Ontogeny of the Human Complement System: In Vitro Biosynthesis of Individual Complement Components by Fetal Tissues

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ABSTRACT The human fetal liver is capable of synthesizing the biologically active form of the second (C2) and fourth (C4) components of complement as early as 8 wk after conception, and the inhibitor of C1 (C1 INH) as early as 11 wk after conception. Biologically active C3 was produced in vitro by fetal liver obtained at 14 wk gestation. These conclusions were based on the observations that isolated fetal livers produced biologically active C2, C3, C4, and C1 INH, that this production was temperature dependent and reversibly inhibited by well-known inhibitors of protein synthesis, and that ^{14}C-labeled amino acids were incorporated into proteins immunochemically identical with these proteins. The data suggested that a large mononuclear cell was the cell type in the fetal liver that synthesized C2 and C4.

INTRODUCTION

It is known that the developing fetus can synthesize immunoglobulins and is capable of mounting an effective cellular and humoral immune response to specific antigens (1, 2). With a few notable exceptions, however, the ontogeny of the complement system, particularly in man, has not been investigated. The few studies of the ontogeny of human complement that are available have suggested that human fetal tissues are capable of synthesizing the first (C1), third (C3), and fourth (C4) components of complement, and that this capacity appears early in embryogenesis. For example, we have shown that segments of large and small intestine, obtained from a human fetus at 19 wk gestation, were capable of producing functionally active C1 in vitro (3). It has also been shown that human fetal tissues are capable of synthesizing proteins immunochemically identical with C3 (2, 4) and C4 (4), although no direct evidence was obtained that these components are produced by the fetus in a functionally active form. Recent developments in complement research have made it possible to detect in vitro the biosynthesis of biologically active complement components. As a consequence, the tissues and, in some cases, the cell types that synthesize most of the complement components have been identified (5). In earlier studies, we showed that fragments of lung, spleen, and lymph node, and bone marrow and peritoneal exudate cells obtained from adult guinea pigs are capable of synthesizing the hemolytically active form of the second component of complement (C2) in short-term tissue culture (6). Furthermore, it was demonstrated that macrophages isolated from these tissues synthesize C4, as well as C2 (7). The methods developed in the course of these studies were applied to an investigation of the ontogeny of individual complement components in the human fetus.

In this report, evidence will be presented that the liver is the major site of synthesis of C2, C3, and C4 and the natural inhibitor of C1 (C1 INH)[1] in the human fetus. This conclusion was based on the observations that liver obtained from human fetuses at gestational ages ranging from 8 to 22 wk produced C2, C3, C4, and C1 INH in vitro, that this production was highly temperature dependent, was reversibly inhibited by puromycin, cycloheximide, and actinomycin D, and that ^{14}C-labeled amino acids were incorporated into proteins immunochemically identical with C2, C3, C4, and C1 INH. Smaller amounts of C2 and C4 pro-

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[1] *Abbreviations used in this paper:* C1 INH, C1 inhibitor, VBS, veronal-buffered saline.

duction were detected in short-term cultures of spleen and thymus, but not in muscle, lung, kidney, or amniotic cell cultures.

METHODS

The source and preparation of reagents for the hemolytic assay of biologically active complement components have been described in detail (8). Methods for the hemolytic titration of human C4 and C2 and the preparation of rat CEDTA (rat serum diluted 1/5 in EDTA buffer, final molarity of EDTA 0.01) are described in reference 9. Quantitative determinations of C1 INH activity were performed as given in reference 10. Hemolytic assays of C3 activity were performed in the following way: 0.2 ml of a suspension of EAC14 (7.5×10^7 ml) was mixed with 0.2 ml of the sample and 0.4 ml of a mixture containing functionally purified human C2, C5, C6, and C7. After the mixtures were incubated at 30°C for 30 min, 0.4 ml of a solution containing human C8 and C9 was added. The resulting mixtures were then incubated at 37°C for 1 hr, then centrifuged, and the optical density of the supernatant fluid read at a wavelength of 412 nm. Veronal-buffered saline-dextrose ($\mu = 0.075$) buffer was used throughout. Controls in which the sample was omitted yielded less than 2% lysis. The number of hemolytically effective C3 molecules in each sample was calculated from the extent of lysis by methods described in reference 8.

