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Immunology

# Cell-free synthesis of the fourth component of guinea pig complement (C4): Identification of a precursor of serum C4 (pro-C4)

(precursor molecule/protein biosynthesis/liver polysomes/guinea pig albumin)

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**ABSTRACT** **Polysomes (S-20) from homogenates of guinea pig liver synthesized serum albumin and a precursor of the fourth component of guinea pig complement (C4) *in vitro*. The C4 precursor (pro-C4) accounted for approximately 0.2% and albumin 4% of the radiolabeled protein precipitable by trichloroacetic acid and not bound to polysomes. Pro-C4 is a single polypeptide chain (molecular weight 200,000) which is then converted to C4, a three-chain (molecular weights 95,000, 78,000, and 31,000) structure linked by interchain disulfide bridges. Pro-C4 was also detected intracellularly in short-term tissue cultures of guinea pig liver. C4 was found in the medium harvested from these cultures.**

The fourth component of complement (C4) is one of several serum proteins that act to mediate inflammation. Human C4 is a glycoprotein with a molecular weight of 209,000 consisting of three subunits linked by disulfide bridges (1, 2). Its concentration in normal serum is about 480 μg/ml (3), or approximately 0.7% of the total serum protein. Guinea pig C4, although functionally and immunochemically distinct (4, 5), is similar in size and subunit structure to the corresponding protein in human serum*.

Evidence has been obtained that guinea pig liver, spleen, and peritoneal macrophages are sites of synthesis of C4 (6–8). Studies of complement biosynthesis by isolated tissues and cells have made it possible to define, in part, the biochemical basis for genetic and microenvironmental control of synthesis rates. The cell-free synthesis of a few plasma proteins, including rat serum albumin (9), parathyroid hormone (10), human placental lactogen (11), and $\alpha_{2\mu}$ globulin (12), has been accomplished. A cell-free system for synthesis of C4 would be useful for investigation of the molecular basis of control of its synthesis.

The purpose of this report is to describe a method for the cell-free synthesis of guinea pig C4 and to present evidence that C4 is released from the polysome in a precursor form. Precursor C4 is a single polypeptide chain (molecular weight 200,000) which is then converted to a three-chain structure linked by interchain disulfide bridges.

## MATERIALS AND METHODS

Phenylmethylsulfonylfluoride, reduced glutathione, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), guanosine 5'-triphosphate, creatine phosphokinase, phosphorylase A, and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO. Adenosine 5'-triphosphate (dipotassium salt) and phosphocreatine (dipotassium salt) were from Calbiochem, La Jolla, CA. Ribonuclease-free sucrose and ovalbumin were from Schwartz/Mann, Orangeburg, NY. Bovine gamma globulin (BGG) was from Miles Laboratories, Kankakee, IL. [$^3$H]Leucine (NET-135H, 57 Ci/mmol) and [$^{14}$C]leucine (NEC 279, 270 mCi/mmol) were from New England Nuclear, Boston, MA.

In order to minimize ribonuclease activity, buffers were autoclaved for 20 min at 125°, and all heat-labile compounds were dissolved in autoclaved water. Gloves and sterile disposable pipettes were used. Glassware was soaked 8 hr in chromic acid, rinsed with distilled water, and dried for 8 hr at 135°.

**Antisera.** Preparation of guinea pig antiserum to guinea pig C4 made in C4-deficient guinea pigs, guinea pig antiserum to ovalbumin, and their globulin fractions are described elsewhere*.

Rabbit antiserum to guinea pig C4 was prepared by three injections (10 days apart) of washed immunoprecipitates (1.5 mg) of guinea pig C4 and guinea pig antiserum to C4. Serum was obtained on day 30 and absorbed twice with serum from C4-deficient guinea pigs, yielding a monospecific antiserum to $\beta_{1E}$ globulin (C4).

Guinea pig antiserum to BGG was a gift from Dr. David H. Katz (Boston, MA), and its globulin fraction was prepared. Rabbit antiserum to guinea pig albumin was purchased from ICN Biochemicals, Plainview, NY. Rabbit antiserum to porcine albumin was purchased from Microbiological Associates, Bethesda, MD.

