The Permeability to Calcium of Pigeon Erythrocyte 'Ghosts' Studied by using the Calcium-Activated Luminescent Protein, Obelin

By ANTHONY K. CAMPBELL and ROBERT L. DORMER

Department of Medical Biochemistry, The Welsh National School of Medicine,
Heath Park, Cardiff CF4 4XN, U.K.

(Received 22 May 1975)

1. Obelin, the Ca^{2+} -activated luminescent protein from the hydroid *Obelia geniculata*, was sealed inside pigeon erythrocyte 'ghosts' in order to investigate effects on their permeability of different methods of preparation and of the bivalent cation ionophore A23187. 2. Changes in free Ca^{2+} within the 'ghosts' were studied by following the rate of luminescence of obelin. The possibility that the obelin might have been released from the 'ghosts' during an experiment was investigated by studying the release of inulin and pyruvate kinase from the 'ghosts'. Less than 10% of the inulin or pyruvate kinase sealed within the 'ghosts' was released under any of the experimental conditions. 3. Triton X-100 (0.1–10%, v/v) made the 'ghosts' highly permeable to Ca^{2+} . In the presence of 1 mM- Ca^{2+} and Triton, 95–100% of the obelin was utilized within 10–20 s. 4. A time-course of resealing 'ghosts' at 37°C showed that over a period of 90 min, the 'ghosts' became gradually less permeable to Ca^{2+} . 'Ghosts' which remained at 0°C retained only a small concentration of obelin and ATP, and were highly permeable to Ca^{2+} . 5. Erythrocyte 'ghosts' resealed for 30 min at 20°C rather than 37°C were more permeable to Ca^{2+} , as shown by the fact that 92% of the obelin in the 'ghosts' was utilized during the first 60 s after the addition of 1 mM- Ca^{2+} , as opposed to 44% for 'ghosts' resealed at 37°C. 6. Haemolysis at pH 6.0 rather than 7.0 resulted in 'ghosts' which were highly permeable to Ca^{2+} after resealing for 60 min at 37°C. Of the obelin in the 'ghosts', produced by haemolysis at pH 6.0, 90% was utilized in the first 60 s after the addition of 1 mM- Ca^{2+} compared with 23% for 'ghosts' produced at pH 7.0. 7. The bivalent cation ionophore A23187 increased the permeability of the 'ghosts' to Ca^{2+} . Maximum effects of the ionophore (16 µg/ml) were obtained by preincubating the 'ghosts' with the ionophore A23187 (16 µg/ml) in the presence of a low concentration of Mg^{2+} and in the absence of Ca^{2+} .

It has been proposed that cyclic nucleotides and Ca^{2+} are intracellular mediators of hormone action and secretion (Robison *et al.*, 1971; Rasmussen *et al.*, 1972). The concentration of free Ca^{2+} in the cell is probably in the range 0.1–10 µM, compared with a total cell $[\text{Ca}^{2+}]$ in the region of 1 mM (Baker, 1972; Ashley & Caldwell, 1974). The role of Ca^{2+} in the mechanism of hormone action and secretion has been investigated *in vitro* by studying Ca^{2+} fluxes (Friedmann & Park, 1968; Werner *et al.*, 1972) or the effect of addition of EGTA* (Exton *et al.*, 1972; Katocs *et al.*, 1974), local anaesthetics (Kissebah *et al.*, 1974; Siddle & Hales, 1974) or the ionophore A23187 (Selinger *et al.*, 1974). None of these studies have been able to show definitively whether a particular hormone increases or decreases free cytoplasmic Ca^{2+} , or what the time-course of such a change might be.

* Abbreviations: EGTA, ethanedioxybis(ethylamine)-tetra-acetate; Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)-ethanesulphonic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid; ATPase, adenosine triphosphatase.

Changes in free Ca^{2+} have been studied in single barnacle muscle fibres (Ashley & Ridgeway, 1970; Ashley *et al.*, 1974), the giant axon of the squid (Baker *et al.*, 1971) and the squid giant synapse (Llinas & Nicholson, 1975) after microinjection of the Ca^{2+} -activated luminescent protein aequorin. Aequorin has also been injected into smaller cells such as *Spirostomum* (Ettienne, 1970) and salivary-gland cells of *Chironomus* (Rose & Lowenstein, 1975). There have been no reports of similar studies using small cells such as those from mammals or birds. Pigeon erythrocytes, in common with other avian erythrocytes, produce large amounts of cyclic AMP when stimulated by adrenaline (Davoren & Sutherland, 1963; King & Mayer, 1974). The aim of the present paper is to show that, by using techniques similar to those for human erythrocytes (Schwoch & Passow, 1973), pigeon erythrocyte 'ghosts' can be prepared containing a Ca^{2+} -activated luminescent protein and that this system can be used to study changes in $[\text{free } \text{Ca}^{2+}]$ within the 'ghosts'. Aequorin cannot be readily obtained in the United Kingdom. Obelin, a protein

similar to aequorin, extracted from the hydroid *Obelia geniculata*, was used, since it can be obtained in sufficient quantities to carry out physiological experiments (Campbell, 1974; Ashley *et al.*, 1975; Moisesescu *et al.*, 1975).

