Biochem. J. (1981) **194**, 551–560
Printed in Great Britain

Direct measurement of the increase in intracellular free calcium ion concentration in response to the action of complement

Anthony K. CAMPBELL, Richard A. DAW, Maurice B. HALLETT and J. Paul LUZIO*
*Department of Medical Biochemistry, Welsh National School of Medicine, Heath Park, Cardiff CF4 4XN, Wales, U.K. and *Department of Clinical Biochemistry, University of Cambridge Clinical School, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, U.K.*

(*Received 8 April 1980/Accepted 21 October 1980*)

1. The effect of rabbit anti-(pigeon erythrocyte) antibodies plus human complement on the concentration of intracellular free Ca^{2+} in sealed pigeon erythrocyte 'ghosts' was investigated with the photoprotein obelin. 2. The addition of human serum, as a source of complement, to 'ghosts' coated with antibody caused a rapid increase in intracellular free Ca^{2+} after a lag of 20–40s, as detected by an increase in obelin luminescence. 3. The increase in obelin luminescence could not be explained by release of obelin into the medium. It was also Ca^{2+}-dependent in that extracellular EGTA abolished the effect and intracellular EGTA inhibited it and required the complete terminal complex ($C\overline{56789}$). No effect was seen with $C\overline{5678}$. 4. The concentration of intracellular free Ca^{2+} before addition of complement was approx. 0.3μM. This increased to a maximum of 5–30μM after complement addition and then remained constant for at least 1–2min. 5. Antibody plus complement induced a rapid increase in $^{42}K^+$ efflux and an inhibition of cyclic AMP formation. 6. When partially purified complement components (C5b–9) were used in 'reactive lysis' it was possible to inhibit the release of macromolecules from pigeon erythrocyte 'ghosts' by extracellular EGTA. 7. It was concluded that the increase in intracellular free Ca^{2+} concentration caused by anti-cell antibody plus complement occurred before cell lysis and may be involved in the mechanism of complement-induced cell injury.

The existence of the complement proteins in serum and their ability to induce cell lysis in the presence of anti-cell antibodies has been known for many years (Osborn, 1935; Lachmann, 1975). Much work has concentrated on the complement components necessary to form a complex in the cell membrane (Lachmann *et al.*, 1973; Michaels *et al.*, 1976; Dourmashkin, 1978; Sims & Lauf, 1978; Podack *et al.*, 1979) that is thought to be the primary cause of the events leading to cell lysis. Relatively little attention, however, has been paid to changes in the intracellular concentration of ions and metabolites, and whether such changes may mediate some of the phenomena associated with immune damage to cells. In particular, apart from a study of antibody and complement on ^{45}Ca uptake by L cells (Shearer *et al.*, 1976), the possibility that changes in intracellular free Ca^{2+} concentration might play an important role in this type of cell injury has not been investigated.

The concentration of free Ca^{2+} in most cells is thought to be of the order of 0.1μM [see Ashley & Campbell (1979) for reviews and references]. In view of the large electrochemical gradient of Ca^{2+} across the plasma membrane a relatively small increase in its permeability to Ca^{2+} induced by a physiological stimulus (Rasmussen & Goodman, 1977) or during cell injury is likely to cause a large fractional change in intracellular free Ca^{2+} concentration that may effect many reactions within the cell. We have shown that a large increase in intracellular Ca^{2+} concentration is an early event after the reaction of complement with antibody bound to cells (Campbell *et al.*, 1979*a*,*b*,*c*; Luzio *et al.*, 1979). The aim of the present studies was to quantify the effect of anti-cell antibody plus complement on the concentration of intracellular calcium ions and investigate its consequent effect on cell metabolism. In particular the complement components necessary for the increase in intracellular

Abbreviations used: spec. pure, spectroscopically pure; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-ethanesulphonic acid.

0306-3283/81/020551-10$01.50/1

Ca^{2+} have been defined. Attempts have also been made to examine the effect of the rise in intracellular Ca^{2+} on K^+ flux, cyclic AMP formation and the lytic mechanism itself.

Pigeon erythrocytes and sealed 'ghosts' were used in the experiments described since they contain an adenylate cyclase that can be activated by β-adrenoreceptor agonists and inhibited by concentrations of Ca^{2+} in the range 1–10μM (Campbell & Siddle, 1976; Campbell & Dormer, 1978).

Experimental

Chemicals

ATP (disodium salt) and phosphoenolpyruvate (potassium salt) were obtained from Boehringer Corp. (London), London, U.K. Rabbit muscle pyruvate kinase (EC 2.7.1.40) and L-adrenaline were obtained from Sigma Chemical Co., Poole, Dorset, U.K. EGTA was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Spec. pure KCl, NaCl and $MgSO_4$ were obtained from Johnson Matthey Chemicals, London. Cyclic [^{3}H]AMP (40–60Ci/mmol), [^{3}H]inulin (25Ci/mmol) and ^{42}KCl (100μCi/ml) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Bovine serum albumin was obtained from Armour Pharmaceutical Co., Eastbourne, U.K. ^{125}I-labelled albumin (140μCi/mg of protein) was prepared by the chloramine-T method. All other chemicals were AnalaR grade from BDH Chemicals, Poole, Dorset, U.K.

Antisera

Antisera to pigeon erythrocytes were produced in rabbits by subcutaneous injection of pigeon erythrocytes as previously described (Campbell *et al.*, 1979*a*). The serum was stored frozen at −20°C after heating it at 56°C for 30min to inactivate complement (Lachmann, 1975). Serum from non-injected rabbits was used as a souce of non-immune serum.

Complement

A pool of normal human serum stored at −70°C was used as a source of complement. Human serum free of C9, which was non-lytic in the presence of anti-cell antibody, was prepared by incubation of 1ml of human serum with 250μl of rabbit anti-(human C9) antibody (Behringwerk Ag, Marburg/Lahn, West Germany) at 37°C for 30min and centrifuging (12000**g**, 5min) to remove the precipitate. Lytic activity of this C9-deficient serum could be restored by addition of C9 in the form of a preparation of a mixture of $C\overline{56}$ + C9.

