The Isolation and Characterization of Bovine C4a, an Activation Fragment of the Fourth Component of Complement

By Nuala A. BOOTH,* R. Duncan CAMPBELL,† Marjorie A. SMITH and John E. FOTHERGILL

Department of Biochemistry, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, Scotland, U.K.

(Received 15 March 1979)

The fourth component of bovine complement, C4, was cleaved specifically by subcomponent C1s to produce two fragments, C4a and C4b. The smaller, C4a, was isolated in pure form and is a peptide of 9500 mol.wt. containing approx. 84 amino acids and no detectable carbohydrate. C4a has an amino acid composition that is comparable with the anaphylatoxins C3a and C5a, containing six cysteine residues/mol and a high proportion of basic residues. The amino acid sequence of the first thirteen residues shows four identities with the porcine C3a sequence. There is almost complete identity between the C4a sequence and that of the α -chain of human C4, indicating that this region is highly conserved. This evidence also clearly establishes that C4a is cleaved from the *N*-terminal of the α -chain of C4.

The classical pathway of complement activation (reviewed by Fothergill & Anderson, 1978; Porter & Reid, 1978) is initiated by antibody–antigen complexes. Activation of the first component, C1, results in the formation of an active proteinase, subcomponent C1s. This cleaves both component C4 and component C2, giving rise to the C4,2 complex or C3-convertase.

In early reports on the interaction of C4 and C1s, it was observed that the addition of C1s to serum caused rapid inactivation of C4 (Lepow *et al.*, 1956; Haines & Lepow, 1964). However, C4 was only capable of binding to sensitized erythrocytes if activated C1 was bound (Müller-Eberhard & Biro, 1963). This apparent discrepancy was resolved by the finding that the inactivation of C4 by C1s occurred only in free solution and consisted of the same reaction as the activation of C4 by EAC1 cells (Müller-Eberhard & Lepow, 1965). This reaction produced C4 with an increased negative charge and a decreased sedimentation coefficient. It was capable of forming complexes with other activated C4 molecules, with unsensitized erythrocytes or with γ -globulin (Müller-Eberhard & Lepow, 1965). That there was another product of the reaction was not discovered until 1970, when C4a, a

low-molecular-weight peptide, was demonstrated (Budzko & Müller-Eberhard, 1970; Patrick *et al.*, 1970). C4a, with a mol.wt. of 8000–16000, could be distinguished from C4b by ultracentrifugation, gel filtration or polyacrylamide-gel disc electrophoresis, but both groups of workers found that acidification of the inactive C4 was essential for dissociation of the fragments.

By the use of SDS/polyacrylamide-gel electrophoresis, it was demonstrated that C4a was cleaved from the α -chain of C4 (Schreiber & Müller-Eberhard, 1974). In this, C4a resembles C3a (Hugli, 1975a) and C5a (Fernandez & Hugli, 1976), both of which are cleaved from the α -chain of C3 and C5 respectively. Both of these peptides, known as anaphylatoxins, have potent biological activities (reviewed by Hugli & Müller-Eberhard, 1978), and the possible biological activity of C4a was therefore of interest. Patrick *et al.* (1970) found human C4a to be inactive in a number of systems, but some activity in contracting uterine muscle was observed (Budzko & Müller-Eberhard, 1970).

Investigations of the structure of human C4a were hampered by the small quantities of the peptide in both these studies. Now that purified bovine C4 is available in relatively large quantities (Booth *et al.*, 1979), the isolation and characterization of bovine C4a has been undertaken as a preliminary to more detailed structural studies.

Materials and Methods

Materials

Materials were obtained from the following sources: *N*-(2-aminoethyl)-3-aminopropyl–glass, *p*-phenylene di-isothiocyanate, amino acid phenyl-

Abbreviations used: the nomenclature of complement components and subcomponents is that recommended by the World Health Organisation (1968). Activated components are indicated by a bar, e.g. C1s. SDS, sodium dodecyl sulphate; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; h.p.l.c., high-pressure liquid chromatography.