Experimental and Results

Materials

Chemicals. ATP (disodium salt), phosphoenolpyruvate (potassium salt) and pyruvate kinase (EC 2.7.1.40) were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. Tris and gelatin type I were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. DEAE-cellulose (DE52) was obtained from Whatman Biochemicals Ltd., Maidstone, Kent, U.K. Sephadex G-75 and G-25 were from Pharmacia Ltd., London W.5, U.K., and Chelex 100 (200–400 mesh) was from Bio-Rad, Bromley, Kent, U.K. The ionophore A23187 was a kind gift from Eli Lilly and Co., Indianapolis, Ind., U.S.A. [^{14}C]EDTA (19.8 mCi/mmol) and [*carboxy*- ^{14}C]inulin (2.16 $\mu\text{Ci}/\text{mg}$) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other reagents were of analytical grade from BDH Chemicals, Poole, Dorset, U.K.

Pigeons. Homer pigeons (*Columba livia domestica*) were used throughout.

Hydroids. Colonies of *Obelia geniculata* attached to fronds of the brown seaweed *Laminaria* species *digitata*, *hyperborea*, *ochroleuca* and *saccharina* were collected by skin divers in Plymouth Sound during the period June–September 1974.

Extraction and purification of obelin

The hydroids were carefully cut off the seaweed; the obelin was extracted in 40 mM-EDTA–200 mM-Tris, pH 7.0, and partially purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation as previously described (Campbell, 1974). The obelin was purified and concentrated further by gel filtration on a Sephadex G-75 column (3 cm $\times$ 30 cm) equilibrated in 10 mM-Hepes–1 mM-EDTA, pH 7.0, followed by elution from a DEAE-cellulose column (10 cm $\times$ 1 cm) equilibrated in 10 mM-Hepes–0.1 mM-EDTA, pH 7.0, containing a salt gradient of 50–500 mM-NaCl.

The obelin from the DEAE-cellulose column was then passed down a Sephadex G-25 column (5 cm $\times$ 1 cm) equilibrated in 10 mM-Hepes–10 μM -EDTA, pH 7.0. To estimate the EDTA concentration in the effluent from the Sephadex G-25 column, a trace (approx. 1 μCi) of [^{14}C]EDTA was added to the solution of obelin put on the column. A 50 μl sample of this solution, together with a 50 μl sample of effluent from the Sephadex G-25 column, was added to Triton–toluene scintillant (Siddle *et al.*, 1973) and the radioactivity measured in a Packard Tri-Carb scintil-

lation counter. The ratio of the radioactivities in the two samples was used to calculate the concentration of EDTA in the effluent from the Sephadex G-25 column. When this solution was added to the media described below, the final concentration of EDTA was always less than 0.2 μM . Samples from the Sephadex G-25 column were freeze-dried after the addition of gelatin to a final concentration of 0.1 mg/ml (Campbell, 1974). The freeze-dried solid was stored in a desiccator at -70°C with no detectable loss in activity over a period of at least 6 months. This procedure resulted in a 200–1000-fold concentration of the active obelin from the crude extracts, with a yield of 10–20%, and a purification of 40–50-fold.

Assay of luminescence

Obelin luminescence, as with aequorin, is stimulated by Ca^{2+} and requires no other substrates or co-factors. Once the protein has luminesced there is no known method of re-activating it. The standard assay for total obelin was to place a sample of obelin in 0.5 ml of 200 mM-Tris–0.1 mM-EDTA, pH 8.9, in a plastic tube (LP3; Luckam Ltd., Labco Works, Burgess Hill, Sussex, U.K.). The tube was placed in front of a highly sensitive, low-dark-current photomultiplier tube (P4232B, from Twentieth Century Electronics Ltd., Centronics, New Addington, Croydon CR9 0BG, U.K.; dark current 0.07 nA at 1190 V, overall sensitivity 200 A/lm; or 9757A from E.M.I. Electronics Ltd., Hayes, Middx., U.K., dark current 0.21 nA at 920 V, overall sensitivity 200 A/lm) in a specially constructed light-tight apparatus (Campbell, 1974). The photomultiplier tube was connected to a scalar (Ekco Electronics Ltd., Maidenhead, Berks., U.K.; M5060A) and the total counts were recorded during the first 10 s after the addition of 0.5 ml of 10 mM- CaCl_2 . The CaCl_2 was added by injecting it from a syringe while the tube containing the obelin was in front of the photomultiplier tube. The background count in the absence of obelin was approx. 200 counts/10 s. The rate of luminescence was also measured by connecting the photomultiplier output to a rate meter (Ekco Electronics; M5190) which in turn was connected to a chart recorder (26000 X–Y, from Bryans Ltd., Mitcham, Surrey, CR4 4UL, U.K.). By knowing the maximum output of the rate meter, it was possible to calibrate the chart recorder in c.p.s. None of the substances used in the experiments described below affected the total number of counts. Only Mg^{2+} and univalent cations affected the rate of Ca^{2+} -stimulated luminescence.

Ca^{2+} contamination in the medium

In an attempt to decrease Ca^{2+} contamination in the resealing solutions, all glassware, pipettes and

plastic containers were rinsed in 1–10% (v/v) HCl, followed by rinsing in double-glass-distilled water. In spite of this the rate of obelin luminescence in 10mM-Tes pH7.0, suggested that the free Ca^{2+} concentration was 1–5 μM . The free Ca^{2+} concentration in this Tes buffer could be lowered to approx. 0.1 μM , assessed by obelin luminescence, by passing the Tes down a Chelex 100 column (3cm $\times$ 20cm). The Chelex was first washed with 1M-HCl, followed by 1M-NaOH (three times), and then washed with double-glass-distilled water. After 3 days, when the pH of the effluent was pH9.0, 10mM-Tes was passed down the column. The effluent had a pH of 6.8 and contained 1mM- Na^+ and less than 10 μM - K^+ as measured by atomic absorption spectrophotometry. This buffer was used in all experiments except that described in Fig. 2.