Sheep erythrocytes were purchased from Microbiological Associates, Inc. (Bethesda, Md.); guinea pig and rat C were purchased from Suburban Serum Laboratories (Silver Spring, Md.); and human C was obtained from normal donors. Functionally purified human C3, C5, C6, C7, C8, and C9 were purchased from Cordis Corp. (Miami, Fla.).

Human fetal tissues were obtained at therapeutic abortion from fetuses delivered by hysterotomy.[2] Gestational ages of the fetuses were estimated by comparing crown to rump measurements with normal values given in reference 11. The tissues were immediately placed in ice-cold medium 199 (Microbiological Associates, Inc.) containing 50 U penicillin, 50 μg of streptomycin, and 50 U mycostatin per ml (M199). The tissues were minced, and the fragments were then washed three times in ice-cold M199. The tissue fragments were then incubated in M199 at O°C for 1 hr to permit release of preformed C2 and C4 and then washed once again in cold M199. This procedure reduced base line levels of preformed C2 and C4 to less than 7×10^7 effective C2 and C4 molecules per 100 mg tissue. For studies of C2 and C4 biosynthesis, measured portions (35–50 mg wet wt) of each of the tissues were transferred to 30-ml plastic tissue culture flasks (Falcon Plastics, Div. of Bioquest, Oxford, Calif.) and then incubated in 3.5 ml of M199 supplemented with 10% heat-inactivated (2 hr, 56°C) fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) (M199 FCS). The biosynthesis of functionally active C3 and C1 INH was examined by incubating measured portions of minced and washed liver in 3.0 ml of serumless medium (Neuman and Tytell) obtained from Grand Island Biological Co.

Individual cell suspensions were prepared from liver fragments by aspirating the tissue through progressively smaller needles (No. 18 to No. 26). The cells were then collected by centrifugation at 100 *g* for 15 min at 0°C, washed three times, and resuspended to a concentration of approximately 1×10^8 nucleated cells per ml. In one series of experiments, 0.5 ml portions of the cell suspension were fractionated on a discontinuous albumin gradient (15–33%) by centrifugation at 900 *g* for 30 min at 4°C according to published methods (12). Fractions were then removed with a sterile Pasteur pipette washed twice in M199, and each fraction was then resuspended in M199 FCS to a concentration of about 1×10^6 cells per ml. All single cell preparations were cultured in M199 FCS (final volume 2.0 ml) in 35×10 mm plastic Petri dishes (Falcon Plastics).

The incorporation of ^{14}C-labeled amino acids into complement proteins was investigated by incubating the tissue fragments for 72 hr at 37°C in a humidified atmosphere (95% air, 5% CO_2) in M199 FCS in which ^{14}C-labeled leucine (130 mCi/mmole), lysine (180 mCi/mmole), arginine (130 mCi/mmole), and valine (120 mCi/mmole) (protein ^{14}C-labeling mixture, Schwarz BioResearch Inc., Orangeburg, N. Y.) at a final concentration of 1.0 μCi/ml were substituted for the corresponding unlabeled amino acids. The tissue fragments were then removed and the media were dialyzed 3 days at 4°C against veronal-buffered saline (VBS). Each sample was then concentrated 15-fold in a Diaflo model 50 cell (Amicon Corp., Lexington, Mass.) equipped with a UM-10 membrane. A portion of each of the concentrated samples was then mixed with an equal volume of normal human serum and placed in wells cut in 1% agarose opposite specific rabbit antibodies to human C2, C3, C4, C1 INH, and transferrin.[3] After a 48 hr incubation, the immunodiffusion plates were washed for 48 hr in 0.15 M NaCl and then an additional 48 hr in distilled water. The plates were then dried and exposed to Kodak X-ray film for 4–6 wk.

Actinomycin D, puromycin, and cycloheximide were purchased from Nutritional Biochemicals Corporation (Cleveland, Ohio), and each was used at a concentration of 1–2 μg/ml in M199 FCS in experiments to test the effect of known inhibitors of protein synthesis on complement production.

RESULTS

C2 and C4 biosynthesis. Preliminary studies indicated that the amount of C2 and C4 production by fetal liver was at least 20–40 times the production of C2 and C4 by equal weights of spleen and thymus. Furthermore, there was no detectable production of C2 or C4 by muscle, kidney, lung, or amniotic cells. Accordingly, fetal liver was used in all of the following experiments.