* Present address: Department of Medicine, University of Aberdeen, Medical School, Foresterhill, Aberdeen AB9 2ZD, Scotland, U.K.

† Present address: MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, Oxford OX1 5QU, U.K.

thiohydantoin standards, Pierce and Warriner, Chester, U.K.; phenyl isothiocyanate, acetonitrile, *N*-methylmorpholine, trifluoroacetic acid, Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K.; Sephadex G-100 and G-25, Pharmacia Fine Chemicals, Uppsala, Sweden; iodo[2-¹⁴C]acetate, The Radiochemical Centre, Amersham, Bucks., U.K.; μ -Bondapak C₁₈, Waters Associates, Hartford, Cheshire, U.K.

Bovine C4 was isolated as described previously (Booth *et al.*, 1979), and C1s as described in the following paper (Campbell *et al.*, 1979b). Purified human C1r was prepared by the method of Ziccardi & Cooper (1976).

Cleavage of component C4

Purified C4 in 28mM-phosphate buffer, pH 6.6, containing 2mM-EDTA and 150mM-NaCl, was incubated with C1s (Booth *et al.*, 1979) in a weight ratio of 100:1 for 30 min at 37°C. The reaction was terminated by rapid cooling, followed by desiccation or acidification.

Isolation of C4a

The C4 digest, containing 40–50 mg of protein, was acidified to pH 3.5 with 0.5M-HCl, applied to a column (2.5cm × 100cm) of Sephadex G-100 and eluted with 0.15M-NaCl, pH 3.5 (Budzko & Müller-Eberhard, 1970). The acidification procedure caused the formation of a protein precipitate, which was removed before gel filtration. The fractions containing C4a were localized by SDS/polyacrylamide-gel electrophoresis and pooled. After concentration by freeze-drying, C4a was desalted in a column of Sephadex G-25 equilibrated with 1% (w/v) NH₄HCO₃. The material was freeze-dried, dissolved in water at a concentration of 1 mg/ml and stored at –20°C.

Haemolytic assay of C4 activity

C4 haemolytic activity was assayed as described previously (Booth *et al.*, 1979). Results are expressed in CH₅₀ units, the reciprocal of the final dilution of test material that lyses 50% of the available cells.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. C4a concentrations were measured by quantitative amino acid analysis.

Electrophoresis in polyacrylamide gel

Electrophoresis was performed in the presence of SDS in 5 or 10% (w/v) polyacrylamide gels as described by Weber *et al.* (1972).

Electrophoresis at pH 4.5 was by the method of Reisfeld *et al.* (1962), with 5.8% polyacrylamide gels.

Gels were stained for protein with Coomassie Brilliant Blue and for carbohydrate by periodic acid/Schiff's reagent as described by Campbell *et al.* (1979a).

Amino acid analyses

These were carried out as described by Campbell *et al.* (1979a).

N-Terminal amino acid determination and automatic sequencing

The *N*-terminal amino acid of C4a was identified by dansylation and t.l.c. of the dansyl-amino acid on polyamide sheets (Gray, 1972).

