STUDIES OF THE ACTIVITY IN CHICKEN SERUM THAT STIMULATES THE LYMPHOID DEVELOPMENT OF THE EMBRYONIC CHICKEN THYMUS IN ORGAN CULTURE

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Some properties of the thymus stimulating activity in chicken serum (TSAS) which is essential for the lymphoid development of the embryonic chicken thymus in organ culture, have been studied and a partial purification has been performed. TSAS resisted heating for 30 min at 60°C but not higher temperatures. During dialysis and ultrafiltration of serum a loss of TSAS down to a constant level of about 50 per cent of the original activity was obtained. TSAS was sensitive to treatment at pH 5 or lower but resisted alkaline pH up to at least pH 10. When serum was fractionated with ammonium sulphate little TSAS precipitated at 2.43 M ammonium sulphate or lower. At higher concentrations TSAS was found in the precipitates, but even at 4.05 M significant activity remained in the supernatant. TSAS was recovered from a fraction dominated by the serum albumins after gel filtration on Sephadex G-200 and G-100. Further fractionation by ion exchange chromatography on DEAE-Sephadex separated the TSAS from serum albumin. These results suggest that part of the TSAS is associated with one or more specific factors with a molecular weight of approximately 70,000. The effect of the partly purified TSAS on the growth and morphologic development of the embryonic thymus in organ culture was studied.

One important function of the thymus as a central lymphoid organ is the production of lymphoid cells with specific functional characteristics. By seeding these cells to the peripheral lymphoid organs (8, 10, 18) and apparently also by secreting a humoral lymphopoietic factor (9, 12, 28) the thymus influences the development and maintenance of the morphology and function of peripheral lymphoid organs.

The external and internal regulation of thymic development and lymphopoiesis is poorly understood. However, there is evidence that an external control of the thymus is accomplished by mediators from the pituitary gland, either directly or indirectly via other endocrine organs such as the thyroid gland (22). Pituitary growth hormone exerts a thymotropic effect either alone or together with thyroxine and also influences the peripheral lymphoid organs (13, 19, 22). A specific thymotropic hormone of pituitary origin has been suggested but not yet identified (5). Whether the target cells of this pituitary control are the epithelial or lymphoid components of the thymus or both is not...
known. A feed-back mechanism between the thymus and the pituitary gland has also been indicated (2, 21, 22). The inhibitory action of glucocorticosteroid hormones on thymic lymphopoiesis is well documented (3). Regulatory effects of sex hormones on the thymus have also been suggested (6, 7). Recently thyrocalcitonin has been shown to inhibit and parathormone to stimulate thymic lymphopoiesis (14).

The evidence for an autonomous internal regulation of the lymphopoiesis in the thymus, in particular the importance of the thymus epithelium in this, have been reviewed by Metcalf (15, 16) and Metcalf & Moore (17).

We have previously described an organ culture system for the embryonic chicken thymus (1). This organ culture system appears suitable for the examination of potential thymotropic factors. It permits the reproducible morphological development of thymic anlagen from a stage with only relatively few lymphoid precursor cells to a stage containing large numbers of predominantly small lymphoid cells (1, 24, 26). We have also presented results indicating that the lymphoid cells differentiating in the organ cultures, are able to react to lymphocyte mitogens in vitro (24, 27).

The organ culture technique included a complex culture medium, RPMI 1640, supplemented with heat inactivated chicken serum only. Data showing that the chicken serum was essential for both initiation and maintenance of lymphoid development in the thymic anlagen have been presented (26). This thymus growth stimulating activity in serum (TSAS) may be due to specific factors of physiological significance. The purpose of the present investigation was therefore to define some of the properties of the stimulating activity in serum necessary for the lymphoid development of the embryonic thymus in organ culture. Parts of these results have been briefly reported elsewhere (27).

**MATERIALS AND METHODS**

**Organ culture technique:** The White Leghorn chicken embryos were of DeKalb Chix strain 161 or Babcock B-300. Details of the incubation conditions for the eggs, the procurement of the thymic anlagen from 10-day-old embryos, and the organ culture technique have been given elsewhere (1, 26). The culture medium in the present investigation was RPMI 1640 (Flow Laboratories, Irvine, U.K.) with supplements as indicated. Heat inactivated chicken serum (CS), 10 per cent (v/v) in the medium, was used as a positive control in all experiments. The culture time was six days where not otherwise indicated. Medium changes were not performed because they did not improve the lymphoid development of the anlagen.

