Clin. exp. Immunol. (1984) **55**, 189–196.

Quantitative determination of complement components produced by purified hepatocytes

G. RAMADORI, H. RASOKAT,* R. BURGER,* K. H. MEYER ZUM BÜSCHENFELDE & D. BITTER-SUERMANN* *I. Med. Klinik und Poliklinik and* **Institut für Med. Mikrobiologie, Johannes Gutenberg-Universität, Mainz, Federal Republic of Germany*

(Accepted for publication 12 August 1983)

SUMMARY

In this report we describe, on a quantitative basis, the secretion of complement components by hepatocytes. Primary cultures were established after isolation of the cells from guinea-pig liver and the synthesis of C3, C5, C4 and C2 was measured. The cells were isolated by collagenase perfusion of the liver followed by differential centrifugation. The contamination of the hepatocyte suspension with non-parenchymal cells was less than 1%. At 24 h after plating the cells the kinetics of complement production were measured. C3 and C5 content in the culture medium harvested at different time intervals was determined by a sensitive ELISA. Secretion of C2 and C4 was measured haemolytically using C2 or C4 deficient guinea-pig serum. Under the conditions used hepatocytes secreted C3 at a rate of about 100 ng/10^6 cells/h with a plateau of secretion after 24 h of culture corresponding to about 350,000 molecules/cell/h. C5 secretion was detectable after 3–6 h of culture. The C5 secretion rate was about 15 ng/10^6 cells/24 h. The functional activity of C4 and C2 in the supernatants amounted to about 80 SFU/cell/h if the culture medium was changed every 3 h but dropped significantly if the medium was changed every 12 h. The decrease of the haemolytic activity became stronger if the medium was changed every 24 h. Cycloheximide reversibly inhibited the complement production. Our results show that guinea-pig hepatocytes synthesize considerably more C3 and C5 compared to peritoneal macrophages supporting the hypothesis that hepatocytes provide the major source of plasma complement.

Keywords hepatocyte primary culture complement biosynthesis

INTRODUCTION

It is known that the liver is responsible for 19% of total body protein synthesis in the rat; in man the export proteins account for about 50% of the 48 g proteins synthesized by the liver per day; albumin accounts for half of this protein synthesis (Kirsch, 1982). Some plasma proteins are only synthesized by the liver e.g. albumin or fibrinogen. Other plasma proteins are provided by the liver to a major portion (Davidson, 1982). This is especially true for complement as revealed by studies performed in liver transplanted patients (Alper, Johnson & Moore, 1969; Torisu *et al.*, 1972), by studies performed *in vitro* with human liver (Colten, 1972) and by studies with hepatoma cells of rat (Strunk, Tashjan & Colten, 1975) and human origin (Morris, Colten & Bing, 1982a; Morris *et al.*, 1982b). Besides the liver several cell populations were shown to produce complement components. Monocytes and macrophages synthesize C1, C2, C3, C4, C5, factor B, factor D, factors I and H

Correspondence: Professor K. H. Meyer zum Büschenfelde, I. Med. Klinik und Poliklinik Universitätsklinik, Langenbeckstr. 1, 6500 Mainz, Federal Republic of Germany.

(Einstein *et al.*, 1977; Cole *et al.*, 1980; Whaley, 1980; Fey & Colten, 1981; Zimmer *et al.*, 1982; Newell & Atkinson, 1983), fibroblasts synthesize C1 and C3 (Al-Adani & O'D McGee, 1976; Skok *et al.*, 1981; Stecher, Morse & Thorbecke, 1967), whereas epithelial cells produce C1 (Morris *et al.*, 1978). The quantitative contribution of the extrahepatic synthesis is not clear. It is conceivable that this secretion is mainly important locally, i.e. in the microenvironment of the cells. This would mean that its contribution to the plasma complement compartment might be very small. Some authors consider the liver as the main source of at least some complement components (Alper & Rosen, 1976). Saunders & Edidin (1974) detected C4 in hepatocytes by means of the immunofluorescence technique. Biochemical studies concerning the structure of C4 have been performed using C4 as produced by primary culture of mice hepatocytes (Karp, Shreffler & Atkinson, 1982). So far, no studies have been performed to quantify the capacity for complement production by purified liver cells. This paper describes in quantitative terms the complement synthesis by guinea-pig hepatocytes.