Preparation of pigeon erythrocyte 'ghosts'

A pigeon was anaesthetized with ether and bled from the wing vein. Blood (5–10ml) containing 200–1500 units of heparin (5–10mg) was centrifuged at 4°C at 27000g for 5min in a MSE superspeed 50 ultracentrifuge. The erythrocytes were washed once in medium A (10mM-Hepes or -Tes, 140mM-NaCl, 5mM-KCl, 2mM-MgCl₂), adjusted to pH7.4 with NaOH. The cell pellet was haemolysed for 15min at 0°C in 6mM-NaCl–3mM-MgCl₂ at a final pH of 7.0 or 6.0, adjusted with solid Tris or HCl. After centrifuging for 5min at 27000g the pellet was washed four or five times with 6mM-NaCl–3mM-MgCl₂ at pH7.0 or 6.0 (total volume of washes = 60ml). The final, faintly pink, pellet was resuspended in 5–10ml of 10mM-Hepes or -Tes, 6mM-NaCl, 2mM-MgCl₂, pH7.4 with or without an ATP-regenerating system {2mM-ATP (disodium salt), 10mM-phosphoenolpyruvate (potassium salt), 19 units of pyruvate kinase/ml [centrifuged for 2min in a Beckman microfuge in order to remove the (NH₄)₂SO₄]}. The pH was adjusted with KOH. Obelin was added and after 5min at 0°C a small volume of 3M-KCl was added to give a final concentration of 150mM. The suspension was incubated for up to 90min at room temperature (approx. 20°C) or at 37°C. After centrifuging at 27000g for 5min at 4°C, the pellet was washed three times in medium A (total volume of washes = 15–20ml). The ATP concentration in the final wash was undetectable (less than 40nM) and the obelin concentration less than 1% of that in the final suspension. The 'ghosts' were resuspended in 0.5–1.0ml of medium A and the final volume was measured. The number of 'ghosts'/ μl of suspension was measured with a haemocytometer. Comparison with the number of cells in the original blood showed that there was no evidence of cell fragmentation. The 'ghosts' were stored for up to 4h at 0°C.

Leakiness of 'ghosts' to inulin and pyruvate kinase

To investigate the possibility that luminescence of the obelin might be explained by its gradual release from the 'ghosts' into the medium, the release of two macromolecules, inulin (mol.wt. approx. 5000; information sheet, The Radiochemical Centre, Amersham) and pyruvate kinase (mol.wt. approx. 230,000; Bergmeyer, 1965), was measured. Pigeon erythrocyte 'ghosts' were prepared as described above. The resealing medium contained an ATP-regenerating system (19 units of pyruvate kinase/ml) and 1mg of [*carboxy*-¹⁴C]inulin/ml (2.16 $\mu\text{Ci}/\text{mg}$). A portion (50 μl) of the final 'ghost' suspension containing 5000–10000 d.p.m. of inulin and 15–30 μunits of pyruvate kinase was added to 0.5ml of medium A (10mM-Tes, 140mM-NaCl, 5mM-KCl, 2mM-MgCl₂, pH7.4), and 0.5ml of medium A containing 2mM- Ca^{2+} was then added. To measure release of inulin and pyruvate kinase from the 'ghosts', the medium containing the 'ghosts' was centrifuged for 2min in an Eppendorf microfuge at 4°C. The supernatant was removed and the pellet resuspended in 0.2ml of 6mM-NaCl–3mM-MgCl₂. Samples (10–50 μl) were taken for assay of pyruvate kinase and added to 1ml of medium containing 85.6mM-triethanolamine, 15mM-MgSO₄, 100mM-KCl, 1.2mM-phosphoenolpyruvate (potassium salt), 1.6mM-ADP (disodium salt), 0.25mM-NADH and 4.8 units of lactate dehydrogenase. The decrease in E_{340} was followed at 30°C in a Unicam SP.8000 recording spectrophotometer. Inulin was measured in Triton–toluene scintillant (Siddle *et al.*, 1973) in a Packard Tri-Carb scintillation counter. Quenching was corrected for by the method of internal channels-ratio. In all experiments, 6–12% of the inulin and less than 10% of the pyruvate kinase were found in the supernatant after its separation from the 'ghosts'. The addition of 0.5ml of medium A containing 2mM- Ca^{2+} immediately after adding the 'ghosts' to 0.5ml of medium A, or after preincubation of the 'ghosts' with ethanol (2 $\mu\text{l}/\text{ml}$), or the bivalent cation ionophore A23187 (16 $\mu\text{g}/\text{ml}$) (for conditions see Fig. 5), resulted in the release of an additional 2–4% of inulin and no detectable pyruvate kinase. After the addition of 0.5ml of medium A containing 2mM- Ca^{2+} and Triton X-100 (2%, v/v) more than 95% of the inulin and more than 90% of the pyruvate kinase was released into the medium.

Measurement of ATP

The ATP content of the 'ghosts' was measured by using a firefly luciferase assay (Siddle *et al.*, 1973) after acidifying 50 μl of suspension with 50 μl of 10% (w/v) trichloroacetic acid and extracting the trichloroacetic acid with 4 $\times$ 500 μl of diethyl ether. ATP standards were taken through the same procedure. ATP recovery in the medium was measured in 50 μl samples taken at defined intervals.

Obelin recovery and incorporation into 'ghosts'

Samples of incubation medium were taken at defined times during resealing and added to 0.5 ml of 200 mM-Tris-0.1 mM-EDTA, pH 7.0. The total counts during the first 10 s after the addition of 0.5 ml of 10 mM-CaCl₂ were recorded. In order to assay the obelin content of the 'ghosts', 10 μ l of 'ghost' suspension was added to 100 μ l of 1 mM-EDTA or 1 mM-EGTA, pH 7.0. Then 0.5 ml of 200 mM-Tris-0.1 mM-EDTA, pH 8.9, was added and the total counts during the first 10–20 s after the addition of 0.5 ml of 10 mM CaCl₂ were recorded (Fig. 1).