**Evaluation of lymphoid development:** The effect of the different medium supplements on the lymphoid development of the thymic anlagen was determined, as previously described (26), by counting the number of lymphoid cells obtained from homogenized thymic anlagen. Four cultures were used for each treatment. These were either pooled at harvest or harvested separately and the cell yield was expressed as the mean number of cells per anlage.

**Chicken serum:** The procurement of serum from 5–8 weeks old chickens (CS) has been dealt with elsewhere (26). Whole serum and the various serum preparations were stored at –85°C.

**Heat inactivation:** The serum was usually heat inactivated at 56°C for 30 minutes. To examine the heat resistance of the thymus stimulating activity, CS was incubated at respectively 56, 60, 80 and 100°C for 30 minutes. After centrifugation, any precipitate was discarded and the supernatant collected.

**Sterile filtration:** Whole serum was always aseptically handled and needed no further sterilization. If necessary, sterilization of serum preparations was done by filtration through 0.45 μ Millipore filters.

**Dialysis:** Whole serum or fractions of serum were dialysed in Visking 23/32 cellulose dialysis tubing (Union Carbide, New York, N.Y., U.S.A.) for 2 days against four changes of 100-fold volumes of phosphate buffered saline (PBS), pH 7.4 (0.05 M sodium phosphate and 0.09 M NaCl). The dialysis tubing is assumed to retain molecules with a Mw larger than 6,000 (4).

**Test of different concentration procedures:** Concentration of diluted serum was in four experiments performed by ultrafiltration through an SM 16 305 collodion membrane (Sartorius-Membranfilter, Göttingen, Germany). This membrane retains molecules with a Mw larger than 12,000. In five experiments the concentration procedure was carried out by ultrafiltration through Diaflo UM-2 or UM-10 filters (Amicon, Lexington, Mass., U.S.A.) with cut-off levels of 1,000 and 10,000, respectively. In two other experiments the concentration was performed by lyophilization.

Concentration of serum fractions obtained at the different separation steps was performed by ultra-
filtration through either collodion membranes or UM-10 filters.

**Test of pH-sensitivity:** The pH-sensitivity of TSAS was tested by incubating samples of heat inactivated CS for 30 min with an equal amount of buffer of pH-values ranging from 2 to 10. The following buffers in 0.1 M concentration were used to achieve the desired pH: Citrate-HCl, pH 2-4; citrate-phosphate, pH 5 and 6; glycine-NaOH, pH 9 and 10. The pH of the buffer-serum mixture was always checked. After the incubation the sera were centrifuged, the supernatants dialysed against PBS, reconcentrated to the original serum volume, sterile filtered and tested as medium supplements in organ culture in two experiments.

**Ammonium sulphate precipitation:** Fractionation of chicken serum by sequential precipitation of protein from serum half diluted with PBS, pH 7.0, was performed with ammonium sulphate (\(\text{(NH}_4\text{)}_2\text{SO}_4\)) at a final concentration of 1.50, 1.66, 2.03, 2.45, 3.08 and 4.05 M at 4°C under continuous stirring for two hours. At each step both precipitates and supernatants were collected after centrifugation. The supernatants were concentrated to half the original serum volume, dialysed and restored to the original serum volume with PBS. The precipitates were washed once with the (\(\text{(NH}_4\text{)}_2\text{SO}_4\)) solution and were then redissolved in PBS, dialysed and returned to the original serum volume.

**Gel filtration on Sephadex G-200:** Whole serum was fractionated by gel filtration at 12°C on Sephadex G-200 using a K 25/100 column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with PBS, pH 7.4. Five-ml fractions were collected after elution by upward flow at a rate of 3.5 ml/cm² × h. After determination of the protein concentration, fractions were pooled as indicated in Fig. 6 and concentrated to 20 mg protein per ml, dialysed against PBS, and tested in organ culture as medium supplements.

**Gel filtration on Sephadex G-100:** The supernatant from chicken serum precipitated with 2.43 M \(\text{(NH}_4\text{)}_2\text{SO}_4\), as described above, was concentrated twice. It was then simultaneously desalted and separated by gel filtration on Sephadex G-100 using a K 50/100 column (Pharmacia). The gel bed was equilibrated with PBS, pH 7.4. Ten-ml fractions were collected by downward elution at a flow rate of 5.1 ml/cm² × h. Fractions were pooled as indicated in Fig. 5 and concentrated to about 1.6 times the original serum volume.

Chicken serum supernatants after \(\text{(NH}_4\text{)}_2\text{SO}_4\)-precipitation at 2.43 M were also separated on a large scale on Sephadex G-100, using a K 100/100 column (Pharmacia) equilibrated with 0.05 M tris-HCl buffer, pH 8.0, ionic strength 0.10. One fraction, corresponding to the second peak (the “albumin peak”) in the elution diagram (Fig. 5), was saved. It was concentrated to approximately the original serum volume for further separation.