Functionally pure complement-component fractions prepared as described by Lachmann & Hobart (1978) were a kind gift from Professor P. J. Lachmann. The components used were preparations of the complex $C\overline{56}$, C7, a $C\overline{56}$ preparation containing C9 ($C\overline{56}$ + C9) and a C7 preparation containing C8 (C78). All preparations were assayed for content of terminal complement components by using reactive lysis of guinea-pig erythrocytes (Lachmann & Hobart, 1978). From these data it was possible to use different preparations, e.g. $C\overline{56}$ and $C\overline{56}$ + C9, with the common components, i.e. $C\overline{56}$, at the same effective concentrations. In all experiments with pigeon erythrocyte 'ghosts' the amounts of components used were sufficient to cause >75% release of [^{3}H]inulin within 30min at 37°C if the entire complement sequence ($C\overline{56789}$) was present, and the 'ghosts' were incubated in medium A containing 1mM-$CaCl_2$.

Obelin

The Ca^{2+}-activated photoprotein obelin was extracted and purified from the hydroid *Obelia geniculata* as previously described (Campbell & Dormer, 1975, 1978; Campbell *et al.*, 1979*b*).

Preparation of sealed pigeon erythrocyte 'ghosts'

Sealed pigeon erythrocyte 'ghosts' containing obelin were prepared as previously described (Campbell & Dormer, 1978). The 'ghosts' were resealed in 10mM-Tes/6mM-NaCl (spec. pure)/2mM-$MgSO_4$ (spec. pure)/2mM-ATP (disodium salt)/10mM-phosphoenolpyruvate (potassium salt)/rabbit muscle pyruvate kinase (approx. 20units/ml), adjusted to pH7.4 with KOH, containing obelin (10^9–10^{10} luminescence counts, see below), 0.1–1mg of bovine serum albumin/ml, [^{3}H]inulin (approx. 1μCi/ml) and 150mM-KCl for 30–60min at 37°C. The resealed 'ghosts' were centrifuged, washed and resuspended in medium A (10mM-Tes/140mM-NaCl/5mM-KCl/2mM-$MgCl_2$) at pH7.4.

Assay of obelin

Obelin was assayed as previously described (Campbell & Dormer, 1978). To estimate the quantity of active obelin inside the 'ghosts' at any time during the experiment a solution of medium A + 1mM-$CaCl_2$ containing the 'ghosts' was placed in front of the photomultiplier tube and an equal volume of the same medium containing 1–3% (v/v) Triton X-100 (scintillation grade) added. The total luminescence counts over the first 10s after addition of Triton X-100 were measured. Normal grade Triton X-100 produced a low, but significant luminescence, dependent on obelin, that continued for many minutes.

$^{42}K^+$ release from 'ghosts'

'Ghosts' were loaded with $^{42}K^+$ by incubation of the whole suspension in ^{42}KCl (10–50μCi/ml) for 3–16h at 4°C. Just before the efflux experiment was

conducted the 'ghosts' were centrifuged, washed in medium A and incubated with antibody plus complement at 37°C under the same conditions as for the obelin measurements. At defined intervals the 'ghost' suspension was centrifuged in an Eppendorf micro-centrifuge for 10s and the $^{42}K^+$ in the supernatant measured by Cerenkov counting. Results were expressed as $^{42}K^+$ release (%) relative to time zero.

Assay of cyclic AMP and ATP

Samples were taken for assay of cyclic AMP (40μl), and measured by radioimmunoassay and for ATP (10μl) by firefly luciferase assay, as previously described (Campbell & Siddle, 1976).

Results and discussion

Effect of anti-(pigeon erythrocyte) antibody plus complement

Anti-(pigeon erythrocyte) antibody plus complement induced a large increase in obelin luminescence from within pigeon erythrocyte 'ghosts' that began to occur 20–40s after addition of the complement (20–50μl of human serum) and reached a peak 20–50s later (Fig. 1, traces *a* and *b*). No rapid increase in luminescence was observed when antibody was used alone, when antibody plus heat-inactivated human serum was used as complement source (Fig. 1, trace *c*) or when no antibody was added (Fig. 1, trace *d*). However, it was found that a small increase in obelin luminescence could be detected when non-immune rabbit serum + 50–100μl of human serum was used. The absence of any detectable increase in luminescence with antibody alone was in contrast to the increase in the uptake of ^{45}Ca induced by antibody in L cells reported by Shearer *et al.* (1976).

Preincubation of the 'ghosts' with anti-(pigeon erythrocyte antibody) for 1–30min resulted in the same luminescence response after addition of a source of complement. It was therefore concluded that maximum antibody binding occurred within 1min. The time of onset (t_1) and time to peak (t_2) of the increase in obelin luminescence were markedly dependent on the concentration of human serum used, whereas they were little affected by antibody concentration over the range 5–50μl in 1ml.

Requirement for C9

To investigate whether C9 was required to induce the increase in obelin luminescence a serum defi-

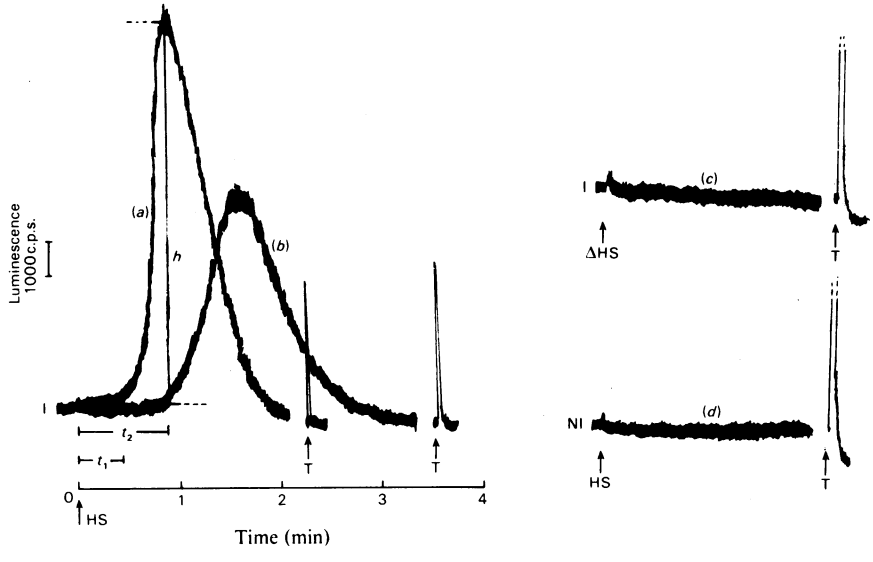

Fig. 1. *Effect of anti-(pigeon erythrocyte) antibody plus complement on obelin luminescence from pigeon erythrocyte 'ghosts'*