**Guinea Pig Liver S-20.** A modification of the technique of Richardson *et al.* (13) was used. Hartley male guinea pigs (approximately 900 g) were kill by exsanguination. The livers were removed, immediately placed in ice-cold homogenization buffer (0.25 M sucrose/50 mM KCl/5 mM $MgCl_2$/3 mM reduced glutathione/50 mM Hepes at pH 7.3), and maintained at 0–4° throughout the balance of the procedure. The tissues were rinsed several times in homogenization buffer, finely minced, and then homogenized in two volumes of the same buffer by five strokes of the loose pestle of a Dounce homogenizer. The homogenate was centrifuged for 8 min at 20,000 × $g_{av}$, and the upper $\frac{4}{5}$ of the supernatant was removed and centrifuged again (8 min, 20,000 × $g_{av}$). The supernatant (6.5 ml) was then applied to a 2 × 35 cm Sephadex G-25 (coarse) column that had been equilibrated with column buffer (homogenization buffer without sucrose) and was eluted at a rate of 2 ml/min. Fractions (3 ml each) were collected, and the four most turbid fractions (eluting at approximately the void volume) were pooled and stored at −90° in small aliquots.

**Incubation Conditions.** A portion of the S-20 preparation (0.30 ml) was incubated for 90 min at 25° in a mixture containing 5 mM $MgCl_2$, 100 mM KCl, 2 mM ATP, 0.5 mM GTP, 15 mM phosphocreatine, 10 μg/ml of creatine phosphokinase, 19 amino acids (40 μM) excluding leucine, 10 μM [$^3$H]leucine (500 μCi/ml), 1.8 mM reduced glutathione, and 30 mM Hepes

Abbreviations: BGG, bovine gamma globulin; C4, fourth component of complement; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; TCA, trichloroacetic acid; pro-C4, precursor of C4.

* R. E. Hall and H. R. Colten (1977) *J. Immunol.*, in press.

Table 1. Synthesis of C4 and albumin by guinea pig liver under cell-free conditions and in tissue culture

| | Total protein, cpm × $10^{-3}$[a] | | | |
|---|---|---|---|---|
| | Before cfg.[b] | After cfg.[b] | C4, cpm × $10^{-3}$[c] | Albumin, cpm × $10^{-3}$[c] |
| Cell-free[d] | | | | |
| Complete | 1690 | 940 | 1.81[e] | 38.5[f] |
| − Energy | 30 | 5 | 0.08 | 0.10 |
| + RNase (20 μg/ml) | 0 | 0 | 0.15 | 0.30 |
| + Cycloheximide (30 μg/ml) | 430 | 60 | 0.54 | 0.01 |
| Tissue culture[g] | 13.9 | — | 0.47[h] | — |

[a] TCA precipitation, average of duplicate determinations, range ±2%.
[b] cfg. = centrifugation at 100,000 × *g* for 2 hr.
[c] Immunoprecipitates, average of duplicate determinations, range ±5%.
[d] cpm/100 μl of original cell-free reaction mixture. Energy system: GTP, ATP, creatine phosphokinase, phosphocreatine.
[e] Background of 1680 subtracted, equivalent to amount of radioactivity trapped by BGG–anti-BGG precipitate.
[f] Background of 2680 cpm subtracted, equivalent to amount of radioactivity trapped by porcine albumin–anti-porcine albumin precipitate.
[g] cpm/100 μl of 5-fold concentrated medium harvested after 3 hr in culture.
[h] Background of 120 cm subtracted as in footnote[e].

(pH 7.3), in a final volume of 0.50 ml in sterile Brinkmann microfuge tubes (Curtin Matheson Products, Medford, MA).

**Assay of Total Protein Synthesis.** A procedure similar to that of Roberts and Paterson (14) was followed. Aliquots of 5 μl were spotted on Whatman 3 MM filter paper discs, allowed to dry, and placed in a beaker containing 10% trichloroacetic acid (TCA)/10 mM leucine for 10 min. The solution was replaced with 5% TCA/10 mM leucine, and the discs were incubated at 90° for 15 min, then rinsed sequentially with 5% TCA/10 mM leucine twice, ethanol, ethanol/ether (1:1), and ether. The discs were transferred to scintillation vials, dried under a heat lamp, and shaken in a mixture of 130 μl of water and 700 μl of NCS solubilizer (Amersham) for 2 hr at room temperature. Scintillation solution (0.5% 2,5-diphenyloxazole (PPO) 0.01% *p*-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in toluene) was added and the vials were stored at 4° for 24 hr. Radioactivity was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer.