**Ion exchange chromatography:** The “albumin peak” material from the gel filtration on Sephadex G-100 was separated by ion exchange chromatography on DEAE-Sephadex A 50 (Pharmacia). The ion exchange resin was equilibrated in 0.05 M tris-HCl buffer, with 0.073 M NaCl, pH 8.0, ionic strength 0.10 (2.73 mho). A sample containing approximately 1 g protein in the same buffer was applied to 100 ml resin in a K 25/50 column (Pharmacia). The column was eluted with 500 ml starting buffer followed by two continuous linear gradients of 0.05 M tris-HCl buffer with increasing salt concentration. The first gradient was formed from the starting buffer and buffer with 0.17 M NaCl, ionic strength 0.20 and the second from the latter buffer and buffer with 0.475 M NaCl, ionic strength 0.50. Fractions of 3 ml were collected at a flow rate of 40 ml/h. After determination of the protein concentration and the conductivity, the fractions were pooled as indicated in Fig. 6, concentrated to 20 mg protein per ml, dialysed against PBS, and tested in organ culture as medium supplements.

**Protein determination:** The protein concentration in serum and serum fractions was determined from the absorbance at 280 nm using bovine serum albumin in distilled water as standard.

**Immunoelectrophoresis:** The various serum fractions were analysed by immunoelectrophoresis in agar, using an LKB 6800 A immunoelectrophoresis equipment (LKB, Stockholm, Sweden). Rabbit antiserum to whole chicken serum served as developing reagent.

**Expression of the thymus stimulating activity:** The thymus stimulating activity (TSAS) in different serum fractions and in serum after various treatments was related to that of heat inactivated (56°C, 30 min) whole chicken serum in every experiment and expressed as the mean number of cells per anlage obtained with the test material in per cent of the number of cells obtained per anlage with the 10 per cent control CS.

Student's t-test was used to evaluate the significance of the difference between group means. The calculations were performed on the number of lymphoid cells per thymic anlage after logarithmic transformation.

**Morphological examination:** The morphological development of thymic anlagen cultured with different medium supplements was examined in smears and sections.

Smears made from thymic cells suspended in 50 per cent CS were air-dried, fixed in methanol and May-Grunwald-Giemsa (MGG) stained. Sections for light and electron microscopy were prepared in one experiment. The thymic tissue was fixed in an ice-cold mixture of 1 per cent formaldehyde and 2 per cent glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.4, and embedded in Epon. Sections for light microscopy, 1 μ thick, were stained with 1 per cent toluidine blue in an
**TABLE 1. The Effect of Heating for 30 min on the Thymus Stimulating Activity of Chicken Serum**

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Protein conc. (mg/ml)</th>
<th>Stimulatory activity (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>51</td>
<td>76.0±4.7 (6)</td>
</tr>
<tr>
<td>56</td>
<td>51</td>
<td>100.0 (6)</td>
</tr>
<tr>
<td>60</td>
<td>51</td>
<td>74.7 (2)</td>
</tr>
<tr>
<td>80</td>
<td>20</td>
<td>4.3 (2)</td>
</tr>
<tr>
<td>100</td>
<td>13</td>
<td>7.4 (2)</td>
</tr>
</tbody>
</table>

* Expressed as per cent of the thymus stimulating activity of chicken serum heat inactivated at 56° C for 30 min (mean ± S.E.M.). Figures within parenthesis represent the number of experiments.

aqueous solution of 1 per cent borax. Ultrathin sections were stained with uranyl acetate followed by lead citrate and examined in a Jeol JEM 100 B electron microscope.

**RESULTS**

Heat inactivation: The effect of heating at different temperatures on the thymus stimulating activity in serum (TSAS) was tested in two complete experiments. Four further experiments were performed to specifically study the effect of heat inactivation at 56° C for 30 min. As illustrated in Table 1 heat inactivation of CS at 56° C for 30 min caused a significant increase of the TSAS ($t = 4.75$; d.f. = 5; $P < 0.01$). Heating at 60° C resulted in a loss of activity in comparison with heating at 56° C but not when related to untreated serum. After heating at 80° C and 100° C, protein precipitates were formed which decreased the protein concentration to approximately 40 per cent and 25 per cent, respectively, of the original concentration. The TSAS decreased proportionally more than the total protein concentration. Thus TSAS was relatively thermo stable up to 60° C but was inactivated or precipitated at still higher temperatures.