Portions (50μl: approx. 10^8 'ghosts') of sealed pigeon erythrocyte 'ghosts' were incubated in 0.5ml of medium A + 1mM-$CaCl_2$ (see the Experimental section) containing 5μl of rabbit anti-(pigeon erythrocyte) serum (traces *a*, *b* and *c*) or 5μl of non-immune (NI) rabbit serum (trace *d*) for 5min at 37°C. The tube containing the 'ghosts', maintained at 37°C, was placed in front of the photomultiplier tube and 0.5ml of medium A + 1mM-$CaCl_2$ containing 50μl (traces *a* and *d*), 20μl (trace *b*) or 50μl of heat-inactivated (trace *c*) human serum added (HS ↑). The rate of obelin luminescence was recorded on a chart recorder; *h* = height. After 3–4min Triton X-100 (T ↑) was added at a final concn. of 0.3–1% (v/v) to stimulate the remaining active obelin. The percentage of obelin utilized before addition of Triton was (%): *a*, 95; *b*, 90; *c*, 15; *d*, 20.

cient in C9 (see the Experimental section) was used in the presence of anti-(pigeon erythrocyte) antibody (Fig. 2). No detectable effect was observed until a source of C9 was added, since $C\overline{56}$ + C9 but not $C\overline{56}$ alone was able to restore the effect. Although the anti-C9 antibody has been shown by using immunoelectrophoresis and Ouchterlony plates not to bind C5 or C6 (Heimberger & Karges, personal communication) it was not possible under the conditions of these experiments to unequivocally state how much of the terminal complex was required, since formation of C9–anti-C9 complexes might cause loss of other components as a result of activation. To do this a combination of functionally pure terminal components was used (see the Experimental section).

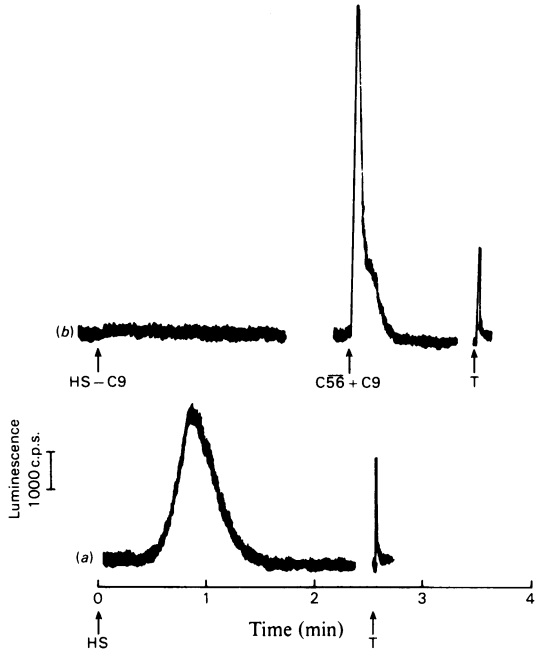

Fig. 2. *Effect of removal of C9 with anti-C9 serum*
Human serum deficient in C9 was prepared as described in the Experimental section. Portions (50 μl) of sealed pigeon erythrocyte 'ghosts' were incubated in 0.5 ml of medium A + 1 mM-$CaCl_2$ (see the Experimental section) containing 10 μl of anti-(pigeon erythrocyte) serum for 5 min at 37°C. The tube containing the 'ghosts', maintained at 37°C, was placed in front of the photomultiplier tube. A 0.5 ml portion of medium A + 1 mM-$CaCl_2$ containing 50 μl of human serum (trace *a*) or 50 μl of human serum deficient in C9 (trace *b*) was added (HS or HS – C9 ↑). The rate of obelin luminescence was plotted on a chart recorder. After 2 min in the case of trace (*b*), 0.5 ml of medium A + 1 mM-$CaCl_2$ containing 50 μl of $C\overline{56}$ + C9 was added. At the end of the experiment Triton X-100 (0.7%, v/v) + $CaCl_2$ (13 mM) was added (T ↑) to stimulate the remaining active obelin. In both cases >90% of the obelin had been utilized just before addition of Triton X-100. In the case of trace (*b*) only 15% of the obelin was utilized before addition of $C\overline{56}$ + C9.

The components C5b–9, in the absence of anti-(pigeon erythrocyte) antibody, bind to the cell membrane and cause lysis, so-called 'reactive lysis' (Thompson & Lachmann, 1970; Lachmann & Thompson, 1970). To ensure that the functional activity of $C\overline{56}$ was the same as that in the $C\overline{56}$ + C9 preparation both reagents were titrated with normal human serum as a source of the other components by using the increase in obelin luminescence as an assay. The effective concentration of $C\overline{56}$ in the $C\overline{56}$ + C9 preparation was found to be nine times that of the $C\overline{56}$ preparation. This was because the removal of C9 by gel filtration to produce $C\overline{56}$ alone causes a dilution of the sample. With these preparations at the same final effective concentration of $C\overline{56}$ it could be shown that there was no detectable effect of $C\overline{56}$ + C78 on pigeon erythrocyte 'ghosts' (Fig. 3*a*), whereas the effect with $C\overline{56}$ + C9 + C78 (Fig. 3*b*) was very similar to that with anti-cell antibody plus human serum. No effect of additional $C\overline{56}$ to $C\overline{5678}$ was observed (Fig. 3*d*). This absolute requirement for C9 is of interest as other workers have reported that C8 is the component mainly responsible for initiating damage of both biological and pure phospholipid membranes and that C9 acts by accelerating the lytic process (Stolfi, 1968; Tamura *et al.*, 1972; Michaels *et al.*, 1976).

The requirement for $C\overline{56}$ was demonstrated by the fact that addition of 'partially pure' $C\overline{56}$ containing no detectable C9 to human serum increased obelin luminescence in the 'ghosts' in the absence of anti-(pigeon erythrocyte) antibody, and by the fact that ($C\overline{56}$) + ($C\overline{56}$ + C9) produced a larger effect than ($C\overline{56}$ + C9) in the presence of C78 (data not shown). The requirement for C8 was shown by the fact that very little effect was observed with $C\overline{56}$ + C9 + C7 (Fig. 3*c*).

These results are therefore consistent with the increase in obelin luminescence induced in the 'ghosts' by complement being caused by formation of the complete terminal complex, namely $C\overline{56789}$.