N-Terminal-amino acid sequencing of C4a was done with a solid-phase sequencer (Laursen, 1971) constructed in this laboratory. The coupling procedure was based on the use of phenylene di-isothiocyanate (Laursen *et al.*, 1972) with aminoethylaminopropyl-glass beads (Bridgen, 1975). The peptide (100 nmol) was reduced and carboxymethylated with iodo[2-¹⁴C]acetate (Crestfield *et al.*, 1963), desalted, freeze-dried and redissolved in 2.5 ml of *N*-methylmorpholine buffer, pH 9.4 (*N*-methylmorpholine/H₂O, 1:1, pH adjusted to 9.4 with trifluoroacetic acid), containing 1% (w/v) SDS. It was heated at 45°C for 20 min before addition of 200 mg of aminoethylaminopropyl-glass beads to which *p*-phenylene di-isothiocyanate had been coupled (Wachter *et al.*, 1973). The suspension was shaken gently at 45°C for 30 min under an atmosphere of N₂. Excess ethanolamine (1 ml) was added and the incubation continued for a further 30 min. The beads were washed with 3 × 3 ml of methanol, dried under vacuum and saturated with trifluoroacetic acid (0.5 ml). After incubation at 37°C for 20 min, they were washed with 3 × 3 ml of butyl chloride and dried under vacuum. The extent of coupling was determined by liquid-scintillation counting of a sample of beads added to 6 ml of toluene containing 0.6% (w/v) PPO (2,5-diphenyloxazole) and 0.0075% (w/v) POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]. The linked beads were stored under N₂ at –20°C and sequenced by stepwise Edman degradation. The thiazolinones released were converted into amino acid phenylthiohydantoin derivatives by treatment under N₂ with 1M-HCl at 80°C for 10 min and then identified and quantified by h.p.l.c. on a column (4 mm × 30 cm) of μ -Bondapak C₁₈, using a methanol/acetic acid gradient (Bridgen *et al.*, 1976). The amino acid phenylthiohydantoin derivatives were also identified by amino acid analysis after hydrolysis with HI (Bridgen *et al.*, 1975). Release of radioactivity was determined by liquid-scintillation counting.

Results

Cleavage of component C4 by bovine C1s

Incubation of purified C4 (haemolytic activity 6000 CH₅₀ units/mg) with bovine C1s for 30 min at 37°C resulted in complete loss of C4 haemolytic activity (<10 CH₅₀ units/mg). An identical sample treated with buffer under the same conditions suffered no loss of activity.

After treatment a decrease was observed in the apparent molecular weight of the α -chain of C4, from 98 000 to about 89 000, and a new protein band of low molecular weight, corresponding to C4a, appeared (Figs. 1a and 1b).

C4 treated with trypsin in a weight ratio of 50:1 for up to 60 min at 37°C showed no change in chain structure.

Isolation of C4a

Bovine C4a could be isolated by gel filtration on Sephadex G-100 as described for human C4a (Budzko & Müller-Eberhard, 1970). A peak of low-molecular-weight protein was resolved from the bulk of the material (Fig. 2). Little protein was detected in the same position from the control incubation. The C4a pool was free of contaminating protein, as judged by SDS/polyacrylamide-gel electrophoresis (Fig. 1c). Increased yields of C4a could be obtained by washing the precipitate, formed on acidification of the C4+C1s incubation mixture, with 0.15 M-NaCl, pH 3.5, and applying the washes to the Sephadex G-100 column. Approx. 1 mg of purified C4a could be isolated from 40–50 mg of C4.

Molecular weight of C4a

The molecular weight of purified C4a was determined by gel electrophoresis and by gel filtration. C4a

had an apparent mol.wt. of 23 000 on 10% (w/v) polyacrylamide gels in the presence of SDS under non-reducing conditions. Under reducing conditions, the molecular weight decreased to 12 000. Gel filtration on a Sephadex G-100 column calibrated with proteins of known molecular weight yielded a value of 20 500. The difference between the molecular weights of the α -chain of C4 and the chain formed on incubation with C1s is about 9 000, as judged by SDS/polyacrylamide-gel electrophoresis. These results suggest that C4a may dimerize.

Electrophoretic mobility

On polyacrylamide-gel electrophoresis at pH 4.5, purified C4a migrates very rapidly to the cathode, so that it co-migrates with the tracking dye, Pyronin Y. Untreated C4 and C4b remain at the top of the gel.

Carbohydrate content

Polyacrylamide gels loaded with C4a were stained for carbohydrate, but no bands were visible, even on gels that were heavily overloaded.