**pH-sensitivity:** Treatment of CS between pH 2 and 10 gave only insignificant protein precipitation. The effect on TSAS was tested in one experiment.

As demonstrated in Fig. 1, serum treated at pH 6, 9 or 10 showed the same TSAS. The number of cells obtained was 63 per cent of the number of cells obtained by the usual 10 per cent CS control. Treatment of CS at pH 5 resulted in a significant loss of TSAS as compared to pH 6 ($t = 2.55$; d.f. = 6; $P < 0.05$). Further decrease of the pH resulted in a significant successive decrease of the TSAS. The number of cells obtained by CS treated at pH 2 was only 18 per cent of the number obtained by CS treated at pH 6.

**Effects of dialysis and concentration:** These experiments are summarized in Table 2. Dialysis against PBS and ultrafiltration (Collodion and Diaflo membranes) resulted in a marked decrease in TSAS varying between experiments but usually in the order of 45

![Fig. 1. Lymphoid cell yield (log. [no. cells/anlage]) of thymic anlagen cultured in medium supplemented with untreated CS (■) or CS treated at different pH between 2–10 (●). Each point represents the mean ± S.E.M. of four cultures.](image-url)
TABLE 2. The Effect of Dialysis and Different Concentration Procedures on the Thymus Stimulating Activity of Chicken Serum (CS)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of experiments</th>
<th>Stimulatory activity (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile filtration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Millipore, 1.2 and 0.45 μm membranes</td>
<td>1</td>
<td>117.1</td>
</tr>
<tr>
<td>Ultrafiltration§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amicon UM-2 membrane</td>
<td>1</td>
<td>64.3</td>
</tr>
<tr>
<td>Amicon UM-10 membrane</td>
<td>3</td>
<td>57.2 ± 3.1</td>
</tr>
<tr>
<td>Collodion membrane</td>
<td>4</td>
<td>48.1 ± 11.7</td>
</tr>
<tr>
<td>Repeated ultrafiltration§</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Amicon UM-10 membrane: 1st filtration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lyophilization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS, 10 x dilution with distilled water</td>
<td>3</td>
<td>92.3 ± 26.2</td>
</tr>
<tr>
<td>CS, undiluted</td>
<td>2</td>
<td>90.3</td>
</tr>
<tr>
<td>Dialysis</td>
<td>5</td>
<td>60.1 ± 7.0</td>
</tr>
<tr>
<td>Repeated dialysis</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>17 hours against PBS: 1st dialysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Per cent of the thymus stimulating activity of untreated CS control. The figures represent mean ± S.E.M.  
§ Reconcentration after 10 x dilution of CS with PBS.

per cent. Filtration through a Millipore membrane or lyophilization of diluted or undiluted CS gave no significant loss of TSAS.

Effect of dialysis time: To test for the time dependent loss of active material during dialysis the following type of experiment was performed.

Eight 5-ml samples of heat inactivated CS were dialysed under sterile conditions at 4°C against a 400-fold volume of PBS. The dialysis time was 0, 9, 18, 29 hours, and 2, 3, 4, and 5 days. All samples were kept at 4°C until the last dialysis was terminated. The volumes of the samples before and after dialysis did not differ. They were therefore tested in organ culture directly.

The results of three experiments are demonstrated in Fig. 2. There was a significant loss of about 50 per cent of the original activity during the first 2 days of dialysis. The activity remained approximately constant at this level or even increased slightly until day 5. No difference in activity of control CS stored at 4°C and at -85°C during the dialysis period could be detected.

Repeated dialysis and ultrafiltration: Repeated dialysis and repeated ultrafiltration was performed to test whether further losses of the TSAS, remaining after one dialysis or ultrafiltration, occurred.

A 15-ml sample of heat inactivated CS was dialysed for 17 hours against a tenfold volume of PBS at 4°C. Ten ml of the dialysed CS were transferred to a new dialysis bag and were dialysed for another 17-hour period. The remaining 5 ml were stored at 4°C. The procedure was repeated once more. The samples of dialysed CS were tested as medium supplements in one experiment.

Table 2 shows that there was a 35 per cent decrease of TSAS during the first two 17-hour dialysis periods. The third dialysis did not result in a further loss of activity.

Fifteen ml of heat inactivated CS were diluted 10 x with PBS and reconcentrated to the original volume by ultrafiltration through a UM-10 membrane. Five ml were collected and the remaining 10 ml were diluted and reconcentrated as above. The procedure was repeated once more and the three samples of ultrafiltrated CS were tested as medium supplements in one experiment.
Table 2 shows that one ultrafiltration resulted in a loss of TSAS of 39 per cent. After a second filtration the activity decreased by another 20 per cent. The third ultrafiltration did not result in any further decrease of TSAS.