Effect of Triton X-100 on pigeon erythrocyte 'ghosts'

The effect of Triton X-100, at a final concentration of 0.01–10% (v/v) in the presence of 1 mM-CaCl₂, on

Fig. 1. Effect of Triton X-100 on pigeon erythrocyte 'ghosts'

'Ghosts' were prepared from 10 ml of blood as described in the Experimental and Results section. The haemolysis pH was 7.0. The 'ghosts' were resealed with an ATP-regenerating system for 60 min at 37°C. The final 'ghost' suspension (2.5 ml) contained 5.0×10^6 'ghosts'/ μ l. The ATP content of the 'ghosts' was 2.8 pmol of ATP/ μ l. 'Ghost' suspension (10 μ l) + 100 μ l of 1 mM-EGTA, pH 7.0, were added to 0.5 ml of 200 mM-Tris-0.1 mM-EDTA, pH 8.9, and 0.5 ml of 10 mM-CaCl₂ was added ($\circ$). In other experiments 10 μ l of 'ghosts' was suspended in 0.5 ml of medium A (10 mM-Tes, 140 mM-NaCl, 5 mM-KCl, 2 mM-MgCl₂, pH 7.4), and 0.5 ml of medium A + 2 mM-CaCl₂ ($\blacksquare$) + Triton X-100, 0.02% ($\bullet$), 0.1% ($\blacktriangle$), 0.2% ($\triangle$), 2% ($\square$) and 20% (∇) was added. Each point represents the mean of at least two determinations of the total counts recorded on the scalar up to that time. Luminescence was assayed at 20°C.

obelin luminescence in the 'ghosts' is shown in Fig. 1. In 'ghosts' lysed with 1 mM-EGTA or 0.1–10% (v/v) Triton X-100, 95–100% of the obelin luminesced within 10–20 s. Triton X-100 (0.01%, v/v) had no detectable effect on this 'ghost' preparation. To assay the obelin content remaining in the 'ghosts' at any time during the experiments described below, Triton X-100 in 0.5 ml of medium A was added to give a final concentration of 1% (v/v) and the total counts were recorded during the first 10–20 s.

Quenching of luminescence by pigeon erythrocyte lysate

The emission maximum of obelin luminescence is at 475 nm (Morin & Hastings, 1971). This is close to the absorption maximum of haemoglobin. The addition of 10–200 μ l of pigeon erythrocyte lysate, of known haemoglobin concentration, resulted in a decrease in the total number of counts recorded when obelin luminescence was stimulated by Ca²⁺ (Table 1). Haemoglobin was measured by the cyanmethaemoglobin method (Davie & Lewis, 1970). No decrease in luminescence was observed if medium alone was

Table 1. Quenching of obelin luminescence by erythrocyte lysate

'Ghosts' were prepared from 14 ml of pigeon blood. The haemolysis pH was 7.0, and the supernatant (38 mg of haemoglobin/ml) from the initial haemolysis retained for quenching studies. The 'ghosts' were resealed with an ATP-regenerating system at 20°C for 30 min. The final 'ghost' suspension contained 4.77×10^6 'ghosts'/ μ l, and less than 10 mg of haemoglobin/ml. Cell lysate (10–200 μ l) was added to 0.5 ml of 200 mM-Tris-0.1 mM-EDTA, pH 8.9, and then 0.5 μ l of purified obelin was added. The total counts were recorded during the first 10 s after the addition of 0.5 ml of 10 mM-CaCl₂. Each figure represents the mean of at least two determinations.

Pigeon erythrocyte lysate (μ l)	Haemoglobin (mg)	Obelin (counts)
0	0	82800
10	0.38	60300
20	0.76	52100
40	1.52	37900
60	2.28	24700
80	3.04	22800
100	3.80	22500
200	7.60	14000
200 μ l of 6 mM-NaCl-3 mM-MgCl ₂	0	84800
'Ghosts' (μ l)	Haemoglobin (mg)	Obelin (counts)
10	<0.1	81100
20	<0.2	83400
50	<0.5	71000
100	<1	43700
100 μ l of medium A	0	78000

Fig. 2. Rate of obelin luminescence in pigeon erythrocyte 'ghosts'

Ghosts were prepared as described in the Experimental and Results section. The haemolysis pH was 7.0. The 'ghosts' were resealed with an ATP-regenerating system at 20°C for 30 min. The final suspension contained 1.50×10^6 'ghosts'/ μl . 'Ghosts' (50 μl) containing 80000 obelin counts were suspended in 0.5 ml of medium A (10 mM-Hepes, 140 mM-NaCl, 5 mM-KCl, 2 mM-MgCl₂, pH 7.4) at 20°C and 0.5 ml of medium A + 2 mM-CaCl₂ was added ($\uparrow$). The total counts recorded on the scalar showed that 95–100% of the obelin was consumed within 60 s. The rate of luminescence was recorded on the chart recorder. Rate-meter time-constant 0.01 s, 10 mV output equivalent to 1000 c.p.s. t (s) = time to maximum rate of light emission.

added. The haemoglobin concentration in the final 'ghost' suspension was such that it should have produced a quenching of less than 10% in the experiments described below. In the experiment described in Table 1, 20 μl of 'ghost' suspension (equivalent to 50 μl in other experiments) produced no detectable quenching, hence quenching was ignored in all further experiments.