MATERIALS AND METHODS

Animals. Normal guinea-pigs were held under standard conditions.

Isolation of parenchymal liver cells and cell culture conditions. The animals were anaesthetized with Nembutal (60 mg/kg body wt) injected intraperitoneally, the abdomen was opened and the portal vein was cannulated with a 14 gauge catheter. After heparinization the liver was perfused using the two step method as described by Berry & Friend (1969) with following modifications: the liver was perfused *in situ* firstly with a calcium free buffer (Renton, Delcoria & Mannering, 1978) and then with a buffer containing collagenase (0·05%, Boehringer Mannheim, FRG) and soybean trypsin inhibitor (0·005%, Sigma München, FRG). After the perfusion the liver was transferred into a Petri dish and gently teased apart. The cell suspension was filtered through a nylon gauze and gently agitated in a water bath at 37°C for 15 min. Thereafter the cell suspension was again filtered and the cells washed three times (50 g for 3 min each). The cell suspensions used for establishing cell cultures showed a viability of 90–95% as determined by the trypan blue exclusion test and by the determination of the LDH release (LDH opt. Monotest, Boehringer, Mannheim, FRG) in the medium after treating the cells with 0·5% (final concentration) Triton X-100. The contamination with non-parenchymal cells was always below 1%. After the third washing the cell pellet was resuspended in the culture medium (Dulbecco modification of Eagle's medium containing 10 ml penicillin/streptomycin/1, 20mM HEPES, 1 μg/ml insulin, 8 mM D-glucose and with or without 10% heat-inactivated FCS). For the first day of culture litocholic acid was added to the medium (10^{-6}M) in order to inhibit growth of fibroblasts. The Petri dishes used for the cultures were coated with collagen type III from calf skin (Sigma, München, FRG) suspended in 0·2% acetic acid. The cultures were maintained at 37°C in a humidified 5% CO_2/95% air incubator. The presence of non-parenchymal cells in the hepatocyte cultures was studied by means of electronmicroscopy (Meek, 1978) kindly performed by Dr H. P. Dienes, Mainz and by the peroxidase staining (Wisse, 1974). The capacity of the hepatocyte cultures for synthesis was studied by the measurement of the production of the tyrosine aminotransferase (Granner & Tomkins, 1970).

Culture conditions for medium collection. Three millilitres of the cell suspension (10^6 cells/ml) were plated onto Petri dishes (6 cm Ø). Twenty-four hours after plating, the cell cultures were washed four times with 3 ml medium, the last 3 ml were used as control samples (corresponding to time zero of culture).

Cultures were performed in duplicate, and after 3, 6, 9, 12, 24 h of culture, the supernatants were harvested, centrifuged (400*g*, 5 min) to eliminate dead cells and immediately frozen in small aliquots at −70°C. In a second set of cultures held under the same conditions as described above, the medium of each culture was harvested at 3 h and the following repeated medium changes were performed at the same times described above. Inhibition of protein synthesis was performed adding 2–20 μg/ml cycloheximide (Sigma, München, FRG) to the culture medium.