Recombination of ultrafiltrate and concentrate: To test for thymus stimulating material in the ultrafiltrate of CS the following experiment was performed:

100 ml CS was concentrated 6.5 times by ultrafiltration through a UM-10-membrane. Part of the concentrate was reconstituted to its original volume with either filtrate or RPMI 1640. Such reconstituted concentrate and the filtrate were tested as medium supplements in organ culture in two experiments. The effect of filtrate in combination with 0.625 per cent and 10 per cent CS as medium supplements was also tested in one experiment.

The results are shown in Table 3. The observed 33 per cent decrease of the TSAS in the concentrated CS was not statistically significant as compared to untreated CS (t = 1.81; d.f. = 14; P > 0.05). The filtrate showed no stimulating activity. Recombination of ultrafiltrate and concentrate gave a small, but not significant, increase of the thymus stimulating activity. Furthermore there was no significant potentiating effect of filtrate on whole 0.625 per cent or 10 per cent CS.

Ammonium sulphate precipitation: Table 4 gives the results from one of 3 (NH₄)₂SO₄

<table>
<thead>
<tr>
<th>TABLE 3. Test for the Presence of TSAS in Ultrafiltrate of CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium supplement</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>10 % concentrate</td>
</tr>
<tr>
<td>10 % filtrate</td>
</tr>
<tr>
<td>10 % concentrate + filtrate</td>
</tr>
<tr>
<td>0.625 % CS</td>
</tr>
<tr>
<td>0.625 % CS + 10 % filtrate</td>
</tr>
<tr>
<td>10 % CS + 10 % filtrate</td>
</tr>
</tbody>
</table>

* Mean ± S.E.M.; no. of cultures given within parenthesis.
§ Per cent of the thymus stimulating activity of untreated CS control.
TABLE 4. Recovery of the Thymus Stimulating Activity after Ammonium Sulphate Fractionation of Chicken Serum (CS)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein conc. (mg/ml)*</th>
<th>Stimulatory activity (%)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted and conc. CS</td>
<td>32</td>
<td>56.5</td>
</tr>
<tr>
<td>1.50 M, precipitate</td>
<td>12</td>
<td>3.4</td>
</tr>
<tr>
<td>1.50 M, supernatant</td>
<td>20</td>
<td>46.6</td>
</tr>
<tr>
<td>2.43 M, precipitate</td>
<td>9</td>
<td>9.9</td>
</tr>
<tr>
<td>2.43 M, supernatant</td>
<td>18</td>
<td>75.7</td>
</tr>
<tr>
<td>4.05 M, precipitate</td>
<td>10</td>
<td>52.9</td>
</tr>
<tr>
<td>4.05 M, supernatant</td>
<td>3</td>
<td>25.9</td>
</tr>
</tbody>
</table>

* Protein concentration of untreated CS was 40 mg/ml.
§ Per cent of the thymus stimulating activity of untreated CS control.

fractionation experiments. The volumes of the different fractions were equal to that of the original serum sample. The protein concentrations of each fraction are also shown in the table. The two controls included were 10 per cent heat inactivated CS and 10 per cent of CS dialysed, diluted and concentrated in the same way as the fractions from the precipitation procedure. The latter control showed about 50 per cent of the thymus stimulating activity of the former. The precipitates formed at a final concentration of 1.50 M (NH₄)₂SO₄ did not contain any TSAS activity. The latter remained in the supernatant. The same result was obtained in other experiments, not shown in the table, using 1.66 M and 2.03 M (NH₄)₂SO₄. Using 2.43 M (NH₄)₂SO₄, a small part of the activity was found in the precipitate. At the same time the activity appeared to increase in the supernatant. Using 4.05 M (NH₄)₂SO₄ (100 per cent saturation), the main part of the TSAS was found in the precipitate, but significant activity still remained in the supernatant.

The dose-response curve for CS depleted of γ-globulins by (NH₄)₂SO₄-precipitation at 1.42 M is demonstrated in Fig. 3 which shows the number of lymphoid cells per thymic anlage as a function of the log (concentration of agamma-CS) in two experiments. The curve has approximately the same shape as that for whole chicken serum described previously (26). It appears to be biphasic and is approximately linear at higher serum concentrations.