Requirement for Ca^{2+}

To show that the increase in obelin luminescence from the 'ghosts' was caused by an increase in free Ca^{2+} the effect of anti-(pigeon erythrocyte) antibody plus complement on 'ghosts' containing EGTA, a strong Ca^{2+} chelator, was investigated. EGTA inside the 'ghosts' both increased the time of onset and time to peak of luminescence, and reduced the height of the peak itself. However, it did not abolish the effect (data not shown). The source

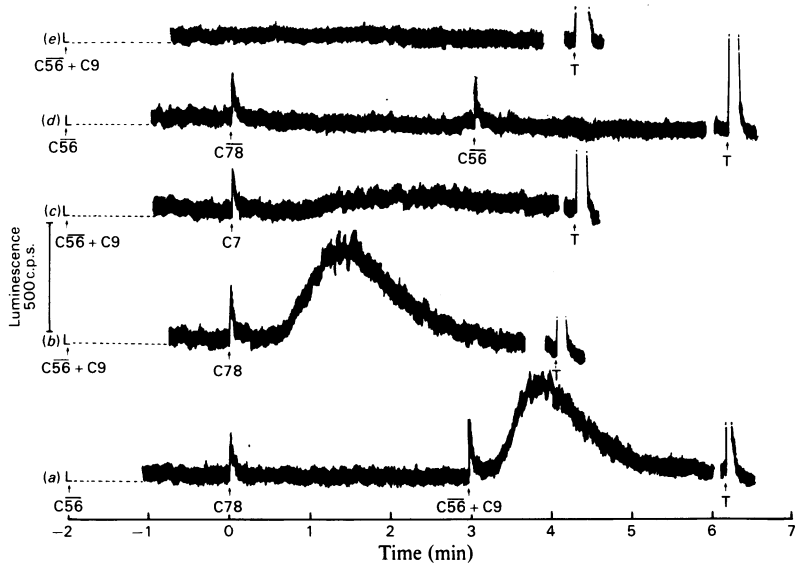

Fig. 3. *Requirement for complement component C9*

Portions (50 μl: approx. 10^8 'ghosts') of sealed pigeon erythrocyte 'ghosts' were incubated in 0.5 ml of medium A + 1 mM-$CaCl_2$ (see the Experimental section) containing 20 μl of $\overline{C56}$ (traces *a* and *d*) or 2 μl of $\overline{C56}$ + C9 (traces *b*, *c* and *e*) for 2 min at 37°C. The tube containing the 'ghosts', maintained at 37°C, was placed in front of the photomultiplier tube and C78 (1 μl; traces *a*, *b* and *d*), C7 (1 μl, trace *c*), $\overline{C56}$ + C9 (2 μl, trace *a*) and $\overline{C56}$ (20 μl, trace *d*) were added all in a final volume of 20 μl. The rate of obelin luminescence was recorded on a chart recorder. At the end of the experiment Triton X-100 (1.5%, v/v) + $CaCl_2$ (20 mM) was added (T ↑) to stimulate the remaining active obelin.

of Ca^{2+} required to stimulate intracellular obelin was from outside the cell, as demonstrated by the fact that extracellular EGTA abolished the effect of C5b-9 on obelin luminescence (Campbell *et al.*, 1980).

Estimation of absolute free Ca^{2+} concentration

Like most luminescent reactions (Campbell & Simpson, 1979) the reaction of the prosthetic group of obelin is first-order with respect to the amount of chromophore (O_t) at any time t as shown by Eqn. 1,

$$\text{light intensity} = (\mathrm{d}hv/\mathrm{d}t)_t = Q.k_t.O_t = (-\mathrm{d}O/\mathrm{d}t)Q \quad (1)$$

where Q = quantum yield of the reaction (see Campbell & Simpson, 1979) and k_t = rate constant at time t (see Campbell *et al.*, 1979*a*,*b*,*c* for further analysis).

Eqn. 1 is valid at all times providing the reaction remains first-order. The rate constant (k_t) for obelin luminescence is dependent on the fractional saturation by Ca^{2+} of the molecules of active obelin present (Fig. 4).

In the present experiments >90% of the photoprotein was consumed in a few minutes after addition of anti-(pigeon erythrocyte) antibody plus complement to the 'ghosts'. To relate the rate of obelin luminescence to absolute intracellular free Ca^{2+} concentrations over the complete time course, when both the intracellular Ca^{2+} concentration and the quantity of active photoprotein were changing, the rate constant of obelin luminescence was determined at different times.

Returning therefore to Eqn. 1 and rearranging gives Eqn. 2,

$$(k_{\text{app.}})_t = k_{\text{app.}} \text{ at any time } t = \frac{(\mathrm{d}hv/\mathrm{d}t)_t}{Q.O_t} \quad (2)$$

where $(\mathrm{d}hv/\mathrm{d}t)_t$ is the luminescence intensity at time t, and $Q.O_t$ is the amount of active photoprotein (luminescence counts) remaining within the 'ghosts' at time t. $Q.O_t$ was estimated by measuring the sum of the areas under the obelin luminescence traces before and after addition of Triton X-100. $(\mathrm{d}hv/\mathrm{d}t)_t$ was measured as the height of a luminescence trace (luminescence c.p.s.). Such an analysis was performed on the traces shown in Fig. 5. The rate constants thus obtained were converted into absolute free Ca^{2+} concentration by using the calibration curve in Fig. 4. In this experiment (Fig. 5) the resting intracellular free Ca^{2+} concentration was estimated to be 0.3 μM and reached a maximum of 8.2 μM (Fig. 5, trace *a*) and 4.7 μM (Fig. 5, trace *b*) 2 min after the addition of human serum in the presence of

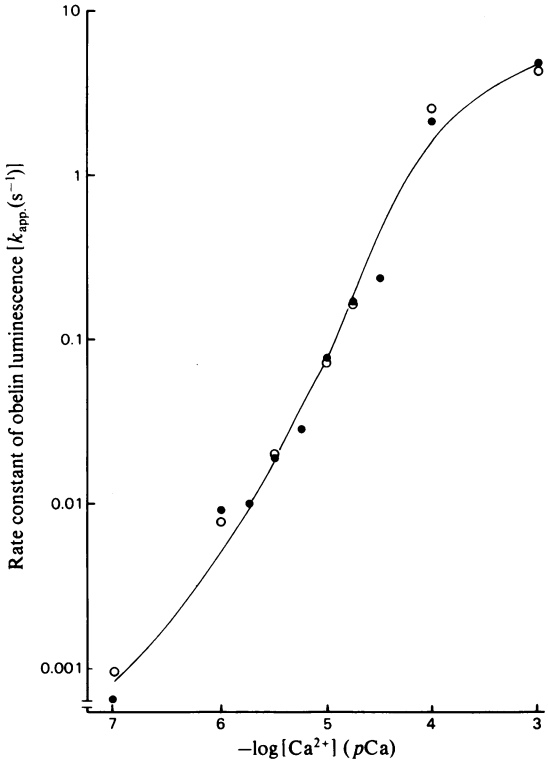

Fig. 4. *Effect of Ca^{2+} concentration on rate constant of obelin luminescence*

