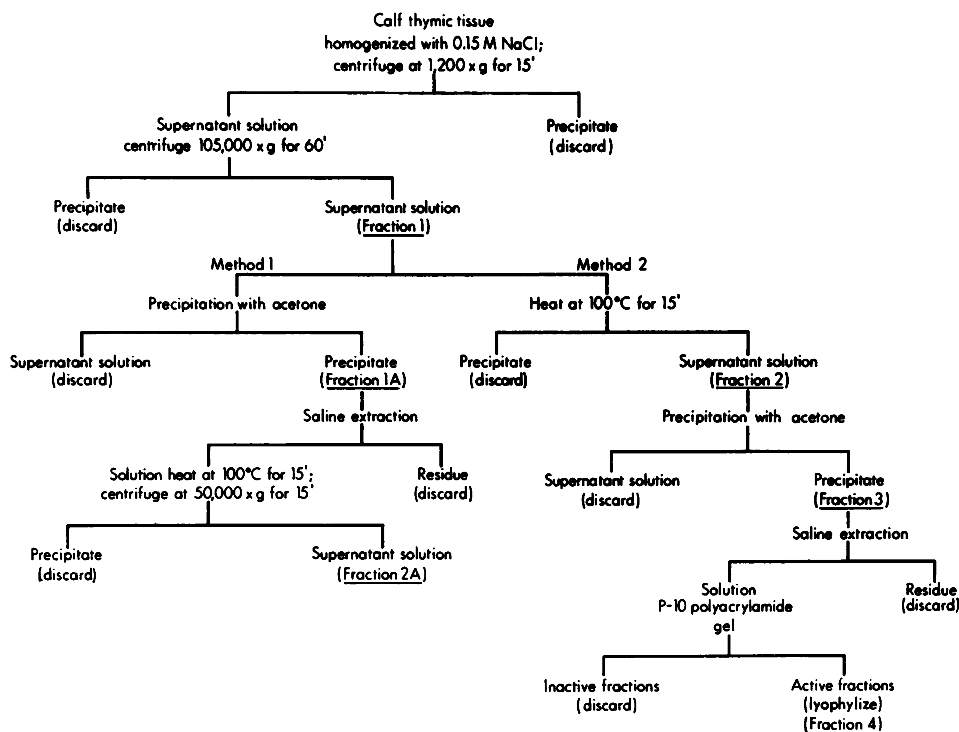

FIG. 1.—Diagram of fractionation procedures.

constant stirring to 10 vol of cold (−20°C) acetone. The precipitate was collected by filtration on a large Büchner funnel, using Whatman #1 filter paper. The precipitate was scraped from the paper into a large volume of acetone (−20°C), broken up with a glass rod, and again collected on the Büchner funnel. This acetone-washing procedure was repeated once more, and the precipitate finally washed on the funnel with several volumes of the cold acetone. The precipitate (*fraction 1A*) was then transferred to a glass dish and dried overnight *in vacuo* over concentrated H_2SO_4 and anhydrous $CaSO_4$. When ground in a mortar with a pestle and extracted with 0.15 *M* NaCl, approximately 90% of this powder was soluble. The small insoluble residue was removed by centrifugation prior to assay or further fractionation of the clear supernatant solution. Further purification was effected by heating the clear 0.15 *M* NaCl extract of fraction 1A in a water bath at 100°C for 15 min and then cooling in an ice bath. A large white voluminous precipitate formed and was removed by centrifugation at 50,000 × *g* for 15 min. The clear supernatant solution was designated as fraction 2A.

An alternative procedure (*method 2*) utilized the heat step at an earlier stage of fractionation. Fraction 1 (above) was subjected to heat at 100°C for 15 min, as above. The clear supernatant solution (*fraction 2*), obtained after removal of the heat-insoluble material, was treated with acetone, as in method 1. The acetone-insoluble precipitate was dried, as above (*fraction 3*). The 0.15 *M* NaCl soluble material from fraction 3 was placed on a polyacrylamide gel column (P-10) and eluted with either 0.15 *M* NaCl or with glass-distilled water. The active fractions were combined, lyophilized (*fraction 4*), and stored in the freezer in a bottle containing anhydrous $CaSO_4$.