Assay for guinea-pig C3. The supernatants were assayed for guinea-pig C3 (GPC3) content using an ELISA as described elsewhere (Zimmer *et al.*, 1982). Briefly, 10 μl IgG anti-GPC3 (800 ng

protein) were incubated in each well of a microtitre plate (Dynatech Lab., Denkendorf, FRG); the surface was then saturated with 1% BSA. Ten microlitres of the supernatants were incubated in the coated wells for 1h. After washing, 10 μl of the peroxidase labelled anti-C3 IgG were added and incubated or 75 min. After washing 20 μl of 50 mM ABTS (2,2′-azinodi-ethylthiazoline-6-sulphonic acid diamonium salt; Serva Heidelberg, FRG) in potassium phosphate (pH 6·0) containing 0·025% H_2O_2 were added. The reaction was stopped after 2 h incubation by adding potassium phosphate (pH 6·0) containing 0·0125% NaN_3 and the absorbance at 414 nm was measured using a Titertek Multiscan MC machine (Flow Lab., Bonn, FRG). As standard, purified GPC3 was used in concentrations between 1 and 100ng C3/ml.

Assay for GPC 5. The supernatants were tested in a C5 specific ELISA using monoclonal antibodies against guinea-pig C5 obtained by standard techniques after immunization with purified C5. The test was performed as follows: monoclonal antibody 224 was absorbed to a microtitre plate and the plate washed with BSA (1%) buffer. Supernatants as source of C5 or a standard of GP C5 prepared as described previously (Meuer *et al.*, 1981) served as antigen. After washing with BSA buffer, a second monoclonal antibody (217) reacting with another epitope of C5 and coupled with β-galactosidase (105031, 2,2 M/1 Boehringer, Mannheim, FRG) was used as indicator reagent; 4-nitrophenyl-β-D-galactopyranoside (Boehringer, Mannheim, FRG) served as substrate of the enzyme. The galactosidase was coupled to the monoclonal antibody using SPDP (Pharmacia, Freiburg, FRG) according to the manufacturers instructions. The optical density was measured at 414 nm wave length after 24 h incubation at room temperature.

Assay for C2 and C4. The presence of C2 and C4 in the culture medium was determined haemolytically. Sensitized erythrocytes (EA) were incubated with the medium samples in the presence of serum obtained from GP genetically deficient in C2 or C4 (Bitter-Suermann *et al.*, 1981; Gaither, Alling & Frank, 1974). The reaction mixture was incubated for 1 h at 37°C. The