Effect of temperature on C2 and C4 synthesis. Fragments of fetal liver were incubated at 37°C (95% air, 5% CO_2) or at 4°C in 3.5 ml of M199 FCS. At timed intervals, 0.1 ml portions of the media were removed, diluted immediately in VBS-sucrose, and the C4 and C2 content estimated. As shown in Fig. 1, incubation of liver at 37°C results in about a 200- to 300-fold increase in C2 and C4 activity. There was little or no production of C2 or C4 by tissues incubated at 4°C. When the tissue was transferred at 40 hr from 4°C to a 37°C incubator, C2 and C4 production resumed; the rates of production of C2 and C4, after warming

[2] Tissues kindly made available by Dr. Shirley Driscoll, Boston Hospital for Women, Lying-In Division, Boston, Mass.

[3] Antisera kindly supplied by Dr. Chester Alper, Children's Hospital, Boston, Mass.

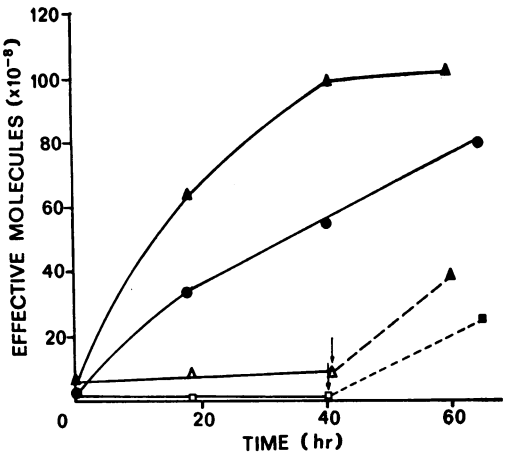

FIGURE 1 Effect of temperature on C2 and C4 production by human fetal (13 wk) liver. C2: (●) tissue incubated at 37°C, (□) incubated at 4°C; C4: (▲) tissue incubated at 37°C; (△) tissue incubated at 4°C. At time indicated by arrows, tissue transferred from 4°C to 37°C.

the tissues, were about the same as the rates of production by tissues maintained at 37°C throughout. The data given in Table I show that the in vitro production of C2 by the liver was independent of the gestational age of the fetus. At 37°C in M199 FCS, C2 was produced at a rate of about 8.9×10^8 effective molecules, and in the older fetuses, C4 was produced at a rate of about 7.2×10^8 effective molecules per 100 mg liver per hr. Liver obtained from fetuses at 11 wk gestation or less, produced C4 at a rate of about 2.5×10^8 effective molecules per 100 mg per tissue per hr.

Effect of inhibitors of protein synthesis on C2 and C4 production. The following experiments were designed to determine the effect of puromycin and actinomycin D, two well-known inhibitors of protein synthesis, on the production of hemolytically active C2 and C4 by isolated fragments of human fetal liver. Measured portions of minced liver (35–50 mg each) removed from

TABLE I

Rate of In Vitro Synthesis of C2 and C4 by Human Fetal Liver

Estimated gestational age	Crown to rump length	Effective molecules (10^{-8})/100 mg liver per hr	
		C2	C4
wk	*cm*		
8	4.0	8.0	2.4
10	6.5	11.0	2.0
11	7.5	7.5	2.6
12.5	9.0	6.2	4.0
13	10.0	12.0	6.8
13.5	10.5	8.4	4.6
14.5	12.0	10.1	11.1
22	21.0	8.0	9.8