Obelin (10^6 luminescent counts) was added to 0.5 ml of Ca^{2+}-free assay medium, prepared with deionized double-glass-distilled water (conductivity $<0.3\,\mu^{-1}$) containing 10 mM-Tes/150 mM-KCl (spec. pure)/30 mM-NaCl (spec. pure)/2 mM-$MgSO_4$ (spec. pure)/bovine serum albumin (0.1 mg/ml, pH 7.4) and held in front of the photomultiplier tube in the thermostatically controlled housing. $CaCl_2$ (0.2 μM–2 mM), dissolved in assay medium, was added to the sample from a syringe (0.5 ml of Ca^{2+}-containing medium into the 0.5 ml of obelin-containing medium) to give a final Ca^{2+} concn. of 0.1 μM–1 mM. The rate of decay of obelin luminescence stimulated by addition of Ca^{2+} was monitored either by using a chart recorder for slow reactions or a transient-recorder, with sweep times of 2 s, for fast reactions. The rate constant, $k_{app.}$, was measured at each Ca^{2+} concn. at either 19.5 (●) or 37°C (○).

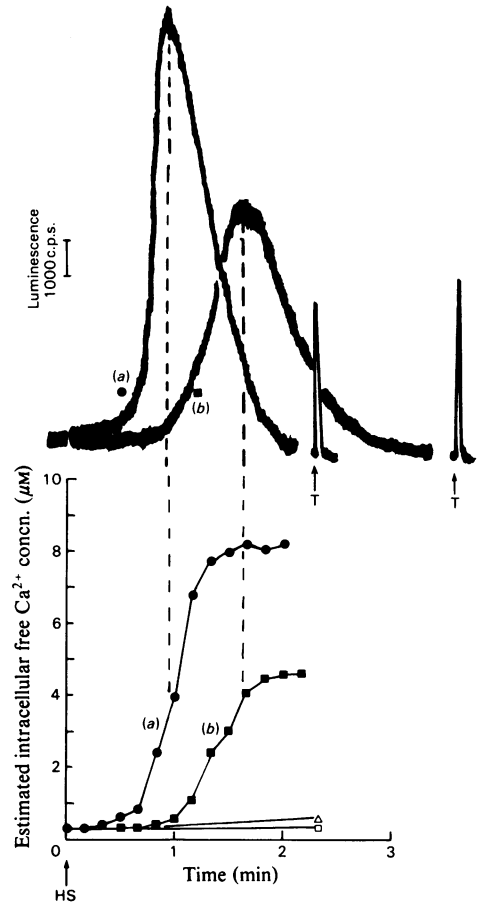

Fig. 5. *Intracellular free Ca^{2+} concentration inside pigeon erythrocyte 'ghosts'*

To estimate the effect of anti-cell antibody plus complement on absolute free Ca^{2+} concentrations the intensity of light emission at defined time intervals was measured from the traces shown in Fig. 1. As described in the Results section division of this value by the total active obelin remaining at each time point gave the rate constant $(k_{app.})_t$. The number of obelin luminescence counts remaining at each time point (t) equals the area under the trace from time (t) to the time at which Triton X-100 was added plus the obelin exposed by Triton + Ca^{2+}. The calculated rate constant $(k_{app.})_t$ was converted into absolute free Ca^{2+} concn. by reading off the value for Ca^{2+} from the standard curve shown in Fig. 4. Traces (*a*) and (*b*) from Fig. 1 are reproduced here to illustrate that the peak in obelin luminescence occurs a few seconds before the maximum in intracellular free Ca^{2+} concn. Symbols [with μl of anti-(pigeon erythrocyte) serum and μl of human serum as a source of complement]: ● (*a*), 5, 50; ■ (*b*), 5, 20; □ (*d*, Fig. 1), 0 (non-immune), 50; △ (*c*, Fig. 1), 5, 50 (heat-inactivated).

anti-(pigeon erythrocyte) antibody. The maximum intracellular free Ca^{2+} concentration occurred approx. 5–20 s after the peak in obelin luminescence (Fig. 5). This is because until the steady-state intracellular free Ca^{2+} concentration was achieved the rate of obelin consumption exceeded the rate of increase of Ca^{2+} binding to obelin.

Further confirmation of the fact that the 'ghosts' were able to maintain a Ca^{2+} gradient in the

presence of complement was obtained from experiments with Triton X-100. The initial rate constant of obelin luminescence in medium A + Ca^{2+} (0.1 or 1 mM) + 1% (v/v) Triton X-100 was $2.8\,s^{-1}$, approx. 10 times that with anti-(pigeon erythrocyte) antibody plus complement (Figs. 1 and 5).

The analysis performed here, as with most biochemical experiments, does not take into account any gross heterogeneity in the 'ghost' population. We have attempted to construct a heterogeneous model based on the Poisson or normal distributions but have been unable to find one to explain the data reported here.

If the Ca^{2+} was penetrating the 'ghost' membranes through a membrane complex produced by complement one might expect the steady-state intracellular free Ca^{2+} concentration to increase as the extracellular Ca^{2+} concentration was increased. Measurement of steady-state intracellular Ca^{2+} concentration versus extracellular Ca^{2+} concentration (Fig. 6) showed that this was true up to 2 mM-extracellular Ca^{2+}. However, at higher concentrations the effect of complement activation on intracellular Ca^{2+} was inhibited. This is similar to effects of extracellular Ca^{2+} on secretory cells (Douglas, 1974). In fact, when the concentration of extracellular Ca^{2+} was 10 mM, the effect of complement was almost abolished (Fig. 6), a condition which also inhibited haemoglobin release from intact cells (data not shown).