Amino acid analysis

The amino acid composition of bovine C4a is shown in Table 1. From the four amino acids present in the lowest amounts, methionine, isoleucine, tyrosine and histidine, all 1.2–1.3 residues per 100 residues, a minimum mol.wt. of 9440 can be calculated for C4a. The most notable features of the amino acid composition are the high cysteine content and the high proportion of basic residues. Since the content of acidic residues is also high, though the

Fig. 1 SDS/polyacrylamide-gel electrophoresis of C4 before (a) and after (b) treatment with C1s on 5% (w/v) polyacrylamide gels in the presence of SDS, under reducing conditions, and of purified C4a on 10% gels (c).

Fig. 2. Separation of C4a on Sephadex G-100. A column (2.5 cm × 100 cm) of Sephadex G-100, equilibrated with 0.15 M-NaCl, pH 3.5, was loaded with 40 mg of C4 treated with 0.4 mg of C1s for 30 min at 37°C (—) or treated with buffer (---).

Table 1. *Amino acid composition of bovine C4a*

	Amino acid composition	
	Residues/100 residues	mol/9440 mol.wt.
Cys	6.8	6
Asp	4.4	4
Thr	5.7	5
Ser	4.7	4
Glu	16.3	14
Pro	5.2	4
Gly	5.9	5
Ala	11.2	9
Val	5.2	4
Met	1.2	1
Ile	1.3	1
Leu	7.1	6
Tyr	1.3	1
Phe	3.7	3
His	1.2	1
Lys	6.8	6
Arg	12.1	10
Trp	ND*	ND

*Abbreviation used: ND, not determined.

peptide is basic, a large proportion of the acidic residues must be in the amide form.

N-Terminal-sequence analysis

N-Terminal amino acid determination by the dansyl method gave aspartic acid or asparagine for bovine C4a.

C4a reduced and carboxymethylated with iodo- $[^{14}\text{C}]$ acetate, was then linked to glass beads by the phenylene di-isothiocyanate method. Since this links the peptide by the amino groups, blanks are obtained for the *N*-terminal residue and for lysine residues during automatic Edman degradation. C4a was linked to glass beads with 51% efficiency. The main problem was insolubility of C4a in the *N*-methylmorpholine buffer, which was partly overcome by the addition of SDS and heating to 45°C. Sequence results are shown in Table 2. The yields of amino acid phenylthiohydantoin derivatives as estimated by the peak heights on h.p.l.c. were satisfactory, but carry-over after residue 11 made the results uncertain. No radioactivity was released until residue 21 and the radioactivity was high in residues 21–25 inclusive. However, significant carry-over could be expected thus far into the sequence, and no more precise location of the cysteine residues is possible.

Discussion

C4a can be cleaved from bovine C4 by bovine $\text{C}\bar{4}$ s and can be isolated in pure form and in reasonable yield (40–50% on a molar basis with respect to C4).

Table 2. *Sequence analysis of the N-terminal residues of C4a*

Reduced and radioactively carboxymethylated C4a coupled to glass beads (51 nmol) was subjected to automatic Edman degradation. The yields ranged from 16 to 22% for steps 2–7 down to 6.8% for steps 8–13, as judged by peak heights of the phenylthiohydantoin derivatives on h.p.l.c. Release of radioactivity was not detected. Amino acid phenylthiohydantoin derivatives were identified by h.p.l.c. (a) or amino acid analysis (b).

Residue no.	(a)	(b)	Proposed sequence
1	—	—	Asx
2	Val/Met	Val	Val
3	Asn+Asp	Asp	Asn
4	Phe/Ile	Phe	Phe
5	Glu+Gln	Glu	Gln
6	—	—	Lys
7	Ala	Ala	Ala
8	Phe/Ile	Ile	Ile
9	—	His	His
10	Glu	Glu	Glu
11	—	—	Lys
12	Leu	—	Leu
13	Gly	—	Gly

The procedure used was based on that of Budzko & Müller-Eberhard (1970). This has the disadvantage that at pH 3.5 some protein precipitates. Even though C4a itself is freely soluble at this pH, losses are incurred due to washing of the precipitate and re-running of the supernatant material on Sephadex G-100. This procedure has now been superseded by ion-exchange on DEAE-Sephadex A-50 at pH 6.6 (M. A. Smith & J. E. Fothergill, unpublished work).