**Gel filtration on Sephadex G-200:** Chicken serum was fractionated on Sephadex G-200. The elution pattern of protein is shown in Fig. 4. Four peaks were obtained. The eluted material representing the four peaks were pooled as indicated in the figure. The four fractions were concentrated and tested as culture medium supplements in three separate experiments. Controls in these experiments were 10 per cent heat inactivated CS and CS diluted with elution buffer and reconstituted by collodion membrane ultrafiltration. The lower part of Fig. 4 demonstrates the thymus stimulating activity in the four fractions. The main part, 42 ± 6 per cent of the activity of the 10 per cent CS control, was found in fraction three. Fraction two contained 9 ± 4 per cent, while fractions one

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**Fig. 3.** Effect of the concentration of chicken serum precipitated with 1.42 M (NH₄)₂SO₄ on the lymphoid cell yield (No. cells/anlage) of thymic anlagen in organ culture (two experiments). Each point represents the mean of four cultures.
Fig. 4. Elution pattern of chicken serum after gel filtration on Sephadex G-200. The four fractions (I-IV), tested as medium supplements in organ cultures are indicated.

The lower part of the figure shows the thymus stimulating activity of the fractions related to the activity of whole CS (%). Mean ± S.E.M. of three experiments. Whole serum diluted and re-concentrated in the same way as the serum fractions showed 33 per cent activity.

and four showed little activity, 1 ± 0.5 per cent and 4 ± 1 per cent respectively. The diluted and re-concentrated control serum demonstrated a loss of 67 per cent of the TSAS.

Gel filtration on Sephadex G-100: The supernatant from chicken serum precipitated with 2.43 M (NH₄)₂SO₄ resolved into two large protein peaks after gel filtration on Sephadex G-100 (Fig. 5). The main constituent of the first peak was probably haptoglobin and the second peak albumin, as revealed by immunoelectrophoresis. The eluant was pooled into six fractions as shown in Fig. 5 and each was tested as medium supplement in organ culture. Thymus stimulating material was recovered from fraction four which corresponded to the albumin containing peak in the elution pattern. All other fractions, including the haptoglobin fraction, showed little stimulating activity. The active fraction four contained transferrin and at least four other minor components, besides albumin, as revealed by immunoelectrophoresis.

 Ion exchange chromatography: The albumin containing fraction from the separation on Sephadex G-100 (fraction 4) was further separated by ion exchange chromatography on DEAE-Sephadex A50 in four experiments. The material resolved into three peaks (Fig. 6 A). The results of the immunoelectrophoretic analysis (Fig. 7) revealed that

Fig. 5. Elution pattern after gel filtration on Sephadex G-100 of the supernatant from whole chicken serum precipitated with 2.43 M (NH₄)₂SO₄. The six indicated fractions (I-VI) were tested as medium supplements in organ culture.

The lower part of the figure shows the thymus stimulating activity related to the activity of whole CS (%). One experiment for fractions I, III, V and VI; mean ± S.E.M. of three experiments for fractions II and IV.
the first was dominated by transferrin and the third by albumin. The second contained 4–5 unidentified components but was virtually free of albumin. The material from these separations was tested in organ culture in 6 experiments.

Fig. 6 A shows the result of one typical experiment. The transferrin peak (fraction 1) consistently failed to stimulate thymic growth. The second peak (fraction 2) showed a small but significant stimulating activity, but this was preferentially located to the first two fractions (3 and 4) of the albumin peak. The remaining fractions (5 and 6) of this peak showed significantly less activity. Fig. 6 B shows that broadening of the eluting gradient over peak 3 resulted in a separation of the thymus stimulating activity from the albumin.

B. Same as Fig. 6 A but with a broadened elution gradient. The first fraction, containing transferrin, excluded from the figure.

The effect of varying the concentration of the active fraction (fraction 3 in Fig. 6 A or fraction 2 in Fig. 6 B) on the development of the thymus anlagen in organ cultures was studied. The fraction contained 20 mg protein per ml and was tested in concentrations ranging from 1.25 to 20 per cent (v/v) in the culture medium. Three experiments were done.

The results of one of the two experiments using fraction 2 in Fig. 6 B is shown in Fig. 8. The number of lymphoid cells per anlage increased in an almost linear fashion with the logarithmic increase of the concentrations of
Fig. 7. Immunelectrophoretic patterns of CS fractions from separation on DEAE-Sephadex A50. 
(a) Upper well first and lower well second half of the “transferrin” peak (fraction 1, Fig. 6 A).
(b) Upper well active, albumin-free fraction (fraction 2, Fig. 6 B) compared to whole CS in lower well. 
(c) Upper well active albumin-free fraction (fraction 2, Fig. 6 B), lower well inactive albumin fraction (fraction 3 in Fig. 6 B).