**Immunoprecipitation.** Three volumes of 0.67% deoxycholate/0.67% Triton X-100/6.7 mM leucine/100 mM KCl/50 mM Tris·HCl (pH 7.5) were added to one volume of incubation mixture, and then centrifuged at 100,000 × $g_{av}$ for 2 hr at 2° to sediment polysomes. Immunoprecipitation of C4–anti-C4 and BGG–anti-BGG (which served as control) were performed as described* except that the immunoprecipitation mixtures contained 100 μl of polysome-free supernatant, 75 μl of 0.5% sodium deoxycholate/0.5% Triton X-100/100 mM KCl/50 mM Tris·HCl (pH 7.5), 20 μl of pseudoglobulin* (carrier C4), and 5 μl of BGG (1 mg/ml). Either anti-C4 (10 μl) or anti-BGG (15 μl) was then added. Immunoprecipitation of guinea pig albumin and control porcine albumin were performed in a similar manner except that immunoprecipitation mixtures contained 45 μl of polysome-free supernatant, 152 μl of wash solution, and 3 μl of porcine albumin (1 mg/ml) to which either antiserum to guinea pig albumin (30 μl) or antiserum to porcine albumin (5 μl) was added. The amount of antiserum in each instance was chosen to provide 2-fold antibody excess and to yield 30 μg of protein precipitate. Carrier guinea pig albumin was not added because the supernatant contained sufficient albumin for immunoprecipitation. Radiolabeled C4 in tissue culture media was assayed by immunoprecipitation*.

For immunoprecipitation, using the double-antibody technique, rabbit antiserum to guinea pig C4 (10 μl, 1:80 dilution) was incubated with cell-free products (37°, 30 min), followed by a second incubation with 10 μl of goat anti-rabbit gamma globulin (37°, 30 min). For controls, normal rabbit serum was substituted for antiserum to C4 in the first incubation. Precipitates (30 μg) were harvested and washed with detergent as described above.

**Sodium Dodecyl Sulfate ($NaDodSO_4$)/Polyacrylamide Gel Electrophoresis.** The procedure of Fairbanks *et al.* (15) was followed for the preparation of and electrophoresis in 5.6% polyacrylamide/1% $NaDodSO_4$ gels. The methods of sample preparation, standard molecular weight markers, Coomassie blue staining of gels, and slicing and determining radioactivity in gels are described elsewhere*.

Radiolabeled C4 was eluted by shaking 2-mm gel slices in 1 ml of 1% $NaDodSO_4$/50 mM Tris·HCl (pH 7.5) at 37° for 18 hr. Bovine serum albumin (30 μg) was then added to the labeled C4 and the mixture was precipitated with an equal volume of 20% TCA. The precipitate was collected by centrifugation (10 min; 10,000 × $g_{av}$) and washed twice with 1 ml of acetone.

**Tissue Culture of Guinea Pig Liver Fragments.** Tissue culture of minced guinea pig liver in the presence of $^{14}C$-labeled valine, isoleucine, leucine, and lysine, the harvesting and concentration of medium, and assay of immunoprecipitable C4 are described elsewhere*.

For assay of intracellular immunoprecipitable C4, the liver fragments were rinsed three times with cold medium 199 after the original culture medium was removed. Then 400 mg of liver fragments were homogenized with 10 strokes of a Ten-Broeck homogenizer in 8 ml of cold 0.5% sodium deoxycholate/0.5% Triton X-100/100 mM KCl/50 mM Tris·HCl (pH 7.5) to which 80 μl of 200 mM phenylmethylsulfonylfluoride in ethanol had been added to retard proteolysis. The homogenate was centrifuged at 10,000 × $g_{av}$ for 10 min at 4°. Aliquots (700 μl) of the supernatant were examined for radioactivity precipitable by anti-C4 or control material precipitable by anti-ovalbumin using 20 μl of pseudoglobulin (carrier C4) or 8 μl of ovalbumin (0.5 mg/ml) as described* except that incubations required 3 hr at room temperature to complete the immunoprecipitation because of the larger volume.