Permeability of 'ghosts' to Ca^{2+}

The relative permeability of the 'ghosts' to Ca^{2+} was assessed by measuring the rate of consumption of obelin in the 'ghosts' after the addition of Ca^{2+} at a final concentration of 1 mM. After the addition of Ca^{2+} to 'ghosts' suspended in medium A (10 mM-Tes, 140 mM-NaCl, 5 mM-KCl, 2 mM-MgCl₂, pH 7.4), the rate of luminescence was plotted on the chart recorder and the total number of counts produced every 10 s recorded. The obelin utilized after any given time was calculated by summing the total counts recorded up to this time and expressing them as a percentage of the total counts originally added. The latter was measured either separately or by the addition of Triton X-100 (final concentration 1%, v/v) at the

end of the experiment, and by using the sum of all the counts recorded during the experiment as the 100% value for total obelin. The two methods resulted in values within 10% of each other.

An illustration of the chart-recorder trace of the rate of obelin luminescence from 'ghosts' incubated with 1 mM-CaCl₂ is shown in Fig. 2. After the addition of Ca^{2+} , a peak in the rate of luminescence occurred within the response time of the chart recorder (100–200 ms), and decayed within 1–2 s. This peak could not have been caused by obelin contamination from the original resealing medium. It may have been due to obelin released from the 'ghosts' when the final pellet was resuspended or when the medium containing Ca^{2+} was added. The initial peak was followed by a slow increase and then a decrease in the rate of luminescence. This decrease could at least be partly explained by a loss of active obelin resulting from its utilization within the ghosts. The time to the peak of this second phase was measured in all experiments. A long time to this maximum, together with a slow rate of utilization of obelin, were interpreted as showing that the 'ghosts' were relatively impermeable to Ca^{2+} . The noise on the chart-recorder traces appeared to arise from electrical noise in the apparatus.

Table 2. Time-course of resealing pigeon erythrocyte 'ghosts' at 37°C

'Ghosts' from 10 ml of pigeon blood were prepared as described in the Experimental and Results section. The haemolysis pH was 7.0. Samples (1 ml) were taken at defined times during resealing, centrifuged immediately at 4°C at 27000g for 5 min and resuspended in 1.5 ml of medium A (10 mM-Tes, 140 mM-NaCl, 5 mM-KCl, 2 mM-MgCl₂, pH 7.4). This suspension remained at 0°C until the end of the experiment (90 min), when all the 'ghosts' were washed three times in medium A and resuspended in medium A to give a final volume of 0.7 ml. The total obelin at the start of resealing was 1.73×10^9 counts. The time (*t*) to maximum rate of luminescence was measured from the chart-recorder traces as described in Fig. 2. The obelin and ATP content of the 'ghosts' and recoveries during resealing were measured as described in the Experimental and Results section. Each value represents the mean of at least two determinations. Obelin luminescence was assayed at 18°C.

Time of reseat (min)	Obelin recovery in medium (%)	ATP recovery in medium (%)	'Ghosts'/ μ l	ATP (pmol/ μ l of 'ghosts')	Obelin (counts/ μ l of 'ghosts')	Obelin in 'ghosts' after 4 h at 0°C (%)	<i>t</i> (s)
0	100	100	8.7×10^5	1.5	500	19.1	5.5
15	48.8	1.8	1.28×10^6	8.6	8990	40.7	8.7
30	14.1	—	1.34×10^6	5.2	5270	60.0	22.1
45	3.8	0.4	1.61×10^6	5.0	5370	80.3	43.9
60	1.7	0.2	1.63×10^6	2.7	2900	72.8	49.2
90	0.7	0.06	1.50×10^6	0.7	1230	83.7	≈ 60

Fig. 3. Time-course of resealing pigeon erythrocyte 'ghosts' at 37°C

'Ghosts' were prepared as described in Table 2. 'Ghosts' (50 μ l) resealed for 0 (Δ), 15 min ($\square$), 30 min ($\circ$), 45 min ($\blacktriangle$), 60 min ($\blacksquare$), or 90 min ($\bullet$), at 37°C were suspended in 0.5 ml of medium A (10 mM-Tes, 140 mM-NaCl, 5 mM-KCl, 2 mM-MgCl₂, pH 7.4) at 18°C. Then 0.5 ml of medium A + 2 mM-CaCl₂ was added and the counts were recorded every 10 s. After 60 s, the remaining active obelin was measured by recording the total counts after the addition of 0.5 ml of medium A + 1 mM-CaCl₂ + 3% Triton X-100.

Time-course of resealing 'ghosts' at 37°C

After haemolysis of pigeon erythrocytes at pH 7.0, the 'ghosts' were resealed for up to 90 min at 37°C (Table 2). Loss of obelin activity in the suspension after 60 min varied between 70 and 98% in different experiments. This may have been due to Ca²⁺ released from the 'ghosts'.

A small quantity of obelin was found in 'ghosts' which remained at 0°C during the resealing (zero time,

Table 2). There was apparently little or no spontaneous resealing at 0°C, since more than 95% of the obelin was consumed within 60 s after the addition of Ca²⁺ to the 'ghosts' (Fig. 3), and the second peak in the rate of luminescence occurred within 6 s (Table 2). The largest concentration of obelin was found in 'ghosts' resealed for 15 min, although the 'ghosts' appeared to become increasingly impermeable to Ca²⁺ up to 90 min, as shown by the decrease in the rate of utilization of the obelin (Fig. 3) and the increase in the time at which the second peak occurred (Table 2). The decrease in total obelin in the 'ghosts' resealed for between 15 and 60 min was thought to be accounted for mainly by the inactivation of obelin during resealing. In fact the percentage decrease in total obelin in the 'ghosts' was less than that of the whole suspension.