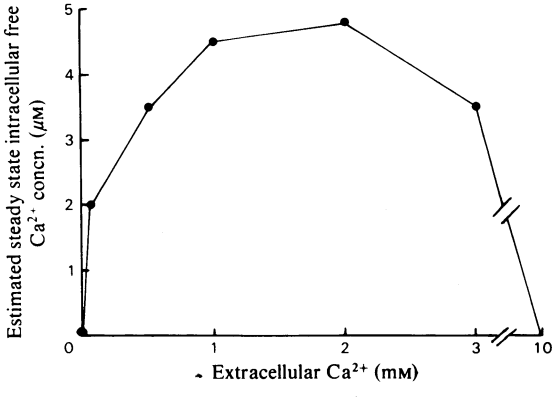

Fig. 6. *Effect of extracellular Ca^{2+} concentration on intracellular free Ca^{2+} in the presence of anti-(pigeon erythrocyte) antibody plus complement*

Portions (50 μl: approx. 10^8 'ghosts') of sealed pigeon erythrocyte 'ghosts' were incubated in 0.5 ml of medium A (see the Experimental section) containing various Ca^{2+} concns. from 0 (1 mM-EGTA) to 10 mM and 10 μl of $C\overline{56}$ + C9 for 5 min at 37°C. The tube containing the 'ghosts', maintained at 37°C, was then placed in front of the photomultiplier and 0.5 ml of medium A containing the same Ca^{2+} concn. and 1.3 μl of C7,8. The rate of obelin luminescence was plotted on a chart recorder and the maximum rate constant $(k_{app.})_t$ from the trace measured and converted into absolute free Ca^{2+} concns. as described in Figs. 4 and 5. The maximum intracellular free Ca^{2+} concn. was then plotted against extracellular Ca^{2+} concn. Each point represents the mean of two determinations.

Effect of anti-(pigeon erythrocyte) antibody plus complement on $^{42}K^+$ efflux and cyclic AMP formation

Several cells, including some mammalian erythrocytes, have been shown to contain a Ca^{2+} activated K^+-conducting pathway in the cell membrane (Meech, 1976, 1979; Lew & Brown, 1979). Pigeon erythrocytes contain an adenylate cyclase that can be activated by adrenaline and inhibited by concentrations of Ca^{2+} in the range 1–10 μM (Campbell & Siddle, 1976; Campbell & Dormer, 1978). Since anti-(pigeon erythrocyte) antibody plus complement had been shown to raise the intracellular Ca^{2+} concentration before significant lysis occurred (Campbell *et al.*, 1979*a*), the effects on cyclic AMP formation and $^{42}K^+$ efflux were investigated, though the latter is known to be difficult to measure accurately because of the relatively rapid exchange of K^+ across biological membranes (Lew & Brown, 1979).

Anti-(pigeon erythrocyte) antibody plus complement caused an inhibition of the adrenaline-stimulated rise in cyclic AMP formation (Table 1) in a similar manner to that shown with intact cells (Campbell *et al.*, 1979*a*).

Measurement of the percentage obelin consumption in 'ghosts' and, under identical conditions, $^{42}K^+$

Table 1. *Effect of anti-(pigeon erythrocyte) antibody plus complement on cyclic AMP formation*

Portions (50 μl: approx. 10^8 'ghosts') of sealed pigeon erythrocyte 'ghosts' were incubated in 0.5 ml of medium A + 1 mM-$CaCl_2$ (see the Experimental section) containing 5 μl of anti-(pigeon erythrocyte) serum for 1 min at 37°C. A 0.5 ml portion of medium A + 1 mM-$CaCl_2$ containing 25 μM) of human serum and adrenaline (final concn. 55 μM) was then added. After a defined time interval a small volume of perchloric acid was added (final concn. 0.28 M). Cyclic AMP was then assayed as described in the Experimental section. Each figure represents the mean of two separate incubations.

Time after addition of adrenaline (min)	Cyclic AMP (pmol/10^6 'ghosts')	
	Control	Antibody + complement
0	<1	<1
2	6.9	4.7
5	19.1	14.9
15	42.0	16.4

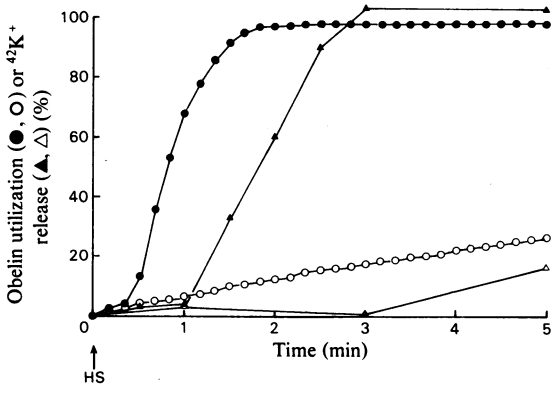

Fig. 7. *Effect of anti-(pigeon erythrocyte) antibody plus complement on release of $^{42}K^+$ from the 'ghosts'*
Portions (50 μl: approx. 10^8 'ghosts') of sealed pigeon erythrocyte 'ghosts' prepared containing obelin and $^{42}K^+$ as described in the Experimental section were incubated with 0.5 ml of medium A + 1 mM-$CaCl_2$ containing 10 μl of anti-(pigeon erythrocyte) serum for 5 min at 37°C. A 0.5 ml portion of medium A + 1 mM-$CaCl_2$ containing no additions (○, △) or 100 μl of human serum (●, ▲) was then added (HS ↑). The percentage consumption of obelin (●, ○) together with percentage $^{42}K^+$ (▲, △) release were then measured as described in the Experimental section. Each point represents the mean of two determinations.

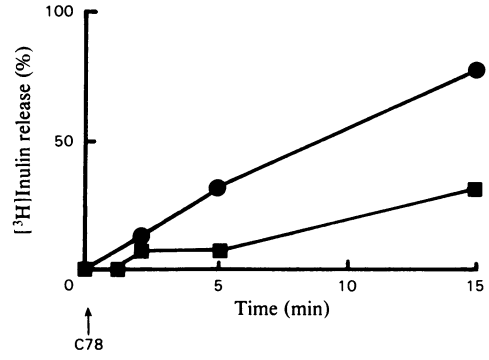

Fig. 8. *Inhibition of reactive lysis by extracellular EGTA*
Portions (50 μl: 5×10^7 'ghosts') of sealed pigeon erythroctye 'ghosts' containing [^{3}H]inulin (6000 d.p.m.) were incubated in 0.5 ml of medium A (see the Experimental section) + 1 mM-$CaCl_2$ (●) or 2 mM-EGTA (■) + 10 μl of $\overline{C56}$ + C9 for 5 min at 37°C. A 0.5 ml portion of medium A + 1 mM-$CaCl_2$ (●) or 2 mM-EGTA (■) + 0.7 μl of C78 was then added and the percentage of inulin released into the medium measured after centrifugation by scintillation counting. In the absence of complement the total inulin release after 15 min was 15%. Results represent the mean of two determinations.