## RESULTS

Preliminary experiments indicated that the S-20 incorporated [$^3H$]leucine into protein for 90 min at 25° and that maximum incorporation occurred in buffer containing 5 mM magnesium and 100 mM potassium. As shown in Table 1, incorporation was inhibited by omitting the energy-generating system, by adding ribonuclease, or by adding cycloheximide. About 44% of the

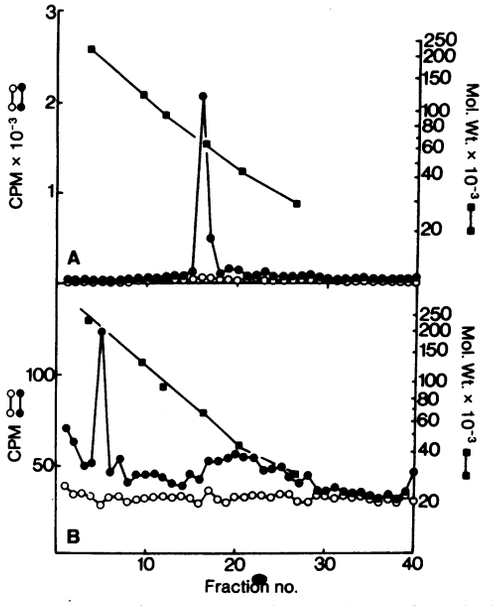


FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis in the presence of dithiothreitol (50 mM) of immunoprecipitates of cell-free reaction products. (*A*) Anti-guinea pig albumin (●), anti-porcine albumin (○). (*B*) Anti-guinea pig C4 (●), anti-BGG (○). Standard molecular weight (Mol. Wt.) markers run on a parallel gel.[Hall and Colten (1977) *J. Immunol.*, in press].

total TCA-precipitable radioactivity remained bound to polysomes. After polysomes were removed by centrifugation, approximately 0.2% of the radiolabeled protein was specifically precipitated by guinea pig antibody to C4 and 4% by antibody to guinea pig albumin. Immunochemically defined C4 protein was also detected by incubating with rabbit antiserum to guinea pig C4 followed by precipitation of immune complexes with goat antiserum to rabbit gamma globulin (cpm = 4283). Control precipitates using normal rabbit serum yielded 1680 cpm (radioactivity was distributed uniformly in $NaDodSO_4$/polyacrylamide gels). Of the total TCA-precipitable radiolabeled material secreted by liver within 3 hr in tissue culture, about 3% was precipitated by anti-C4 antibody.

The immune precipitates of C4 that was not bound to polysomes and of albumin were dissolved in electrophoresis buffer containing 1% $NaDodSO_4$ and electrophoresed on 5.6% polyacrylamide/1% $NaDodSO_4$ gels (Fig. 1). Analysis of anti-C4-precipitable material from cell-free reaction mixtures after reduction with dithiothreitol revealed a peak of approximately

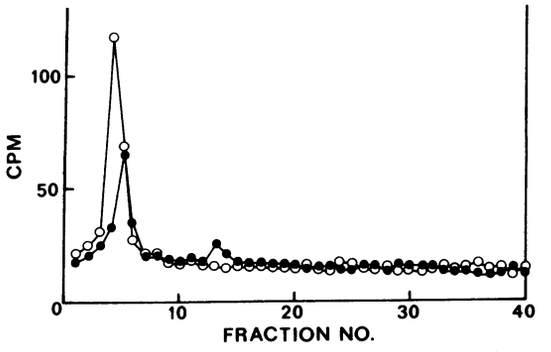


FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis in the absence of dithiothreitol of anti-C4-precipitable material eluted from $NaDodSO_4$/polyacrylamide gels (see *Materials and Methods*). $^3H$-labeled cell-free product (●) combined with $^{14}C$-labeled liver tissue culture product harvested from medium (○).

Table 2. Molecular size and subunit composition of guinea pig C4[a]

| Source of C4 | Molecular weight (× $10^{-3}$) Nonreduced | Reduced |
|---|---|---|
| Serum | 205[b] | 95; 78; 31[b] |
| Cell-free | 200 | 200 |
| Liver tissue culture | | |
| Extracellular medium | 205 | 100; 84; 31 |
| Intracellular | 200 | 200 |

[a] Molecular sizes were determined under reducing and nonreducing conditions on $NaDodSO_4$/polyacrylamide gel electrophoresis (*Materials and Methods*). Differences between serum C4 and that secreted by liver cells in culture in estimates of sizes of $\alpha$ (5%) and $\beta$ (7%) chains were not significant because each C4 preparation was analyzed on an individual gel.

[b] Hall and Colten (1977) *J. Immunol.*, in press.

200,000 daltons plus a small amount of poorly resolved material in the 30,000–70,000 dalton range. Radioactivity trapped in control BGG–anti-BGG precipitates was distributed evenly throughout the gel. The radiolabeled albumin peak migrated in a position corresponding to 68,000 daltons.