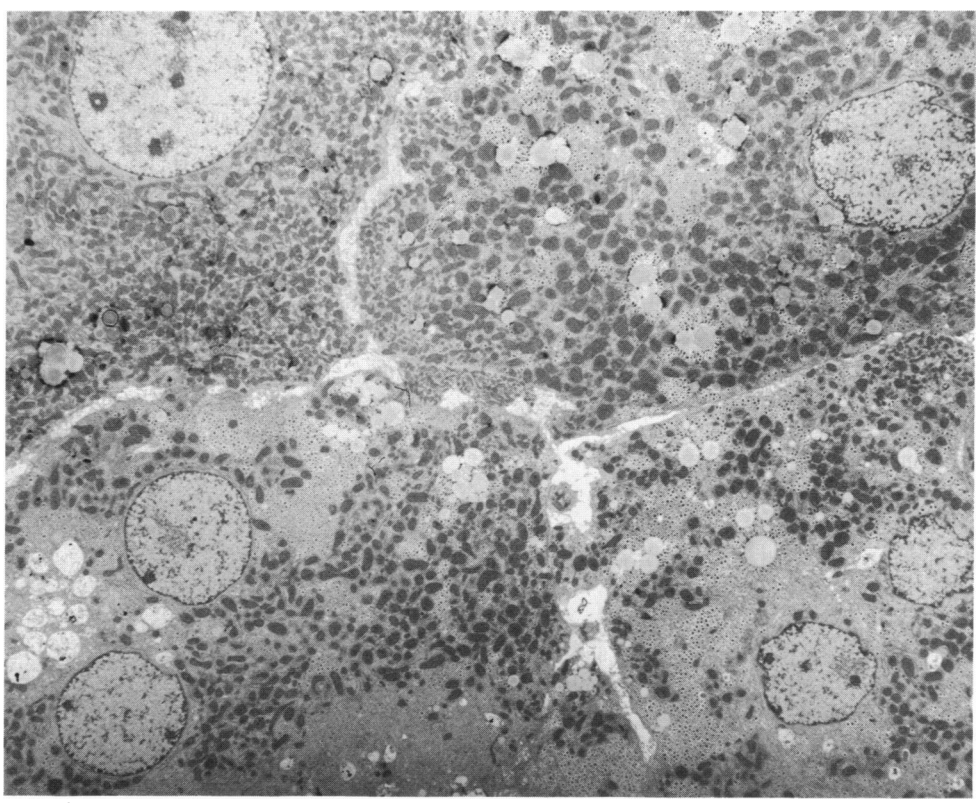

Fig. 1. Electronmicroscopic picture of a GP hepatocyte monolayer 24 h after plating (× 2,254).

haemoglobin release was determined after adding 1 ml veronal buffer by measuring absorbance of the supernatant at 412nm. For the extent of the lysis the number of site forming units (SFU) was calculated according to Gaither *et al.* (1974).

RESULTS

Morphology of the GP hepatocyte cultures

As shown in the Fig. 1, the hepatocytes in the 24 h old monolayer cultures have kept original polygonal form, the junctional complexes were reestablished and biliary poles can be detected by electronmicroscopy. The peroxidase staining detected one positive cell (Kupffer cell) per about 200–300 hepatocytes. The same results were obtained by electronmicroscopy. No other cells were detected.

Biosynthesis of C3 and C5 by GP parenchymal liver cells

The results of three separate experiments, each of them performed in duplicate, are reported in Fig. 2. The C3 amounts were determined in the culture medium which was harvested and replaced with fresh medium at 3, 6, 9, 12 and 24 h after beginning the study. On each panel of the figure the calculated sum of the C3 content in each sample is reported. The total C3 synthesis in these experiments reached 2,500–3,000 ng/10^6 cells/24 h corresponding to about 100 ng/10^6 cells/h and to 350–400,000 molecules/cell/h. In Fig. 3 a comparison of the results obtained by the two different experiment sets described in Materials and Methods is shown. As it can be seen the total amount of

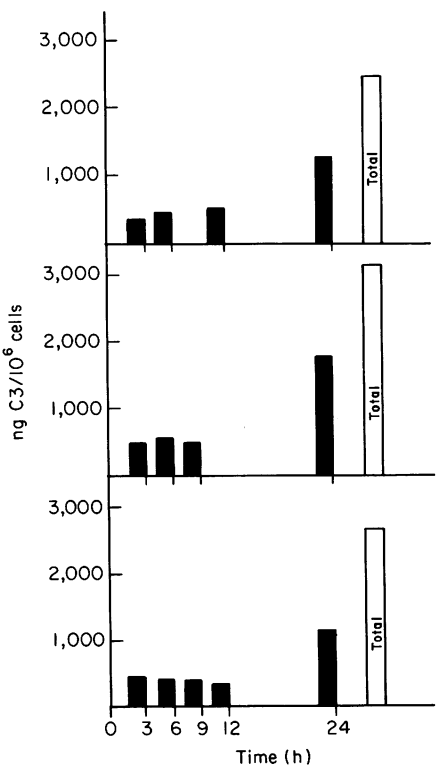

Fig. 2. C3 synthesis by GP hepatocytes. The figure represents three separate experiments each performed in duplicate. The synthesis of C3 was measured in the harvested culture medium at different time intervals (for more details see text). The s.d. was always less than 10%. The solid columns represent the amount of the C3 at the different times. The open columns represent the calculated sum of the individual samples.