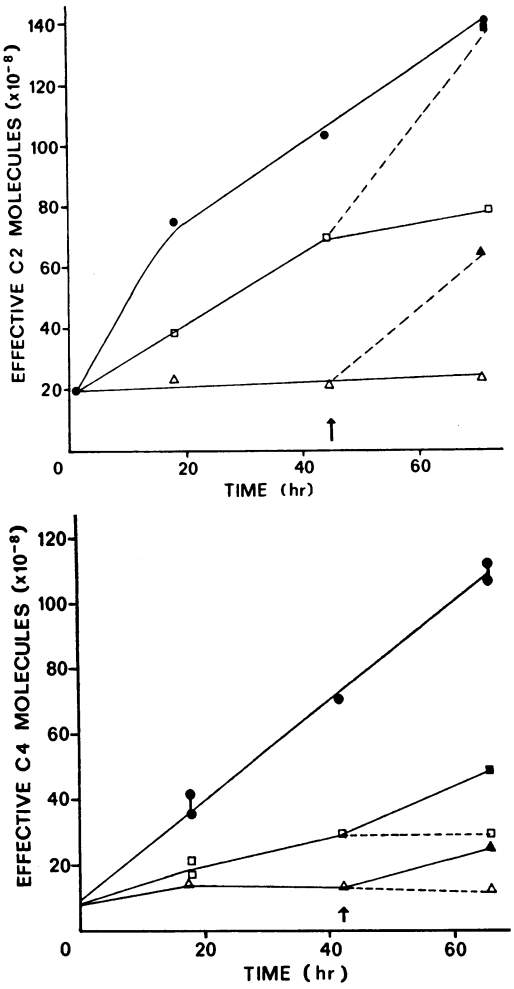

FIGURE 2 (*a*) Effect of actinomycin D and puromycin on the in vitro biosynthesis of C2 by human fetal (18 wk) liver. (●) M199 FCS alone; (□) M199 FCS containing actinomycin D (2.0 μg/ml); (△) M199 FCS containing puromycin (2.0 μg/ml). At time indicated by arrow, tissues washed and incubated in medium lacking inhibitors (solid symbols) or medium containing the appropriate inhibitor (open symbols). (*b*) Effect of actinomycin D and puromycin on the in vitro biosynthesis of C4. (●) M199 FCS; (□) M199 FCS with actinomycin D (2 μg/ml); (△) M199 FCS with puromycin (2 μg/ml). At time indicated by arrow, tissues washed and fresh M199 FCS with inhibitors (open symbols) or lacking inhibitors (solid symbols) added.

a fetus of 18 wk gestation were incubated at 37°C in M199 FCS alone and in M199 FCS containing either puromycin 2 μg/ml or actinomycin D 2 μg/ml. At timed intervals, 0.1 ml portions of the media were removed and assayed for hemolytically active C2 and C4. At 42 hr, each of the tissues was washed three times in M199 FCS. The tissues were then incubated in fresh M199 FCS or in medium containing the appropriate inhibitor at a concentration of 2 μg/ml. The results of these experiments are sum-

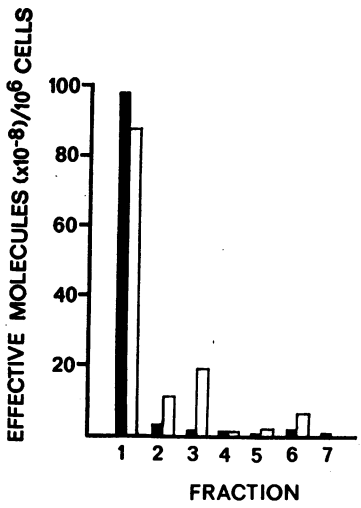

FIGURE 3 Production of C2 and C4 by human fetal (22 wk) liver cells fractionated by centrifugation at 900 *g* for 30 min on discontinuous albumin gradients (15–33%). Fraction 1 = top of gradient. *Solid bars:* C2 production by each fraction measured after incubating the cells in M199 FCS for 60 hr at 37°C (95% air, 5% CO_2). *Open bars:* C4 production by each fraction measured after incubating the cells in M199 FCS for 60 hr at 37°C (95% air, 5% CO_2).

marized in Figs. 2*a* and *b*. Incubation of the tissues in the presence of actinomycin D led to a reduction of 75% and 48% in the amount of C4 and C2 produced, respectively. Puromycin at a concentration of 2 μg/ml inhibited both C2 and C4 production approximately 90%. Inhibition of production of C2 and C4 by each of the inhibitors of protein synthesis was readily reversible. The increased rate of C2 production after release from inhibition by actinomycin D was observed in most, but not all, the experiments designed to test the effect of actinomycin D on C2 and C4 synthesis. The inhibitory effect of puromycin and actinomycin D was not a consequence of interference with either the stability or assay of C2 and C4 since preliminary experiments had indicated that these compounds at concentrations up to 10 μg/ml had no effect on the stability or measurement of preformed C2 and C4.