Bioassay: One of the bioassays used was the *in vivo* procedure described previously,[10, 11] with several modifications. In addition to our continued use of male CBA mice from our own colony, 29-day-old male Swiss Webster mice, reared for germ-free studies, were also employed. At this time, all CBA mice in our colony receive tetracycline in their drinking water (340 μg/ml). The extirpated lymphoid tissue was placed immediately in cold 7% perchloric acid rather than, as

previously reported, in cold 0.15 *M* NaCl. The nucleic acids were then extracted and analyzed.[10, 11]

Assay of thymic preparations by an *in vitro* method was based on a modification of the techniques described by Makman, Dvorkin, and White[15] for examining the effects of steroids on thymocytes. Eight to ten-week-old male New Zealand white rabbits, or male Sprague-Dawley rats of the same age, were sacrificed by exsanguination (rabbits) or decapitation (rats). The mesenteric lymph nodes were removed and placed in ice-chilled Eagle's spinner culture medium,[16] previously equilibrated with 10% CO_2. The cells were teased out gently and passed through a 250-mesh stainless-steel wire screen and then collected by centrifugation at $150 \times g$ for 2 min in a Clay-Adams clinical centrifuge. The supernatant fluid was removed with a pipette, and the cell pellet broken up by gentle tapping on the side of the test tube. The cells were then washed twice with the same culture medium and centrifuged each time at $150 \times g$ for 2 min. The washed cells were suspended in a small volume of medium, counted, and diluted to 1.5×10^7 cells/ml.

The cell suspension was then incubated at 37°C for 30 min with 10% CO_2 in air as the gas phase. Aliquots (0.5 ml) of this cell suspension were then used in each assay tube which received the preparations to be assayed, dissolved in 50 λ of 0.15 *M* NaCl. Incubation was continued for 3 hr with 10% CO_2 in air as the gas phase. The cells were then pulse-labeled for an additional hour with H^3-thymidine- or other labeled precursors of DNA, RNA, or protein. The tubes were then chilled in an ice bath and centrifuged. After removal of the supernatant fluid, the cell pellets were broken up with the aid of a Vortex mixer, and 1 ml of cold 5% trichloroacetic acid was added. The contents of the tube were transferred to a Millipore filter (0.45 μ pore size) with generous washing out of the tube with cold 5% trichloroacetic acid. The filters holding the acid-insoluble fractions were placed in polyethylene vials containing 15 ml of Bray's solution.[17] The vials were shaken thoroughly and then counted in a Packard liquid scintillation counter.

Total protein was determined by the Lowry method[18] on precipitates obtained by treatment of aliquots of cell suspensions with 4 vol of cold 5% trichlororoacetic acid, centrifuging the precipitate, and washing it twice with 95% ethanol. A standard protein curve was prepared utilizing bovine serum albumin.

Results.—Figure 2 illustrates the effects of injection *in vivo* of a saline extract of a thymic fraction (*fraction 1A*) prepared from calf thymus on lymph node weight, and on incorporation of H^3-thymidine into lymphoid structures. Following two subcutaneous injections, separated by a 24-hr interval, there was a marked increase at 48 hr in the incorporation of H^3-thymidine into the total nucleic acids of peripheral lymph nodes, accompanied by a significant increase in node weight. Stimulation of thymidine incorporation was apparent occasionally following one injection, and usually preceded a change in organ weight.

The saline extract of the acetone powder (*fraction 1A*) was similar in activity to the fresh, particle-free $105{,}000 \times g$ thymic extract described previously,[10, 11] except that the former was more stable. Either the acetone powder or a 0.15 *M* NaCl extract of this powder can be stored at −20°C for several months without appreciable loss of biological activity.