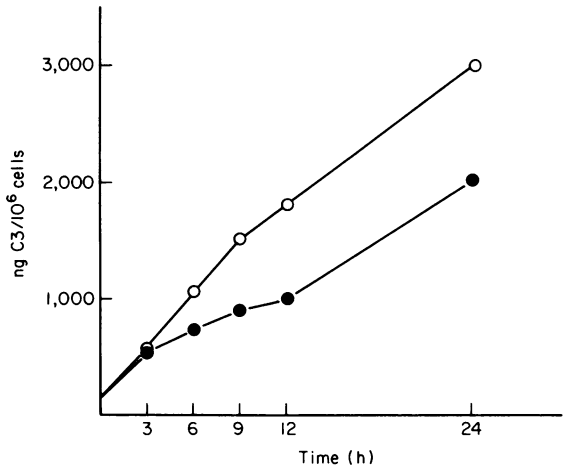

Fig. 3. C3 synthesis by GP hepatocyte cultures. The figure shows the comparison of the results obtained in the two different experiment sets (see text). The circles represent the average of three experiments each performed in duplicate. Closed circles: harvesting of culture supernatant after culture from 0–3 h, or 0–6 h or 0–9 h and so on. Open circles: medium change as in Fig. 2.

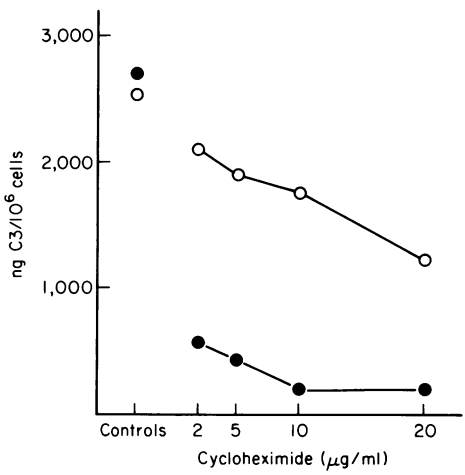

Fig. 4. Cycloheximide inhibition of the synthesis of C3. (●) First 24 h of culture with and without (control) different concentrations of cycloheximide. (○) Second 24 h of culture without cycloheximide.

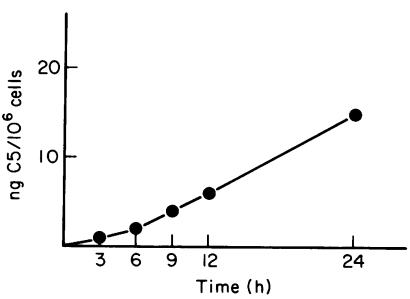

Fig. 5. Synthesis of C5 by GP hepatocyte culture. Represented are the results obtained by means of a C5 specific monoclonal ELISA. Circles represent the accumulated (without medium change) C5 at different time intervals. Three different experiments each of them in duplicate were performed.

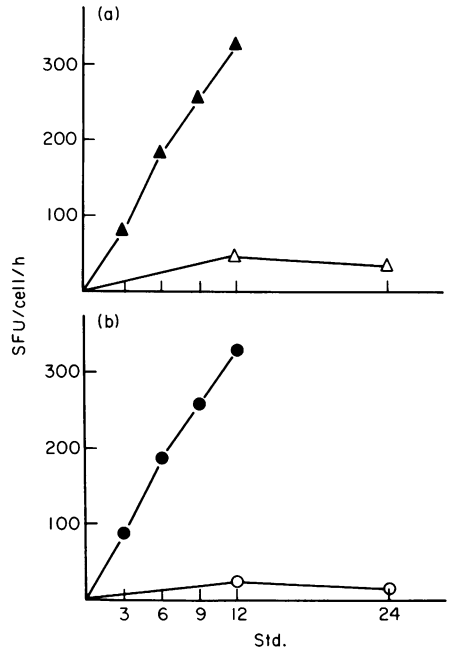

Fig. 6. Determination of the haemolytic activity of C2 (a) and C4 (b) in the culture medium. Closed circles represent experiments with medium change performed every 3 h. The increase at 3 h interval is given as site forming units (SFU). The open circles represent experiments where cultures were run for 12 h or 24 h without medium change.