TABLE II

C1 INH Production by Isolated Liver In Vitro

	C1 INH* (Effective molecules (×10⁻⁸)/100 mg)		
Source of liver	37°C	37°C cyclo-heximide	4°C
Postnatal (15 yr old male)	105.0	35.4	27.0
Fetus (11 wk male)	61.8	ND‡	16.8
Fetus (17 wk female)	117.0	31.8	2.4

* All samples obtained at 70 hr incubation.
‡ ND, not done.

Preliminary attempts to identify the cell capable of synthesizing C2 and C4 by means of a modification of the Jerne plaque technique (6) were uniformly unsuccessful. Therefore, a more indirect method was used; i.e., fractionation of liver cells by centrifugation on discontinuous albumin gradients. The data summarized in Fig. 3 show the results of these experiments. Fractions from the top of the albumin gradients, containing more than 90% mononuclear cells, were the only cell populations capable of significant C2 and C4 production in culture. A small amount of C4 production was noted in fraction 3. This fraction consisted of a heterogeneous population of cells.

Biosynthesis of C1 INH. To detect the biosynthesis of biologically active C1 INH, measured portions of minced liver obtained from fetuses of 11 and 17 wk gestation and from a 14 yr old boy undergoing liver biopsy for acute pancreatitis were incubated in serumless medium. Assay of the media after 15 min and 12 hr of incubation showed no detectable C1 INH activity. At 70 hr, as shown in Table II, a significant amount of C1 INH activity was detected in each of the liver cultures maintained at 37°C. Production of active C1 INH was inhibited by incubating the tissue at 37°C in the presence of cycloheximide or by incubating tissues at 4°C. M199 FCS was found to be an unsuitable tissue culture medium for these studies since preliminary experiments showed that this medium interfered with the biologic assay for C1 INH.

Biosynthesis of C3. Fetal livers obtained at gestational ages ranging from 14 to 18 wk were examined for their capacity to synthesize biologically active C3 in vitro. The results of a representative experiment, given in Fig. 4, show that incubation of fetal liver in

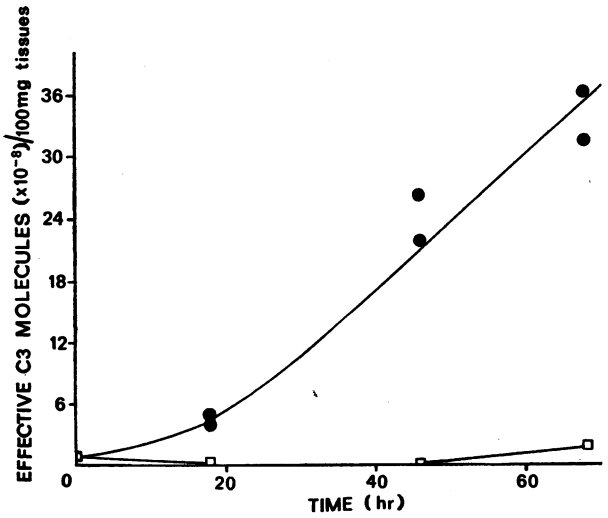

FIGURE 4 Kinetics of in vitro production of hemolytically active C3 by human fetal liver (●) 37°C; (□) 4°C.

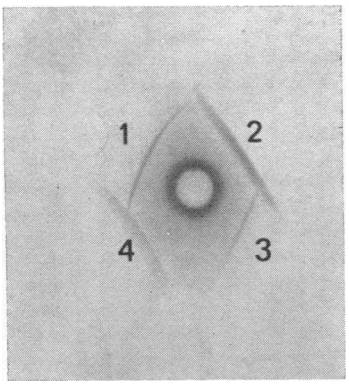

FIGURE 5 Radioimmunodiffusion analysis of incorporation of ^{14}C-labeled amino acids into complement proteins by isolated fragments of human fetal liver. Center well contains medium obtained at 72 hr from culture of fetal (19 wk) liver at 37°C: (1) anti-C3; (2) anti-C4; (3) anti-C1 INH; (4) anti-transferrin.

serumless medium at 37°C for 72 hr led to approximately a 30-fold increase in hemolytically active C3. No C3 was detected in cultures maintained at 4°C. As with the study of C1 INH biosynthesis, it was necessary to use a serumless tissue culture medium to detect production of biologically active C3 since fetal calf serum affects the stability and hemolytic assay for C3.