(Fig. 7) showed that a large increase in obelin luminescence could be detected about 30 s before significant efflux of $^{42}K^+$. It appears from the data shown in Fig. 1, and other similar experiments, that the steady-state intracellular free Ca^{2+} concentration (see Figs. 4 and 5) also occurred before the maximum rate of K^+ efflux. Within 3–5 min under these conditions about 100% of the $^{42}K^+$ had been released. This was in spite of the fact that even in the presence of anti-(pigeon erythrocyte) antibody the 'ghosts' retained approx. 50–75% of their total K^+, measured by atomic absorption spectrometry (Campbell *et al.*, 1979*a*). These experiments suggest that a complex in the cell membrane was formed within 1–2 min as a result of complement action that allows the passage of both ions and molecules of molecular weight at least up to 3000 (inulin). However, the 'ghosts' still appear to be able to maintain both a K^+ and Ca^{2+} gradient for several minutes after the formation of the membrane complex.

The results suggest that the membrane complex may be more selective for Ca^{2+} rather than K^+ since, if the ions were moving by simple diffusion, the larger absolute K^+ gradient (approx. 150–5 mM) compared with Ca^{2+} (approx. 1 mM–0.3 μM) should cause K^+ flux to be faster than Ca^{2+}, even if there is a negative potential across the membrane.

Effect of removal of extracellular Ca^{2+} on complement-mediated cell lysis

To investigate the possible requirement for extracellular Ca^{2+} in cell lysis the release of [^{3}H]inulin from 'ghosts' during reactive lysis (Lachmann & Thompson, 1970; Thompson & Lachmann, 1970) was measured in the presence of 1 mM-$CaCl_2$ or 2 mM-EGTA. The absence of extracellular Ca^{2+} inhibited [^{3}H]inulin release from the pigeon erythrocyte 'ghosts' (Fig. 8).

Effect of anti-cell antibody plus complement on membranes from other cells

The rapid increase in intracellular free Ca^{2+} caused by complement action was observable in several membrane vesicle preparations (Hallett & Campbell, 1980, 1981). However, no effect of C5b–9 could be demonstrated on obelin entrapped within liposomes that were prepared from purified phospholipids containing no antigens and that were highly impermeable to Ca^{2+} (Dormer *et al.*, 1978).

Conclusions

The present paper reports an increase in the luminescence of the Ca^{2+}-activated photoprotein obelin, entrapped within sealed erythrocyte 'ghosts',

as a result of complement activation. The increase in obelin luminescence could not be explained by cell lysis (Fig. 8; Campbell *et al.*, 1979*a*,*b*,*c*). Obelin luminescence was inhibited when EGTA, a Ca^{2+} chelator, was present within the 'ghosts' and abolished when EGTA was added extracellularly. furthermore quantification (Figs. 4 and 5) showed that over the first 1–2 min the concentration of intracellular free Ca^{2+} remained at only a few μM, even when the concentration outside the 'ghosts' was 1 mM. It was therefore concluded that the earliest detectable intracellular event caused by activation of complement at the cell surface was an increase in intracellular free Ca^{2+}.

The time course of the increase in intracellular Ca^{2+} suggested that significant numbers of complement complexes occurred within 10–20 s and that the influx of Ca^{2+} required the complete terminal complex, including C9 (Fig. 3). These membrane complexes appeared to show some selectivity for Ca^{2+} over K^+ (Fig. 7), though like the data of inhibition of cyclic AMP (Table 1) it was not possible to definitively show that any part of the increased K^+ flux was Ca^{2+} activated.

A role for extracellular Ca^{2+} in the formation of the C1–antibody complex has been known for some time (Gigli *et al.*, 1976; Porter & Reid, 1978). Our results (Fig. 8) suggest that an increase in intracellular Ca^{2+} may also be necessary for maximal rate of cell lysis. This possibility may have been ignored in the past since many of the early experiments used EDTA to remove Ca^{2+} (Frank *et al.*, 1964, 1965; Boyle *et al.*, 1976*a*,*b*). However, EDTA binds both Ca^{2+} and Mg^{2+}. When EGTA was used to investigate the requirement for extracellular Ca^{2+} in activation of the complement pathway, Ca^{2+} was added back before measuring cell lysis (Fine *et al.*, 1972). In one study extracellular Ca^{2+} was shown to be required for lysis but the results were interpreted as showing an additional role for C1 (May & Frank, 1973). It is of interest that it has been reported that there is a cation requirement after C9 binding (Frank *et al.*, 1965; Hadding & Müller-Eberhard, 1967).

Increases in intracellular Ca^{2+} lead to shape changes (White, 1974; Marchesi *et al.*, 1976) and membrane vesiculation (Allan & Michell, 1977) in avian erythrocytes, whereas in lymphocytes (Kaiser & Edelman, 1977; Okamoto & Mayer, 1978) and hepatocytes (Schanne *et al.*, 1979) increases in intracellular Ca^{2+} may play a major role in the action of certain cytolytic agents. Complement activation causes membrane vesiculation in rat adipocytes (Richardson & Luzio, 1980) and it would be interesting to know whether this is also dependent on Ca^{2+}.

Non-lytic effects of complement have been demonstrated in cartilage (Fell *et al.*, 1966) and on prostaglandin synthesis and bone resorption (Raisz *et al.*, 1974). The early rise in intracellular Ca^{2+} caused by complement is a good candidate as the mediator of these effects.

We are grateful to the Director and staff of the Marine Biological Association Laboratory, Plymouth for the facilities for the collection of *Obelia geniculata*. We thank Professor P. J. Lachmann for the gift of complement components for reactive lysis and Mr. M. E. T. Ryall for the construction of the luminescence apparatus. We thank Professors G. H. Elder, C. N. Hales and P. J. Lachmann for helpful advice and discusion. This work was supported by grants from the Science Research Council and British Diabetic Association. R. A. D. is the holder of a Studentship for training in research methods from the Medical Research Council.