C3 measured in the first experimental set is considerably lower than the sum of the C3 contents of those cultures, the medium of which was changed in intervals. The synthesis of C3 is reduced to about 15% after adding 5 μg/ml cycloheximide to the culture medium (Fig. 4). The inhibitory effect is almost totally reversible. In Fig. 5 the results of the C5 determinations in the medium of hepatocyte cultures which were interrupted at the time indicated in the figure are reported. The determinations demonstrate that GP hepatocytes in primary culture are able to synthesize C5 in an amount of about 15 ng/10^6 cells/24 h. The synthesis was also reversibly inhibited by cycloheximide (data not shown).

Synthesis of haemolytically active C2 and C4

As reported in Fig. 6 hepatocytes produce about 80 SFU/cell/h of C2 and of C4 if the culture medium is changed every 3 h. The haemolytic activity of the two components drops if the medium is changed only every 12 h and this reduction becomes stronger if the medium is changed every 24 h.

DISCUSSION

The liver consists mainly of hepatocytes, tissue macrophages (Kupffer cells), endothelial cells and fat storing cells (Wisse & Knook, 1979). The Kupffer cells which represents about 90% of the tissue macrophages are believed to originate from the bone marrow (Van Furth, Crofton & Diesselhoff-den Dulk, 1979) and to have many similarities with blood and peritoneal macrophages. It is known that peritoneal and bronchoalveolar macrophages are able to produce most of the complement components of the classical and alternative pathway. In reviewing the literature, to our knowledge there is no information available with regard to the cell population within the liver which is responsible for the synthesis of the bulk of the plasma complement components. In our experiments we measured quantitatively the synthesis of C3 and C5 and haemolytically the production of C2 and

C4 by GP primary hepatocyte cultures. The cell population was demonstrated to be pure and metabolically active under the culture conditions used. Kupffer cells could be detected in the culture only very seldom. Using the ELISA technique we found that GP hepatocyte primary cultures under the conditions used, secreted about 350–400,000 molecules/cell/h of C3. This was reversibly inhibited by cycloheximide. The finding that the sum of C3 found in the cultures where the medium was changed several times during the first 24 h of culture was higher than the C3 content found in the cultures where the medium was changed only every 24 h, suggests that the C3 molecules are catabolized with consequent loss of the antigenic activity. Alternatively, either a deterioration of the culture conditions or the presence of a negative feed back as found for the C4 synthesis in peritoneal macrophages by Auerbach, Baker & Colten (1983) seem reasonable explanations. Pulse chase experiments to clarify these questions are in progress. Using the ELISA, C5 could be already detected after the first 3 h of culture. Compared with the previously reported data (Zimmer *et al.*, 1982) regarding the production of C3 and C5 by peritoneal macrophages, the hepatocytes synthetize 15 times more C3 than the macrophages. A C5 production by the GP macrophages could not be detected at all while hepatocytes synthetize C5 at a rate of about 15 ng/10^6 cells/24h. The determination of C2 and C4 in the culture supernatant showed a constant haemolytic activity if the medium was changed in 3 h intervals and a rapid drop especially of the C4 level if the interval for the medium change increased. Morris *et al.* (1982a) described a similar phenomenon and hypothesized a Cls-mediated cleavage of C2 and C4 in the culture medium of human hepatoma cells. Thus, the secretion rate calculated should be underestimated. Resident GP peritoneal macrophages produce 40 SFU/cell/h C2 and C4 (Zimmer *et al.*, 1982), this is two times less than that produced by cultured hepatocytes.

Our *in vitro* system has the advantage of cultivating normal adult hepatocytes of GP, the complement system of which is well known and characterized. This could be helpful in the study of complement deficiencies in animals (Burger *et al.*, 1983) which may have similarities with human deficiencies (Einstein *et al.*, 1977). The presented results show that hepatocytes probably play the major role in the production of plasma C3, C5, C2 and C4.