The mean cell yield at 20 per cent concentration was 44 per cent of that obtained by 10 per cent CS as medium supplement.

The time dependent development of the thymus in organ cultures supplemented with 10 per cent of the active albumin-free fraction (fraction 2 of Fig. 6 B) was studied in two experiments. The fraction contained 20 mg protein per ml. As shown in Fig. 9, CS and the fraction gave both a rapid increase in the number of lymphoid cells per anlage between day 0–6. With the fraction the number of cells reached a maximum on day 6. The decrease in cell number during the following 6 days (6-12) was not significant ($t = 1.45$), however. With CS the increase of cells continued and a peak value was obtained on day 9. The number of cells per anlage did not differ significantly between the serum fraction. The mean cell yield at 20 per cent concentration was 44 per cent of that obtained by 10 per cent CS as medium supplement.

Morphological examination: Virtually all cells obtained from the thymus anlagen through homogenization were typical lymphoid cells when examined in MGG-stained smears.

The morphology of thymus anlagen developed in vivo or in organ cultures for 8 days was compared. The culture medium was supplemented either with whole CS, the active albumin free fraction (fraction 2, Fig. 6 B) or with an inactive albumin fraction (fraction 5, Fig. 6 B) from the ion exchange chromatography separation. The active fraction or whole CS supported the in vitro development of the initially mainly epithelial anlage into a typical lymphoid tissue with large numbers of lymphocytes (Fig. 10, a, c). The morphology corresponded to that in vivo (Fig. 10 d), although the anlagen were considerably smaller and less densely populated by lymphoid cells. No lymphoid development occurred with the inactive fraction; instead, an atrophy of the epithelial anlagen was observed (Fig. 10 b).

Electron microscopical studies (Fig. 11 a–d) demonstrated that in the anlagen cultured with the active fraction (Fig. 11 a) or whole CS (Fig. 11 c) both the epithelial and lymphoid cells were morphologically well preserved. However, the mitochondria were often swollen and vacuolized, and central areas with degenerative changes were noted in the anlagen. The typical lymphoid cells in these cultures appeared on the average larger, containing more cytoplasm than the lymphocytes developed in vivo (Fig. 11 d). Anlagen cultured with the inactive fraction completely lacked lymphoid cells and vacuolized and pycnotic cells were frequently found (Fig. 11 b).

**DISCUSSION**

Previous work demonstrated that the presence of chicken serum was necessary for the
Fig. 8. Effect of the concentration of the active albumin-free fraction from DEAE-Sephadex A50 separation (fraction 2, Fig. 6 B) on the lymphoid cell yield (No. cells/anlage × 10⁻⁶) of thymic anlagen in organ culture. Each point represents the mean ± S.E.M. of four cultures.

Fig. 9. Time dependent lymphoid development (Log [No. cells/anlage]) of thymic anlage in organ cultures supplemented with 10 per cent active albumin-free fraction from DEAE-Sephadex A50 separation (fraction 2, Fig. 6 B) (■) or 10 per cent whole CS (●). Each point represents the mean ± S.E.M. of four cultures.

lymphoid development of the embryonic chicken thymus in organ culture (1, 26). The present study explored the possibility that the thymus stimulating activity in serum (TSAS) might be due to one or several definable serum factors.

The results show that approximately 50 per cent of the total TSAS was lost during dialysis and ultrafiltration. The loss was not further increased by prolonged dialysis, or by repeated dialysis or ultrafiltration. This may indicate that the TSAS is due to material of both higher and lower Mw than 6,000–12,000, which represents the retention limits for the dialysis and ultrafiltration membranes used. It is perhaps more likely that the loss of TSAS represents inactivation of material of high Mw, rather than escape of material of
Fig. 10. Thymic anlage of 10-day-old chicken embryo after 8 days in organ culture supplemented with:
(a) Active albumin-free CS-fraction (fraction 2, Fig. 6 B) from separation on DEAE-Sephadex A50;
(b) Inactive albumin containing fraction (fraction 5, Fig. 6 B) from same separation; (c) Whole CS.
By way of comparison, the thymus of the 18-day-old chicken embryo is shown (d). Toluidine blue (900 x).

low Mw, because no active material was recovered in the ultrafiltrate outside the membrane. Piez et al. (23) demonstrated a considerable autoproteolytic activity in dialysed human serum, probably due to enzyme inhibitors of low Mw escaping from the serum during the dialysis. A similar mechanism may account for the great loss of TSAS during ultrafiltration or dialysis in the present investigation.