References

Allan, D. & Michell, R. H. (1977) *Biochem. J.* **166**, 495–499

Ashley, C. C. & Campbell, A. K. (1979) (eds.) *Detection and Measurement of Free Ca^{2+} in Cells*, Elsevier/North-Holland, Amsterdam

Boyle, M. D. P., Ohanian, S. H. & Borsos, T. (1976*a*) *J. Immunol.* **116**, 1272–1275

Boyle, M. D. P., Ohanian, S. H. & Borsos, T. (1976*b*) *J. Immunol.* **116**, 1276–1279

Campbell, A. K. & Dormer, R. L. (1975) *Biochem. J.* **152**, 255–265

Campbell, A. K. & Dormer, R. L. (1978) *Biochem. J.* **176**, 53–66

Campbell, A. K. & Siddle, K. (1976) *Biochem. J.* **158**, 211–221

Campbell, A. K. & Simpson, J. S. A. (1979) in *Techniques in Metabolic Research B213*, pp. 1–56, Elsevier/North Holland, Amsterdam

Campbell, A. K., Daw, R. A. & Luzio, J. P. (1979*a*) *FEBS Lett.* **107**, 55–60

Campbell, A. K., Lea, T. J. & Ashley, C. C. (1979*b*) in *Detection and Measurement of Ionized Calcium in Cells* (Ashley, C. C. & Campbell, A. K., eds.), pp. 13–72, Elsevier/North-Holland, Amsterdam

Campbell, A. K., Daw, R. A., Hallett, M. B. & Luzio, J. P. (1980) *J. Physiol.* **306**, 44–45

Campbell, A. K., Hallett, M. B., Daw, R. A., Luzio, J. P. & Siddle, K. (1979*c*) *Biochem. Soc. Trans.* **7**, 865–869

Dormer, R. L., Newman, G. R. & Campbell, A. K. (1978) *Biochim. Biophys. Acta* **538**, 87–105

Douglas, W. W. (1974) *Biochem. Soc. Symp.* **39**, 1–28

Dourmashkin, R. R. (1978) *Immunology* **35**, 205–212

Fell, H. B., Coombs, R. R. A. & Dingle, J. T. (1966) *Int. Arch. Allergy* **30**, 146–176

Fine, D. P., Marney, S. R., Colley, D. G., Sergent, J. S. & Des Prez, R. M. (1972) *J. Immunol.* **109**, 807–809

Frank, M. M., Rapp, H. J. & Borsos, T. (1964) *J. Immunol.* **93**, 409–413

Frank, M. M., Rapp, H. J. & Borsos, T. (1965) *J. Immunol.* **94**, 295–300

Gigli, I., Porter, R. R. & Sim, R. B. (1976) *Biochem. J.* **157**, 541–548

Hadding, U. & Müller-Eberhard, H. J. (1967) *Science* **157**, 442–443
Hallett, M. B. & Campbell, A. K. (1980) *Biochem. J.* **192**, 587–596
Hallett, M. B. & Campbell, A. K. (1981) in *Biomedical Applications of Luminescence* (Kricka, L. & Carter, T., eds.), Marcel Dekker, London, in the press
Kaiser, N. & Edelman, I. S. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 638–642
Lachmann, P. J. (1975) in *Clinical Aspects of Immunology*, (Gell, P. G. H., Coombs, R. R. A. & Lachmann, P. J., eds.), 3rd edn., pp. 323–364, Blackwell, Oxford
Lachmann, P. J. & Hobart, M. J. (1978) in *Handbook of Experimental Immunology* (Weir, D. M., ed.), 3rd edn., pp. 1–23, Blackwell, Oxford
Lachmann, P. J. & Thompson, R. A. (1970) *J. Exp. Med.* **131**, 643–657
Lachmann, P. J., Bowyer, D. E., Nichol, P., Dawson, R. M. C. & Munns, E. A. (1973) *Immunology* **24**, 135–145
Lew, V. & Brown, A. M. (1979) in *Detection and Measurement of Free Ca^{2+} in Cells* (Ashley, C. C. & Campbell, A. K., eds.), pp. 423–432, Elsevier/North-Holland, Amsterdam
Luzio, J. P., Newby, A. C. & Hales, C. N. (1976) *Biochem. J.* **154**, 11–21
Luzio, J. P., Daw, R. A., Hallett, M. B., Richardson, P. J. & Campbell, A. K. (1979) *Biochem. Soc. Trans.* **7**, 1066–1068
Marchesi, V. T., Furthmayr, H. & Tomita, M. (1976) *Ann. Rev. Biochem.* **45**, 667–698
May, J. E. & Frank, M. M. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 649–652
Meech, R. (1976) *Symp. Soc. Exp. Biol.* **30**, 161–191
Meech, R. (1979) *Annu. Rev. Biophys. Bioeng.* **7**, 1–18
Michaelis, D. W., Abramovitz, A. S., Hammer, C. H. & Mayer, M. M. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2852–2856
Okamoto, M. & Mayer, M. M. (1978) *J. Immunol.* **120**, 279–285
Osborn, T. W. B. (1935) *Complement or Alexia*, Oxford University Press, Oxford
Podack, E. R., Biesecker, G. & Müller-Eberhard, H. J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 897–901
Porter, R. R. & Reid, K. B. M. (1978) *Nature (London)* **275**, 699–704
Raisz, L. G., Sandberg, A. L., Goodson, M. J., Simmons, H. A. & Mergenhayer, S. E. (1974) *Science* **185**, 789–791
Rasmussen, H. & Goodman, D. B. P. (1977) *Physiol. Rev.* **57**, 421–509
Richardson, P. J. & Luzio, J. P. (1980) *Biochem. J.* **186**, 897–906
Schanne, F. A. X., Kane, A. B., Young, E. E. & Farber, J. L. (1979) *Science* **206**, 700–702
Shearer, W. T., Atkinson, J. P. & Parker, C. W. (1976) *J. Immunol.* **117**, 973–980
Sims, P. J. & Lauf, P. K. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5669–5673
Stolfi, R. L. (1968) *J. Immunol.* **100**, 46–54
Tamura, N., Shimada, A. & Chang, S. (1972) *Immunology* **22**, 131–140
Thompson, R. A. & Lachmann, P. J. (1970) *J. Exp. Med.* **131**, 629–641
White, J. (1974) *Am. J. Pathol.* **77**, 507–514