We are grateful to Mrs U. Koehler for her technical assistance, to Dr H. P. Dienes from the Institute of Pathology for performing the EM pictures. The work was supported by the DFG, grant Ra 362/1-1 and by grants of SFB 107 Mainz.

REFERENCES

AL-ADANI, M.S. & O'D MCGEE, J. (1976) Clq production and secretion by fibroblasts. *Nature*, **263**, 145.

ALPER, C.A., JOHNSON, A.M. & MOORE, F.D. (1969) Human C3. Evidence for the liver as the primary site of synthesis. *Science*, **163**, 286.

ALPER, C.A. & ROSEN, F.S. (1976) Genetics of the complement system. *Adv. Hum. Genet.* **7**, 141.

AUERBACH, H.S., BAKER, R.D. & COLTEN, H.R. (1983) Molecular mechanism of feedback inhibition of C4 biosynthesis. *Immunobiol.* **164**, 208.

BERRY, N.M. & FRIEND, D.S. (1969) High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. *J. cell Biol.* **43**, 506.

BITTER-SUERMANN, D., HOFMANN, T., BURGER, R. & HADDING, U. (1981) Linkage of total deficiency of the second component (C2) of the complement system and of genetic C2-polymorphism to the major histocompatability complex of the guinea pig. *J. Immunol.* **127**, 608.

BURGER, R., GORDON, J., STEVENSON, G., ZANKER, B., RAMADORI, G., HADDING, U. & BITTER-SUERMANN, D. (1983) An inherited deficiency of the third component of complement (C3) in guinea pigs. *Immunobiol.* **164**, 220.

COLE, F.S., MATTHEWS, W.J., MARINO, J.T., GASH, D.J. & COLTEN, H.R. (1980) Control of complement synthesis and secretion in bronchoalveolar and peritoneal macrophages. *J. Immunol.* **125**, 1120.

COLTEN, H.R. (1972) Ontogeny of the human complement system. *In vitro* biosynthesis of individual complement components by fetal tissues. *J. clin. Invest.* **51**, 725.

DAVIDSON, C.S. (1982) Protein metabolism in relation to liver disease. In *The liver annual 2.* (ed. by I. M. Arias, M. Frenkel & H. H. P. Wilson) pp. 64–73, Excerpta Medica, Amsterdam.

EINSTEIN, L.P., HANSEN, P.J., BALLOW, M., DAVIS, A.E., DAVIES, J.S., ALPER, C.A., ROSEN, F. & COLTEN, H.R. (1977) Biosynthesis of the third

component of complement (C3) *in vitro* by monocytes from both normal and homozygous C3-deficient humans. *J. clin. Invest.* **60,** 963.

FEY, G. & COLTEN, H.R. (1981) Biosynthesis of complement components. *Fed. Proc.* **40,** 2099.

GAITHER, T.A., ALLING, D.W. & FRANK, M.M. (1974) A new one-step method for the functional assay of the fourth component (C4) of human and guinea pig complement. *J. Immunol.* **113,** 574.

GRANNER, D.K. & TOMKINS, G.M. (1970) Tyrosine aminotransferase (rat liver), *Meth. Enzymol.* **17,** 633.

KARP, D.R., SHREFFLER, D.C. & ATKINSON, J.P. (1982) Characterization of the M_r difference between secreted murine fourth component of complement and the major plasma form. Evidence for carboxyl-terminal cleavage of the α-chain. *Proc. Natl. Acad. Sci. USA.* **79,** 6666.

KIRSCH, R.E. (1982) Protein synthesis. In *Clinical Hepatology* (ed. by G. Csomos & H. Thaler) pp. 78–86. Springer-Verlag, Berlin.

MEEK, G.A. (1978) *Practical electronmicroscopy for biologists. 2nd edn.* Wiley Publications, Chichester.