During the course of our many *in vivo* assays, it became increasingly apparent that, although the incorporation of labeled precursors into lymph node DNA was a

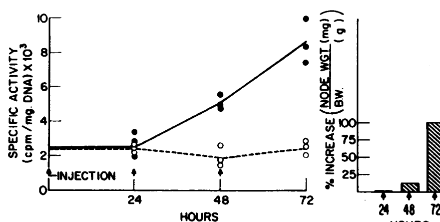

FIG. 2.—Lymph node weights and the incorporation *in vivo* of H^3-thymidine (10 μc per animal) into mouse-pooled axillary, brachial, and inguinal lymph nodes after daily injections (indicated by arrows) of ●—● a calf thymic fraction (*fraction 1A*) equivalent to 2.4 mg protein (each injection) dissolved in 0.25 ml 0.15 *M* NaCl, and ○- -○ 0.15 *M* NaCl, 0.25 ml. Animals sacrificed 24 hr after last injection. Each point represents the pooled lymphoid tissues of four mice.

better index of lymphocytopoiesis than other available bioassays, inherent difficulties were present in the *in vivo* assay. Some of the more general problems concerned the possible antigenicity of the extracts, the limited number of fractions that can be tested in a given period of time, and the deleterious influence of subclinical infection in the mice. These factors led to development of an *in vitro* system which would circumvent some of the shortcomings of the *in vivo* assay. Initially, rat mesenteric nodes were used as a source of cell suspensions; in subsequent studies, rabbit mesenteric nodes have been used because of the need for a larger and more convenient supply of lymph node cells. The *in vitro* assay is described above.

The initial *in vitro* experiments were conducted with 0.15 *M* NaCl extracts of acetone powders prepared from the 105,000 $\times g$ supernatant fraction (*fraction 1A*). Incubation of rat mesenteric lymph node cell suspensions with aliquots of these extracts markedly stimulated the incorporation of a pulse label of H^3-thymidine into cellular DNA. As shown in Figure 3, the incorporation of H^3-thymidine was directly dependent on the amount of extract added to the lymph node cell suspension, generally up to a maximum of 1000 μg of protein per assay vessel. The degree of stimulation fell off at higher protein concentrations, possibly indicating the presence of inhibitory factors in the crude preparations.

Fractions with lymphocytopoietic activity are relatively heat-stable. Heating the saline extract of acetone powder (*fraction 1A*) for periods of up to 30 min at 100°C does not appreciably alter biological activity. This observation greatly facilitated purification, since as much as 90 per cent of the total protein in the high-speed supernatant solutions or in extracts of acetone powders could be removed by heat precipitation, followed by centrifugation. As shown in Figure 1, two methods were employed to utilize the heat step. Initially (*method 1*), the acetone powder extracts were heated at 100°C for 15 min. A precipitate formed which was removed by centrifugation. It was found more useful, however, to introduce the heat step earlier by applying it to the high-speed supernatant fraction (*method 2*) obtained from the initial thymic homogenate. After removal of the large insoluble precipitate which formed, the clear supernatant solution was treated with 10 vol of cold (−20°C) acetone. This facilitated concentration of active material. The resulting acetone powder, after drying as described previously, was extracted with 0.15 *M* NaCl and subjected to gel filtration on a P-10 polyacrylamide column. The elution pattern, using either 0.15 *M* NaCl or glass-distilled water as the fluid phase, is shown in Figure 4. The highest biological activity was found in the peak