Previous studies indicated that both C4 secreted by guinea pig liver fragments in tissue culture and C4 in serum have molecular weights of about 205,000 and dissociate in the presence of dithiothreitol into three subunits of molecular weights 95,000, 78,000, and 31,000*. C4 synthesized under cell-free conditions differed significantly. In the absence of dithiothreitol, it ($^3H$-labeled) migrated slightly faster than C4 secreted by intact cells ($^{14}C$-labeled) when the two were electrophoresed in the same gel (Fig. 2). Moreover, in the presence of dithiothreitol, the mobility of cell-free C4 was unchanged while C4 secreted by intact cells dissociated into three subunits of molecular weights 100,000, 84,000, and 31,000 (Fig. 1 and Table 2). The C4 synthesized under cell-free conditions failed to dissociate under reducing conditions into subunits even after boiling for 2 min in 1% $NaDodSO_4$ alone or for 10 min in $NaDodSO_4$ plus 8 M urea (data not shown). These data suggest that under cell-free conditions C4 is released from the polysome as a single-polypeptide-chain precursor form of the molecule.

To determine whether the precursor form of C4 was synthesized by intact cells, guinea pig liver fragments were incubated in short-term culture and intracellular immunoprecipitable C4 was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The results (Fig. 3) showed that nearly all of the intracellular C4 had a molecular weight of 200,000 and failed to dissociate under reducing conditions, suggesting

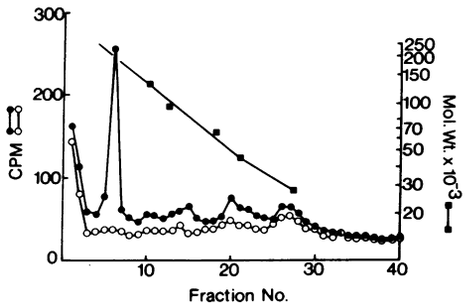


FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis in the presence of 50 mM dithiothreitol of immunoprecipitates of liver homogenate. (●) Anti-C4, (○) anti-ovalbumin. Mol. Wt. = molecular weight.

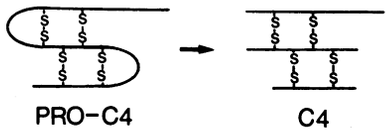


FIG. 4. Model for the conversion of single-chain precursor C4 (pro-C4), produced under cell-free conditions and detected intracellularly, to three-chain C4, isolated from serum and tissue culture medium. The number of disulfide bridges and the positions of the $\alpha$, $\beta$, and $\gamma$ chains are unknown.

similarity between the C4 precursor synthesized by intact cells and under cell-free conditions.

## DISCUSSION

Evidence has been presented for the cell-free synthesis of C4 and albumin by polysome preparations (S-20) obtained from normal guinea pig liver. Synthesis was inhibited by omitting an energy source from the incubation mixture or by the action of ribonuclease or cycloheximide. C4 synthesized under cell-free conditions had a molecular weight approximately 5000 less than C4 secreted by guinea pig liver in culture. The apparent difference in size may be accounted for by the presence of a carbohydrate moiety on the C4 secreted by liver since it is likely, although not yet established, that guinea pig C4 is a glycoprotein. The corresponding protein in human serum is known to be a glycoprotein with a carbohydrate content of about 8% (Dr. Robert Stroud, personal communication).

C4 synthesized by the isolated polysomes does not dissociate under reducing conditions into subunits ($\alpha$, $\beta$, and $\gamma$ chains) characteristic of serum C4 or C4 secreted by liver fragments in culture*. This finding suggests that C4 is synthesized and released from the polysome as a single polypeptide chain and at a later time is converted into a three-chain structure. The possibility that the precursor C4 is an artifact of the conditions required for cell-free synthesis is unlikely since precursor C4 was also detected intracellularly in homogenates of guinea pig liver incubated in short-term tissue culture; i.e., it was synthesized in intact cells. The mechanism and site of conversion of precursor C4 to C4 is not known at the present time.

A proposed model of the precursor form of C4 and its conversion to C4 is shown in Fig. 4. This model is similar to the one described for conversion of proinsulin to insulin (20). Evidence for precursor forms of other proteins has been obtained in studies of the cell-free synthesis of human placental lactogen (11), rat growth hormone (16), insulin (17), parathyroid hormone (10), and collagen (18, 19). With the exception of procollagen (molecular weight 140,000), these proteins are all less than 50,000 molecular weight. Hence, although synthesis of a precursor is not unique to C4, its size is remarkable.

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