Human C3a and C5a can be cleaved from their parent molecules by both trypsin and their respective convertases (Budzko *et al.*, 1971; Cochrane & Müller-Eberhard, 1968). Trypsin also releases a fragment similar to human C4a from human C4 and renders the C4 haemolytically inactive (Budzko & Müller-Eberhard, 1970). However, bovine C4 showed no change in chain structure as judged by SDS/polyacrylamide-gel electrophoresis when treated with trypsin, even after prolonged incubation.

The purified C4a, which appears to contain no carbohydrate, has a minimum mol.wt. of 9440, based on its content of 1 mol each of methionine, isoleucine, tyrosine and histidine/mol. This must also be its true molecular weight, since the α -chain of C4 is decreased by about 9000 on cleavage by $\text{C}\bar{4}$ s. However, estimations of molecular weight by gel filtration on Sephadex G-100 and SDS/polyacrylamide-gel electrophoresis under non-reducing conditions have given values of over 20000. When the polyacrylamide gels were run under reducing conditions, the apparent

mol.wt. decreased to 12000. It is possible that this difference in apparent molecular weight is due to dimerization. Alternatively, it may result from the 'disulphide-knot' structure, which may not bind the usual amount of SDS and could have unusual hydrodynamic properties. This disulphide-knot structure would revert to a more normal open-chain structure after reduction. Dimerization has also been suggested as an explanation for the wide spread of molecular weights reported for human C4a, which range from 6000 to 15500 (Budzko & Müller-Eberhard, 1970; Patrick *et al.*, 1970; Schreiber & Müller-Eberhard, 1974).

The behaviour of purified C4a on acid polyacrylamide-gel electrophoresis seems very similar to that of human C3a (Bokisch *et al.*, 1969) and C5a (Vallota & Müller-Eberhard, 1973), though the anaphylatoxins were not available for direct comparison with C4a in the present study. The basic charge of the three activation peptides suggests similarities in their amino acid compositions, and this has been found (Table 3). All three peptides have high contents of lysine, arginine and glutamic acid, much of it presumably as glutamine. The most striking feature of the compositions, which show general similarity, is the high number of cysteine residues, six/molecules in each. This is a noteworthy observation for C4a, since disulphide-bond formation has profound effects on the stability of the structure of C3a (Hugli, 1975b). The positions of the six cysteine residues, in two cysteinylcysteine sequences and two single residues,

are conserved in porcine and human C3a (Corbin & Hugli, 1976). A disulphide-knot arrangement, similar to that found in the α -subunit of glycoprotein hormones such as lutropin (Chung *et al.*, 1973), is formed by disulphide-bonding between one of the residues of each cysteinylcysteine sequence, the other two being free to form bonds with the two single cysteine residues. This contributes greatly to the stability and highly ordered secondary structure of C3a, which has a total of 40–45% α -helical content (Hugli *et al.*, 1975a). The positions of the cysteine residues in C4a are still unknown, although preliminary results in this study suggest that one or more occur between positions 21 and 25, which corresponds approximately to the cysteinylcysteine sequence at 22, 23 in C3a and at 21, 22 in C5a (Fernandez & Hugli, 1978). Other more definite sequence comparisons can be made between C4a and C3a (Table 4). The *N*-terminal sequences show identity between four of the first thirteen residues when comparing bovine C4a with porcine C3a. Human C3a has a leucine $\rightarrow$ valine replacement at position 12. Three of these identical residues occur in sequence at positions 11–13. It was unfortunate that it was at this point that the sequencing of C4a became unsatisfactory, owing to carry over and could not be continued. No identical residues are present in the first thirteen *N*-terminal residues of bovine C4a and human C5a. The two residues common to this region of human C3a and C5a after manual alignment of the sequences (Fernandez & Hugli, 1978), are not found in bovine C4a.