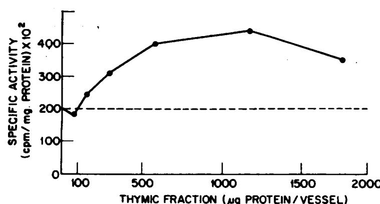

FIG. 3.—Effect of a saline extract of an acetone powder (*fraction 1A*) added *in vitro* on the incorporation of H^3-thymidine into an acid-insoluble form by a suspension of rat mesenteric lymph node cells. The cell suspension was prepared as described in the text. Following a 30-min equilibration period at 37°C, the cells were divided into 0.5-ml aliquots and incubated for 3 hr with various concentrations of the thymic fraction or saline. At 3 hr the cells were pulsed with 2.5 μc of H^3-thymidine, and incubation continued for an additional hr. Subsequent treatment of the cells is described in the text. *Abscissa:* quantity of thymic fraction present in each vessel. *Ordinate:* specific activity of acid-insoluble precipitate in cpm/mg cell protein. Each point represents the mean of three individual incubations. ●——● Incorporation in the presence of thymic preparation; - - - - - - - - controls.

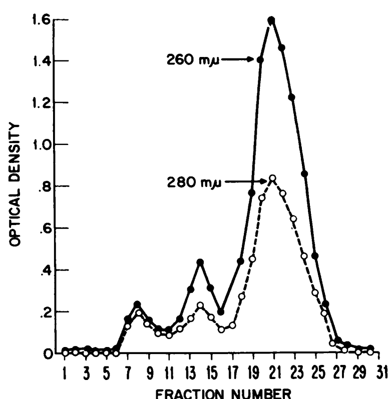

FIG. 4.—Gel filtration of a soluble extract of a thymic fraction (*fraction 3*) on a Bio-Gel polyacrylamide P-10 column, 2 × 46 cm. The eluant was glass-distilled H_2O. Fractions were collected in 6-ml volumes; fractions *19* through *25* contained the major portion of the biological activity.

representing tubes *19* through *25*. The active fractions were then concentrated by lyophilization (*fraction 4*).

The effect of the crude and partially purified thymic fractions *in vitro* on the incorporation of tritiated thymidine into the trichloroacetic acid-insoluble precipitate of rabbit mesenteric lymphoid cells is shown in Table 1. The observation that the high-speed supernatant solution (*fraction 1*) and the heated high-speed supernatant fraction (*fraction 2*) are not active *in vitro* suggests the presence of an inhibitor which was removed by the acetone step.[19]

Table 2 shows the biological activity and total recovery of the thymic lymphocytopoietic factor during a typical fractionation procedure. The activity of the various fractions is expressed in terms of their protein content. It may be noted that fraction 4 was also active in our *in vivo* assay.

The influence of active thymic extracts on the incorporation *in vitro* of other labeled precursors into DNA, RNA, and protein of mesenteric lymph node cells is shown in Table 3. The incorporation of H^3-deoxycytidine, C^{14}-leucine, C^{14}-lysine, and C^{14}-phenylalanine into trichloroacetic acid-insoluble material is greatly increased in the presence of the thymic fraction, while the incorporation of H^3-uridine is relatively unaffected or slightly decreased.

A variety of other preparations, including calf serum, bovine serum albumin, phytohemagglutinin, and thymic extracts prepared as described by others,[20] were also tested *in vitro,* although the data are not presented. Based on comparable protein dosages (i.e., 1–500 μg/incubation vessel), none of the above materials was active, with the exception of calf serum. Large aliquots of calf serum (5% per vessel) produced an approximately 100 per cent increase in the incorporation of H^3-thymidine. In 0.04 mM conc per vessel, uridine, cytidine, deoxycytidine, adenosine, and guanosine had no effect on the incorporation of H^3-thymidine under the experimental conditions described. Whittle[21] has reported that similar concentrations of deoxycytidine added *in vitro* to rat thymocytes stimulated incorporation of P^{32} into cellular DNA.