The TSAS was relatively resistant to heating at 60° C for 30 minutes, but was destroyed at 80° C and 100° C. In fact, heating at 56° C significantly increased the activity. This indicated that the essential thymus stimulating activity is neither associated with a markedly heat stable nor heat labile material.

Treatment of serum at pH 2–10 resulted in decreased TSAS below pH 6. This suggests that TSAS is sensitive to acid but resistant to alkaline pH-values.

Because a dialysis step was used to neutralize the acidified sera, however, it could not be determined whether the TSAS actually was destroyed or an active low Mw component was dissociated and lost through the dialysis membrane.

The high Mw material showing TSAS precipitated at a concentration of (NH₄)₂SO₄ higher than 2.43 M (60 per cent saturation), although some activity remained in the supernatant even at 4.05 M (100 per cent saturation). Therefore the β- and γ-globulins appear to lack thymus stimulating activity. This is also suggested by the results of the fractionation of whole serum on Sephadex G-200, where all TSAS lodged in the albumin fraction. The TSAS was also found in the albumin peak, with little activity in the haptoglobinulin fraction, after Sephadex G-100 fractionation of the serum supernatant from 2.43 M (NH₄)₂SO₄-precipitation. The Mw of the TSAS may therefore be close to that of chicken serum albumin, i.e. 65,000 (20) and at least in the range of 40,000–90,000.

The two major proteins present in the active G-100 peak were transferrin and albumin, as revealed by immunoelectrophoretic analysis. Transferrin has been shown to enhance the DNA synthesis in spleen cell cultures (31), and to increase the proliferative response of lymphocytes to phytohaemagglutinin and antigen (30). It could therefore conceivable also stimulate the thymus in organ culture. Albumin was considered necessary in tissue culture systems for certain
Fig. 11. Ultrastructure of the same thymic anlage as in Fig. 10 a–d (5,300 ×).
types of cells (11). However, the results from fractionation of TSAS by ion exchange chromatography indicated that TSAS is not associated either with transferrin or albumin which lacked significant stimulating activity on the thymus in organ culture.

Thus, the TSAS cannot merely be due to the presence of protein in the organ culture. This is also evident from the fact that the serum globulins in this study or commercial ovalbumin, ovine and bovine serum albumins tested in organ culture (J. F. Sällström, unpublished results) lacked thymus stimulating activity. TSAS appears instead to be a specific factor separate from the major serum proteins. Significant TSAS was not detected in whole or in (NH₄)₂SO₄ precipitated sera from man, horse, foetal calf, rat and mouse (J. F. Sällström, unpublished results). This suggests that TSAS may be either species specific or peculiar to the chicken.

The relative activity per mg protein of the total TSAS did not increase during the separation procedures. Furthermore the apparent yield after ion exchange chromatography was only about 15 per cent. The reasons for this can only partly be the losses at dialysis and ultrafiltration, and major losses at other steps of the separation must therefore occur.

The albumin-free TSAS-fraction from the DEAE-Sephadex separation contained 4-5 proteins detectable by immunoelectrophoresis. It is not known whether TSAS is due to one or more of these unidentified proteins. This TSAS-fraction supported the development of morphologically typical lymphoid cells, ultrastructurally similar to the cells obtained by whole CS.

The number of lymphoid cells per anlage was dependent on the concentration of the TSAS-fraction and increased successively with increasing concentration. Within the tested dose range of the fraction it was not possible, however, to reach cell numbers in the anlagen of the magnitude usually obtained by 10 per cent whole CS. The time dependent development of lymphoid cells in organ culture with the active DEAE-Sephadex fraction was approximately the same as with whole CS during the first 3 days and then apparently reached a maximum earlier and at a lower lymphoid cell number than cultures with whole CS. Whether the demonstrated differences between CS and the active fraction are simply due to low concentration of TSAS in the fraction or the lack of additional thymic growth stimulating factors in this remains to be demonstrated.

Whole serum or different serum factors have been found necessary as medium supplements in most tissue or organ culture systems in various avian or mammalian species. Much work has been done to isolate and characterize such growth stimulating serum factors (for review see 11, 29). Their relation to the thymus growth stimulatory factor in chicken serum remains to be determined.

Further purification and characterization of the component(s) associated with TSAS is under way. It also remains to demonstrate whether purified TSAS represents material that is actually essential for the growth stimulating activity of CS on the embryonic thymus in organ culture. If so, the possible physiological importance of TSAS during the embryonic development of the thymus in vivo and for thymic lymphopoiesis in general is of great interest.

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