MEUER, S., ECKER, U., HADDING, U. & BITTER-SUERMANN, D. (1981) Platelet-serotonin release by C3a and C5a: two independent pathways of activation. *J. Immunol.* **126,** 1506.

MORRIS, K.M., COLTEN, H.R. & BING, D.H. (1978) The first component of complement. A quantitative comparison of its biosynthesis in culture by human epithelial and mesenchymal cells. *J. exp. Med.* **148,** 1007.

MORRIS, K.M., COLTEN, H.R., & BING(1982a) Complement biosynthesis by the human hepatoma-derived cell line Hep G2. *J. clin. Invest.* **70,** 906.

MORRIS, K.M., GOLDBERGER, G., COLTEN, H.R., ADEN, D.P. & KNOWLES, B.B. (1982b) Biosynthesis and processing of a human precursor complement protein. Pro-C3 in a hepatoma-derived cell line. *Science,* **215,** 399.

NEWELL, S.L. & ATKINSON, J.P. (1983) Biosynthesis of C4 by mouse peritoneal macrophages. II. Comparison of C4 synthesis by resident and elicited cell populations. *J. Immunol.* **130,** 834.

RENTON, K.W., DELCORIA, L.B. & MANNERING, G.J. (1978) Effects of polyriboinosinic acid polyribocytidylic acid and a mouse interferon preparation on cytochrome P-450-dependent monoxygenase systems in cultures of primary mouse hepatocytes. *Mol. Pharmacol.* **14,** 672.

SAUNDERS, D. & EDIDIN, M. (1974) Sites of localization and synthesis of Ss protein in mice. *J. Immunol.* **112,** 2210.

SKOK, J., SOLOMON, E., REID, K.B.M. & THOMPSON, R.A. (1981) Distinct genes for fibroblast and serum Clq. *Nature,* **292,** 549.

STECHER, V.J., MORSE, J.H. & THORBECKE, G.J. (1967) Sites of production of primate serum proteins associated with the complement system. *Proc. Soc. exp. biol. Med.* **124,** 433.

STRUNK, R.C., TASHJAN, A.H. & COLTEN, H.R. (1975) Complement biosynthesis *in vitro* by rat hepatoma cell strains. *J. Immunol.* **114,** 331.

TORISU, M., YOKOYAMA, T., KOHLER, P.F., DURST, A.L., MARTINEAU, G., SCHROTER, G., AMENIYA, H., GROTH, C.G. & STARZL, T.E. (1972) Serum complement after orthotopic transplantation of the human liver. *Clin. exp. Immunol.* **12,** 21.

VAN FURTH, R., CROFTON, R.W. & DIESSELHOFF-DEN DULK, M.M.C.(1979) The bone marrow origin of Kupffer cells. In *Kupffer cells and other liver sinusoidal cells* (ed. by E. Wisse & D. L. Knook) pp. 471–480, Elsevier/North-Holland Biochemical Press, Amsterdam.

WHALEY, K. (1980) Biosynthesis of complement components and the regulatory proteins of the alternative complement pathway by human peripheral blood monocytes. *J. exp. Med.* **151,** 501.

WISSE, E. (1974) Observations on the fine structure and peroxidase cytochemistry of normal rat liver Kupffer cells. *J. Ultrastruct. Res.* **46,** 393.

WISSE, E. & KNOOK, D.L. (1979) The investigation of sinusoidal cells. A new approach to the study of liver function. In *Progress in liver diseases* (ed. by H. Popper & F. Schaffner) Vol. VI. pp. 153–171, Grune and Stratton, New York.

ZIMMER, B., HARTUNG, H.P., SCHARFENBERGER, G., BITTER-SUERMANN, D. & HADDING, U. (1982) Quantitative studies of complement component C3 by resident, elicited and activated macrophages. Comparison with C2, C4 and lysosomal enzyme release. *Eur. J. Immunol.* **12,** 426.