There was a considerable variation in the ATP content of the 'ghosts' in different experiments (Table 2, 3 and 4). This was partly due to differences in the ratio of medium to 'ghosts' resulting in differences in the recovery of ATP, at least 99% of which was lost within 60 min.

Resealing 'ghosts' at room temperature

Pigeon erythrocyte 'ghosts' prepared by haemolysis at pH 7.0 were resealed for 30 min at 37°C or 20°C (Table 3). The recovery of obelin in the incubation medium varied between 22 and 87% at 37°C and 90 and 100% at 20°C, the recovery always being greater at 20°C in any particular experiment. In spite of this, the obelin content of 'ghosts' resealed at 20°C was 70% of that in 'ghosts' resealed at 37°C. Similarly the ATP content of 'ghosts' resealed at 20°C was 20% of that of 'ghosts' resealed at 37°C. The greater rate of utilization of obelin after addition of 1 mM-Ca²⁺

Table 3. *Resealing of pigeon erythrocyte 'ghosts' at 20°C or 37°C*

'Ghosts' were prepared from 2 ml portions of pigeon blood as described in the Experimental and Results section. The haemolysis pH was 7.0. Resealing was carried out in the presence of an ATP-regenerating system for 30 min at 20°C or 37°C. The total obelin in each tube at the start of resealing was 1.67×10^8 counts. The final volume of each suspension was 0.66 ml. The obelin and ATP content of the 'ghosts' and recovery in the medium after resealing were measured as described in the Experimental and Results section. 'Ghosts' (50 μl) were suspended in 0.5 ml of medium A (10 mM-Tes, 140 mM-NaCl, 5 mM-KCl, 2 mM-MgCl₂, pH 7.4) at 20°C. The percentage utilization (U) of obelin 60 s after the addition of 0.5 ml of medium A + 2 mM-CaCl₂ was measured. The time (*t*) to maximum rate of light-emission was measured as described in Fig. 2. Each value represents the mean of at least two determinations.

Temperature of reseat (°C)	Obelin (counts/ μl of 'ghosts')	ATP (pmol/ μl of 'ghosts')	U(%)	<i>t</i> (s)
20	700	3.2	92.1	6.2
37	1000	15.9	43.9	22.2

Table 4. *Effect of pH of haemolysis on permeability of 'ghosts' to Ca^{2+}*

'Ghosts' were prepared from 3 ml portions of pigeon blood as described in the Experimental and Results section. The haemolysis pH was 6.0 or 7.0. Resealing was carried out for 30 min at 37°C, in the presence of an ATP-regenerating system. The total obelin in each tube at the start of resealing was 1.90×10^9 counts. The final 'ghost' suspension (1.2 ml) contained 2.0×10^6 'ghosts'/ μl . The obelin and ATP contents of the 'ghosts' were measured as described in the Experimental and Results section. 'Ghosts' (10 μl) were suspended in 0.5 ml of medium A (10 mM-Tes, 140 mM-NaCl, 5 mM-KCl, 2 mM-MgCl₂, pH 7.4) at 20°C. The percentage utilization (U) of obelin 60 s after the addition of 0.5 ml of medium A + 2 mM-CaCl₂ was measured. The time (*t*) to maximum rate of light-emission was measured from Fig. 4 as described in Fig. 2. Each value represents the mean of at least two determinations.

Haemolysis pH	ATP (pmol/ μl of 'ghosts')	Obelin (counts/ μl of 'ghosts')	U(%)	<i>t</i> (s)
6.0	2.8	24300	89.6	3.5
7.0	14.9	56800	40.0	22.6

(Fig. 2 and Table 3) in 'ghosts' resealed at 20°C indicated that they were considerably more permeable to Ca^{2+} than 'ghosts' resealed at 37°C and were thus unsuitable for investigating effects of specific agents on the permeability of 'ghosts' to Ca^{2+} .

Fig. 4. *Effect of pH of haemolysis on permeability of 'ghosts' to Ca^{2+}*

'Ghosts' were prepared as described in Table 4. 'Ghosts' (10 μl), originally haemolysed at pH 6.0 or 7.0 and resealed for 30 min at 37°C, were suspended in 0.5 ml of medium A (10 mM-Tes, 140 mM-NaCl, 5 mM-KCl, 2 mM-MgCl₂, pH 7.4) at 20°C. The rate of obelin luminescence was recorded on the chart recorder after the addition ($\uparrow$) of 0.5 ml of medium A + 2 mM-CaCl₂. After 60 s ($\uparrow$), 0.5 ml of medium A + 1 mM-CaCl₂ + 3% Triton X-100 was added. Rate-meter time-constant 0.01 s, 10 mV output equivalent to 10000 c.p.s.