C4a, like C3a, contains no carbohydrate, whereas C5a contains an oligosaccharide unit attached to the familiar -Asn-X-Ser- sequence, which is not present in C3a.

Table 3. Amino acid composition of (a) bovine C4a, (b) porcine C3a and (c) human C5a

Composition (mol/approx. 9000 mol.wt.)

	(a)	(b)*	(c)†
Cys	6	6	6
Asp	4	6	6
Thr	5	0	3
Ser	4	4	4
Glu	14	11	9
Pro	4	2	1
Gly	5	4	3
Ala	9	5	8
Val	4	2	5
Met	1	4	1
Ile	1	2	5
Leu	6	7	4
Tyr	1	2	2
Phe	3	3	1
His	1	3	2
Lys	6	7	8
Arg	10	9	5
Trp	ND‡	0	0

* From Hugli *et al.* (1975b).

† From Fernandez & Hugli (1976).

‡ Abbreviation used: ND, not determined.

Table 4. *N*-Terminal Sequences of bovine C4a, C3a, C5a and C4 α -chain

Residue	Bovine C4a	Human α -chain C4*	Porcine C3a†	Human C5a‡
1	Asx	Asn	Ser	Thr
2	Val	Val	Val	Leu
3	Asn	Asn	Gln	Gln
4	Phe	Phe	Leu	Lys
5	Gln	Gln	Met	Lys
6	Lys	Lys	Gln	Ile
7	Ala	Ala	Lys	Glu
8	Ile	Ile	Arg	Glu
9	His	Asn	Met	Ile
10	Glu	Glu	Asn	Ala
11	Lys	Lys	Lys	Ala
12	Leu	Leu	Leu	Lys
13	Gly	Gly	Gly	Tyr

* From Gigli *et al.* (1977) and Bolotin *et al.* (1977).

† From Corbin & Hugli (1976).

‡ From Fernandez & Hugli (1978).

The *N*-terminal sequence of bovine C4a showed almost complete identity with that of the α -chain of human C4 (Gigli *et al.*, 1977; Bolotin *et al.*, 1977). This clearly establishes that C4a is cleaved from the *N*-terminus of the α -chain and indicates that this portion of the molecule is highly conserved between species.

We thank Dr. Linda Fothergill and Dr. Rick Harkins for helpful discussions, Mrs. Jean Bathgate, Mr. Brian Dunbar and Mrs. Sandra Short for excellent technical and secretarial assistance, Mr. W. C. Dawson for invaluable help in construction of the sequencer, and W. Donald and Son, Portlethen, Kincardineshire, Scotland, for their generous co-operation in the collection of blood. We are grateful to the Medical Research Council for financial support and a Research Studentship to R. D. C.