Incorporation of ^{14}C-labeled amino acids into complement proteins. Fig. 5 shows the results of radioimmunodiffusion analyses of media obtained from cultures containing fetal liver fragments incubated for 72 hr at 37°C in the presence of ^{14}C-labeled amino acids. These results indicated that radiolabeled amino acids were incorporated by isolated fetal liver into proteins immunochemically identical with C2, C3, C4, and C1 INH. No incorporation of label was detected in controls consisting of fetal liver incubated at 37°C in the presence of puromycin (2 μg/ml) or by tissues incubated at 4°C. The following control was designed to rule out nonspecific labeling of preformed protein: fetal liver fragments were incubated for 72 hr at 37°C in medium lacking radiolabeled amino acids. The tissue fragments were then removed and the medium mixed with the ^{14}C-labeled amino acids (1 μCi/ml). These control mixtures were then handled exactly as the experimental preparations, and no radiolabel was detected in the specific precipitin lines.

DISCUSSION

The ontogeny of several serum proteins has been studied by demonstrating that the fetal tissues are capable of synthesizing these proteins in vitro (2). Modern methods for the study of in vitro biosynthesis of complement have made it possible to utilize a similar approach for an investigation of the ontogeny of serum complement components. The data presented in this report provide direct evidence that in the human fetus the liver is a major site of synthesis of biologically active C2 and C4. Furthermore, the capacity to synthesize these proteins was present in liver obtained as early as 8 wk after conception.

It is interesting to note that the rate of C2 production by fetal liver was independent of the gestational age of the fetus. In contrast, C4 production by fetal livers obtained at 8, 10, and 11 wk gestation was less than one-third the average rate of production by livers that were obtained from older fetuses. At the present time, it is not known whether the increased rate of C4 production in the older fetuses is a consequence of an increased rate of production per cell or whether this increased rate represents recruitment of more cells capable of synthesizing C4. A detailed examination of this question may reveal some of the factors which govern the development of the capacity to synthesize C4.

The results also provided strong evidence that large mononuclear cells in the fetal liver synthesize C2 and C4. In earlier studies, Littleton, Kessler, and Burkholder (13) and we (7) had shown, using a modification of the Jerne plaque technique, that, in the adult guinea pig, a macrophage or macrophage-like cell was the site of C2 and C4 synthesis. Numerous unsuccessful attempts were made to utilize this technique to identify the cell type in human fetal liver that synthesized C2 and C4. This technique was probably unsuitable for the detection of human C2 and C4 production by individual cells in gel because of the rapid decay of human C2 from the SAC142 site (14). Nevertheless, it was possible to show by fractionating fetal liver cells on discontinuous albumin gradients that cell populations rich in large mononuclear cells were capable of synthesizing C2 and C4.

The experiments presented in this report also indicate that the fetal liver is a site of synthesis of biologically active C1 INH. These results are in agreement with the observations of Gitlin and Biasucci that human fetal liver was capable of incorporating ^{14}C-labeled amino acids into C1 INH (2) and those of Johnson, Alper, Rosen, and Craig (15), who found that fluorescent-labeled antibody to C1 INH stained hepatic parenchymal cells in sections of liver obtained from normal human adults, but not from patients with hereditary angioneurotic edema. It should be pointed out that detection of in vitro biosynthesis of biologically active C1 INH requires incubation of the tissues in media that lack serum since fetal calf serum interferes with the assay for C1 INH activity.

Other investigators have demonstrated that human fetal liver is capable of incorporating radiolabeled amino acids into a protein (beta-1-C) immunochemi-

cally identical with C3 (2, 4). In the present report, evidence was presented that isolated fragments of human fetal liver are capable of producing functionally active C3 in vitro. These observations support the conclusion that the fetal liver is a site of C3 biosynthesis, and are in accord with other studies that suggested that the adult human liver is the major, if not only, site of C3 synthesis (16).

Human fetal liver in culture was, in addition, capable of incorporating ^{14}C-labeled amino acids into molecules immunochemically identical with C2, C3, C4, and C1 INH. Incorporation of the radiolabel was temperature dependent and was inhibited by cycloheximide.

The studies presented in this report provide a basis for detailed investigations of the biosynthesis of individual complement components in the genetic complement deficiency states. It is apparent that with such studies it may be possible to localize the specific biochemical abnormalities responsible for these defects and, in some cases, may offer a rational basis for therapeutic trials.

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