Effect of pH of haemolysis on resealing of 'ghosts'

It has been reported that haemolysis of human erythrocytes at pH 6.0 results in a more uniform 'ghost' population, relatively impermeable to cations after resealing (Schwoch & Passow, 1973). The effect of haemolysing pigeon erythrocytes at pH 6.0 and 7.0 was investigated (Table 4, Fig. 4). There was no detectable loss of obelin in the suspension during resealing with 'ghosts' haemolysed and washed at pH 6.0, compared with a loss of 73% with ghosts haemolysed at pH 7.0. This may have been due to displacement and removal of Ca^{2+} from the 'ghosts' at pH 6.0. However, the 'ghosts' haemolysed at pH 7.0 contained more than twice the amount of obelin and five times the amount of ATP compared with 'ghosts' haemolysed at pH 6.0. The percentage utilization of obelin in 'ghosts', haemolysed at pH 6.0, incubated for 60 s with 1 mM- Ca^{2+} was twice that of 'ghosts' haemolysed at pH 7.0 (Table 4). A comparison of the rates of luminescence after addition of Ca^{2+} (Fig. 4) showed that both the initial and second peaks were higher for 'ghosts' haemolysed at pH 6.0 than for 'ghosts' haemolysed at pH 7.0, and the second peaks occurred at 3.5 s and 22.6 s respectively. These data suggested that the 'ghosts' haemolysed at pH 6.0

were both less stable, releasing ATP and obelin on resuspension, and considerably more permeable to Ca^{2+} than 'ghosts' haemolysed at pH 7.0. 'Ghosts' haemolysed at pH 6.0 were therefore unsuitable for studying effects of membrane agents of free Ca^{2+} within the 'ghosts'.

Effect of ionophore A23187 on 'ghosts'

The ionophore A23187 appears to increase the permeability of membranes to bivalent cations (Reed

& Lardy, 1972) and has been shown to stimulate secretion of K^+ from the parotid gland (Eimerl *et al.*, 1974; Selinger *et al.*, 1974). Since the ionophore binds Mg^{2+} and Ca^{2+} , it has been suggested that in order to be most effective it is necessary to preincubate tissues or membranes with the ionophore in the absence of bivalent cations (Eimerl *et al.*, 1974; Selinger *et al.*, 1974). By using the rate of luminescence of obelin as a measure of free Ca^{2+} inside 'ghosts', this has been tested with pigeon erythrocyte 'ghosts' (Figs. 5a and 5b).

'Ghosts', resealed at 37°C for 60 min, were used to investigate the effect of the ionophore. Preincubation of 'ghosts' with ionophore A23187 ($16\text{ }\mu\text{g/ml}$) in the presence of 0.2 mM-Mg^{2+} but in the absence of Ca^{2+} , followed by the addition of Ca^{2+} alone or Ca^{2+} and Mg^{2+} , resulted in more than 95% of the obelin being utilized within 60 s, compared with 24% in the control (Fig. 5a). The effect of the ionophore was markedly decreased if 2 mM-Mg^{2+} was present during the preincubation (Fig. 5a). No effect of the ionophore was observed in the experiment if it was added in the presence of Mg^{2+} and Ca^{2+} . The rate of luminescence recorded on the chart recorder (Fig. 5b) showed that preincubation with the ionophore in the presence of 0.2 mM-Mg^{2+} [Fig. 5b, (i) and (ii)] but in the absence of Ca^{2+} resulted in an immediate rapid increase in the rate of luminescence after the addition of Ca^{2+} . Preincubation in the presence of 2 mM-Mg^{2+} [Fig. 5b, (iii)] resulted in a decrease in the rate of luminescence after the addition of Ca^{2+} but was still detectably greater than that of the control.

Discussion

Obelin, the Ca^{2+} -activated luminescent protein from the hydroid *Obelia geniculata*, was inserted into 'ghosts' prepared from pigeon erythrocytes in order to study changes in free $[\text{Ca}^{2+}]$ within the 'ghosts'.

Obelin luminescence can be stimulated by Ca^{2+} or Sr^{2+} and is inhibited by Mg^{2+} , univalent cations or a decrease in pH (Campbell, 1974; Moisescu *et al.*, 1975). Although an extensive kinetic analysis has yet to be carried out on obelin, it appears that the effects on the rate of obelin luminescence described in the present paper can be explained entirely by changes in

free Ca^{2+} . Apart from the initial peak in the rate of luminescence, obelin luminescence appeared to be occurring from within the 'ghosts' in all experiments. The perhaps unlikely possibility that aequorin may be released from giant cells such as the squid axon has been discussed (Baker *et al.*, 1971). At present it is not possible to rule out release of obelin from 'ghosts' by measuring inactivated obelin. Negligible amounts of inulin (mol.wt. approx. 5000) and pyruvate kinase (mol.wt. approx. 230000) were released from the 'ghosts' under all conditions except in the presence of Triton X-100. It therefore seemed unlikely that any significant quantities of obelin (mol.wt. approx. 30000; Campbell, 1974) were released into the medium.

When the rate of utilization of the luminescent protein is small, such that the amount of active protein remains approximately constant, or when the free Ca^{2+} concentration is constant, it is possible to measure the free Ca^{2+} concentration in experiments *in vivo* (Ashley & Ridgeway, 1968, 1970; Baker *et al.*, 1971). In the 'ghost' experiments described here, the free Ca^{2+} concentration within the 'ghosts' appeared to change during the experiment and significant quantities of the obelin were consumed. Thus it is not possible at present to relate obelin luminescence in the 'ghosts' to actual concentrations of free Ca^{2+} . However, if it is assumed that the free Ca^{2+} concentration remains constant from the second peak in the rate of light emission (Figs. 2, 4 and 5), it may be possible to estimate the free Ca^{2+} concentration within the 'ghosts'.