Preliminary chemical and physical properties of the partially purified products with lymphocytopoietic activity indicated that biological activity is associated with a relatively small, slowly dialyzable protein component of these preparations. The ultraviolet spectra of the most active preparations show absorption at 260 mμ and 280 mμ (Fig. 4). These fractions give positive orcinol and anthrone reactions but no color with diphenylamine. Incubation of fraction 4 at 7.2 with high concentrations of pronase or trypsin for 12–15 hr at 37°C resulted in significant loss of biological activity. Prior to bioassay *in vitro* of the digests, pronase activity was destroyed by heat treatment, and trypsin activity stopped by addition of soybean trypsin inhibitor. Incubation with high concentrations of RNase at pH 7.2 for

TABLE 1

EFFECT OF CRUDE AND PARTIALLY PURIFIED THYMIC FRACTIONS ON THE INCORPORATION OF H^3-THYMIDINE INTO AN ACID-INSOLUBLE FORM BY A SUSPENSION OF RABBIT MESENTERIC LYMPH NODE CELLS *in vitro*

Fractions assayed*	Protein concentration (μg/vessel)	Cpm/mg cell protein†	Change (%)
0.15 *M* NaCl, control	—	42,546	
1	510	45,948	+8
2	80	40,723	−4
1A	2360	90,788	+113
1A	236	117,635	+176
1A	23.6	53,766	+26
2A	285	310,000	+629
2A	28.5	91,533	+115
2A	2.85	47,314	+11
3	241	301,106	+608
3	24.1	96,138	+126
3	2.41	56,053	+32
4	44.3	231,256	+444
4	4.43	117,667	+177
4	0.44	69,282	+63

Incubation procedure as outlined in text.
* See Fig. 1 for method of preparation of fractions.
† Each value represents the mean of at least three individual incubations.

TABLE 2

BIOLOGICAL ACTIVITY AND TOTAL RECOVERY OF THE THYMIC FACTOR DURING FRACTIONATION PROCEDURES

Fractions*	Total protein† (mg)	Activity (units/mg)	Total activity	Recovery‡ (%)
1	10,200	0.9	9,180	—
1A	6,650	15.0	99,750	—
2A	450	155.0	69,750	70
2	1,445	2.0	2,890	—
3	740	110.0	81,400	82
4	50	1360.0	68,000	68

A unit of activity is arbitrarily defined as the amount of protein which produces a 25% stimulation of the incorporation of H^3-thymidine into rabbit mesenteric lymph node cells *in vitro* under conditions described in text.
* See Fig. 1 for preparation of fractions.
† Based on initial thymic tissue weight of 500 gm.
‡ Based on fraction 1A (method 1).

TABLE 3

EFFECT OF PURIFIED THYMIC FRACTION (*Fraction 4*) ON THE INCORPORATION OF LABELED PRECURSORS OF DNA, RNA, AND PROTEIN INTO AN ACID-INSOLUBLE FORM BY A RABBIT MESENTERIC LYMPH NODE CELL SUSPENSION

Experimental group	Precursors*	Cpm/mg cell protein†	Change (%)
Saline control	H^3-thymidine	36,232	—
Fraction 4	"	209,600	+479
Saline control	H^3-deoxycytidine	17,042	—
Fraction 4	"	63,442	+272
Saline control	H^3-uridine	30,653	—
Fraction 4	"	26,737	−13
Saline control	C^{14}-lysine	5,074	—
Fraction 4	"	9,442	+78
Saline control	C^{14}-leucine	28,526	—
Fraction 4	"	48,558	+70
Saline control	C^{14}-phenylalanine	26,621	—
Fraction 4	"	48,800	+83

Incubation procedure described in text.
* The labeled precursors were added in aliquots of 25 λ to the control and experimental vessels in the following concentrations: H^3-thymidine, 2.5 μc; H^3-deoxycytidine, 2.5 μc; H^3-uridine, 1.25 μc; C^{14}-leucine, 0.5 μc; C^{14}-lysine, 0.5 μc; C^{14}-phenylalanine, 0.824 μc. Each experimental vessel contained 50 λ (44 μg of protein) of fraction 4.
† Each value represents the mean of three individual incubations.

2 hr at 37°C did not alter lymphocytopoietic activity. After RNase treatment, there was no increase in perchloric acid-soluble material which absorbs at 260 mμ.