References

- Bokisch, V. A., Müller-Eberhard, H. J. & Cochrane, C. G. (1969) *J. Exp. Med.* **129**, 1109–1130
- Bolotin, C., Morris, S., Tack, B. & Prahl, J. (1977) *Biochemistry* **16**, 2008–2015
- Booth, N. A., Campbell, R. D. & Fothergill, J. E. (1979) *Biochem. J.* **177**, 959–965
- Bridgen, J. (1975) *FEBS Lett.* **50**, 159–162
- Bridgen, J., Graffeo, A. P., Karger, B. L. & Waterfield, M. D. (1975) in *Instrumentation in Amino Acid Sequence Analysis* (Perham, R. N., ed.) pp. 126–128, Academic Press, London and New York
- Bridgen, P. J., Cross, G. A. M. & Bridgen, J. (1976) *Nature (London)* **263**, 613–614
- Budzko, D. B. & Müller-Eberhard, H. J. (1970) *Immunochemistry* **7**, 227–234
- Budzko, D. B., Bokisch, V. A. & Müller-Eberhard, H. J. (1971) *Biochemistry* **10**, 1166–1172
- Campbell, R. D., Booth, N. A. & Fothergill, J. E. (1979a) *Biochem. J.* **177**, 531–540
- Campbell, R. D., Booth, N. A. & Fothergill, J. E. (1979b) *Biochem. J.* **183**, 579–588
- Chung, D., Sairam, M. R. & Li, C. H. (1973) *Arch. Biochem. Biophys.* **159**, 678–682
- Cochrane, C. G. & Müller-Eberhard, H. J. (1968) *J. Exp. Med.* **127**, 371–386
- Corbin, N. C. & Hugli, T. E. (1976) *J. Immunol.* **117**, 990–995
- Crestfield, A. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem.* **238**, 622–627
- Fernandez, H. N. & Hugli, T. E. (1976) *J. Immunol.* **117**, 1688–1694
- Fernandez, H. N. & Hugli, T. E. (1978) *J. Biol. Chem.* **253**, 6955–6964
- Fothergill, J. E. & Anderson, W. H. K. (1978) *Curr. Top. Cell Regul.* **13**, 259–311
- Gigli, I., von Zabern, I. & Porter, R. R. (1977) *Biochem. J.* **165**, 439–446
- Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138
- Haines, A. L. & Lepow, I. H. (1964) *J. Immunol.* **92**, 468–478
- Hugli, T. E. (1975a) *Cold Spring Harbor Conf. Cell Proliferation* **2**, 273–290
- Hugli, T. E. (1975b) *J. Biol. Chem.* **250**, 8293–8301
- Hugli, T. E. & Müller-Eberhard, H. J. (1978) *Adv. Immunol.* **26**, 1–53
- Hugli, T. E., Morgan, W. T. & Müller-Eberhard, H. J. (1975a) *J. Biol. Chem.* **250**, 1479–1483
- Hugli, T. E., Vallota, E. H. & Müller-Eberhard, H. J. (1975b) *J. Biol. Chem.* **250**, 1472–1478
- Laursen, R. A. (1971) *Eur. J. Biochem.* **20**, 89–102
- Laursen, R. A., Horn, M. J. & Bonner, A. G. (1972) *FEBS Lett.* **21**, 67–70
- Lepow, I. H., Ratnoff, O. D., Rosen, F. S. & Pillemer, L. (1956) *Proc. Soc. Exp. Biol. Med.* **92**, 32
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Müller-Eberhard, H. J. & Biro, C. E. (1963) *J. Exp. Med.* **118**, 447–466
- Müller-Eberhard, H. J. & Lepow, I. H. (1965) *J. Exp. Med.* **121**, 819–833
- Patrick, R. A., Taubman, S. B. & Lepow, I. H. (1970) *Immunochemistry* **7**, 217–225
- Porter, R. R. & Reid, K. B. M. (1978) *Nature (London)* **275**, 699–704
- Reisfeld, R. A., Lewis, U. J. & Williams, D. E. (1962) *Nature (London)* **195**, 281–283
- Schreiber, R. D. & Müller-Eberhard, H. J. (1974) *J. Exp. Med.* **140**, 1324–1335
- Vallota, E. H. & Müller-Eberhard, H. J. (1973) *J. Exp. Med.* **137**, 1109–1123
- Wachter, E., Machleidt, W., Hofner, H. & Otto, J. (1973) *FEBS Lett.* **35**, 97–102
- Weber, K., Pringle, J. R. & Osborn, M. (1972) *Methods Enzymol.* **26**, 3–27
- World Health Organisation (1968) *WHO Bull.* **39**, 935–938 [or (1970) *Immunochemistry* **7**, 137–142]
- Ziccardi, R. J. & Cooper, N. R. (1976) *J. Immunol.* **116**, 496–503