Discussion.—Partial purification of thymosin, a soluble lymphocytopoietic factor from calf thymic tissue, has been achieved with the aid of a new *in vitro* assay system. Based on this assay, an approximately 100-fold purification of the active principle has been obtained using ultracentrifugation followed by a heat step, precipitation with cold acetone, and gel filtration on P-10 polyacrylamide.

On the basis of the present evidence, lymphocytopoietic activity appears to be associated with a carbohydrate-containing protein of a particle weight less than 10,000. Activity is relatively stable at 100°C, and decreases significantly during incubation with high concentrations of pronase or trypsin. The most active fractions show absorption at 260 mμ and at 280 mμ, and give a positive orcinol reaction. However, biological activity does not appear to be associated with a soluble RNA. The active principle is soluble in 5 per cent perchloric acid, and incubation with high concentrations of RNase does not result in a loss of biological activity or in an increase in perchloric acid-soluble material which absorbs at 260 mμ.

Active preparations, injected *in vivo* into mice, stimulated the incorporation of H^3-thymidine into the acid-insoluble precipitate prepared from the axillary, brachial, and inguinal lymph nodes of these animals, thus mimicking the *in vitro* effects of these preparations. Fraction 1, although active in an *in vivo* assay, had no activity *in vitro* until treated with acetone. This suggested that an acetone-soluble inhibitor may be present.[19] If this is the case, this inhibitor is inactivated *in vivo* since fraction 1 is very active when injected into mice over a 72-hr interval.

Since the first report by Roberts and White[2] of a thymic fraction rich in nucleoprotein which could stimulate lymphocyte production, a number of other lymphocyte-stimulating thymic extracts have been described.[3–11] With the possible exception of the product prepared in the laboratory of Comsa,[20] it is difficult to assess the lymphocytopoietic significance of the various preparations since they are relatively crude and have been assayed by diverse and variable *in vivo* techniques. However, all of the reported active extracts appear to contain some protein.

The biologically active fractions of Nakamoto[7] and Jankovic *et al.*[9] are also rich in lipids, while the purified preparation of Bezssonoff and Cosma[20] appears to be a glycoprotein. In our *in vitro* assay, fractions prepared according to the procedure of Bezssonoff and Comsa[20] have not stimulated H^3-thymidine incorporation into mesenteric lymph node cells. The lymphocytosis stimulating factor (LSF) reported by Metcalf[22] is, in contrast to the fractions described in this paper, heat-labile and nondialyzable.

Further characterization of thymosin is in progress. The preparation of a cell-free product which stimulates lymphocytopoiesis suggests that this factor may be a thymic hormone, produced in the thymus, and transported via the blood to peripheral lymphoid structures where it stimulates lymphoid tissue proliferation. This hypothesis is now being studied. An inquiry is also being made into the possible relationships of thymosin to previously postulated thymic hormones and to immunological phenomena.

Summary.—A new *in vitro* assay for testing lymphocytopoietic activity of thymic extracts is reported. Utilizing this assay, the preparation and partial purification of a calf thymic lymphocytopoietic factor, for which the name thymosin is sug-

gested, are described. The properties of the most purified fraction suggest that lymphocytopoietic activity is associated with a relatively heat-stable carbohydrate-containing protein with a particle size less than 10,000. This product also stimulates lymphocytopoiesis when assayed *in vivo*. The purified product does not appear to be identical with any of the previously described preparations with lymphocytopoietic activity.

Note added in proof: Since submission of this paper for publication, a further threefold purification of *fraction 4* (Table 2) has been achieved by use of a DEAE-cellulose column. Elution initially with 0.001 *M* phosphate buffer (pH 5.7), and then increasing the pH to 7.4, followed by a gradient up to 1.0 *M* phosphate, yields four fractions. Activity (*in vitro* assay) is associated with the first peak emerging from the column. This lyophilized, active preparation of thymosin is a carbohydrate-containing protein with a 280 mμ/260 mμ ratio of 0.85.

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