The preparation of human erythrocyte 'ghosts' has been investigated by a number of workers. A considerable number of variables in the haemolysis

Fig. 5. Effect of ionophore A23187 on pigeon erythrocyte 'ghosts'

'Ghosts' from 5 ml of pigeon blood were prepared as described in the Experimental and Results section. The haemolysis pH was 7.0. Resealing was carried out for 60 min at 37°C . The resealing medium contained 2.56×10^8 obelin counts. The recovery of obelin after 60 min resealing was 7.6%. The final 'ghost' suspension (1.5 ml) contained 3.3×10^6 'ghosts'/ μl , 3.7 pmol of ATP/ μl , and 736 obelin counts/ μl . Obelin luminescence was assayed at 20°C . 'Ghosts' ($50\text{ }\mu\text{l}$) were suspended in 0.5 ml of medium A (initial Mg^{2+} concentration 0.2 mM or 2 mM). This suspension was incubated for 20 s with ionophore A23187 ($16\text{ }\mu\text{g/ml}$) or ethanol ($2\text{ }\mu\text{l/ml}$). Then 0.5 ml of medium A + 2 mM-CaCl_2 was added (final Mg^{2+} concentration 0.2 mM or 2 mM). The number of counts was recorded every 10 s and the rate of luminescence recorded on the chart recorder. After 60 s the remaining active obelin was measured by recording the total counts after the addition of 0.5 ml of medium A + 1 mM-CaCl_2 + 3% Triton X-100. (a) Percentage utilization of obelin; (b) Rate of obelin luminescence. Rate-meter time-constant 0.01 s, 10 mV output equivalent to 1000 c.p.s., except after the addition of Triton X-100, when 10 mV output was equivalent to 10000 c.p.s. Control, $2\text{ }\mu\text{l}$ of ethanol/ml during preincubation; A23187, $16\text{ }\mu\text{g}$ of A23187/ml during preincubation.

Fig.		[Mg^{2+}] during preincubation (mM)	Final [Mg^{2+}] after addition of Ca^{2+} (mM)
5(a)	5(b)		
○	(i) Control	0.2	0.2
●	(i) A23187	0.2	0.2
□	(ii) Control	0.2	2
■	(ii) A23187	0.2	2
△	(iii) Control	2	2
▲	(iii) A23187	2	2

and resealing medium, including osmolarity, temperature, bivalent cations and pH, affect the enzyme and protein composition and the permeability characteristics of the final 'ghost' preparation (Whittam, 1962; Dodge *et al.*, 1963; Bramley *et al.*, 1971; Bramley & Coleman, 1972; Hanahan *et al.*, 1973; Schwoch & Passow, 1973; Wessels *et al.*, 1973; Wessels & Veerkamp, 1973*a,b*). The haemolysis medium used in the present experiments was based on that of Glynn & Hoffman (1971), except that ATP was omitted.

Resealed avian erythrocytes have not been prepared by many other workers (Harris & Brown, 1971). In keeping with human erythrocyte 'ghosts', pigeon erythrocyte 'ghosts' resealed at 37°C were less permeable to Ca^{2+} than 'ghosts' resealed at 20°C (Schwoch & Passow, 1973). It has been reported that it is difficult to prepare human erythrocyte 'ghosts' which are relatively impermeable to cations if the osmolarity of the haemolysis medium is less than 30–40 mosm (Schwoch & Passow, 1973), and that the optimum haemolysis pH is 6.0. The studies reported in the present paper show that pigeon erythrocyte 'ghosts', relatively impermeable to Ca^{2+} , can be prepared by resealing for at least 60 min at 37°C (Table 2 and Fig. 3) and that haemolysis at pH 6.0 rather than 7.0 resulted in 'ghosts' initially highly permeable to Ca^{2+} (Table 4 and Fig. 4). It has also been reported that resealing human erythrocyte 'ghosts' can result in several populations in the same suspension with different permeabilities to proteins and cations (Schwoch & Passow, 1973). The data obtained for pigeon erythrocyte 'ghosts' do not allow any conclusions to be drawn about the heterogeneity of the final suspension. The presence of 3 mM-Mg $^{2+}$ during haemolysis may be an important factor enabling resealed 'ghosts' to be obtained, whereas the low osmolarity enabled sufficient haemoglobin to be removed to prevent significant quenching of obelin luminescence (Fig. 1). Under certain conditions, 'inside-out' erythrocyte 'ghosts' can be prepared (Steck *et al.*, 1970; Weiner & Lee, 1972; Buckley, 1974). The conditions of preparation of the pigeon erythrocyte 'ghosts' make it unlikely that this was occurring here.

A Ca^{2+} -activated Mg $^{2+}$ -dependent ATPase responsible for maintaining a low intracellular free Ca^{2+} concentration has been identified in human erythrocytes (Schatzmann & Vincenzi, 1969; Schatzmann, 1973). The low osmolarity of the medium used to haemolyse the pigeon erythrocytes may have resulted in loss of the activity of this enzyme (Bramley *et al.*, 1971; Hanahan *et al.*, 1973). The greater recovery of obelin in the 'ghosts' compared with that in the whole suspension (Table 2) might suggest that the free Ca^{2+} concentration within the 'ghosts' was less than that in the medium. Attempts have been made to compare the relative permeability

to Ca^{2+} of 'ghosts' prepared in the presence and absence of ATP. Unfortunately the poor recovery of obelin and low ATP content of the 'ghosts' in these experiments prevented any definite conclusions being reached so far.

The bivalent-cation ionophore A23187 (Reed & Lardy, 1972) has been shown to stimulate K^{+} release from the parotid (Eimerl *et al.*, 1974; Selinger *et al.*, 1974) and secretion of catecholamines from the adrenal (Garcia *et al.*, 1975), to activate fertilization in eggs from several species (Steinhardt *et al.*, 1974), to stimulate Ca^{2+} -dependent K^{+} efflux from rat erythrocytes (Reed, 1973), and to induce Ca^{2+} release from isolated sarcoplasmic reticulum (Entman *et al.*, 1972; Scarpa *et al.*, 1972), and Ca^{2+} and Mg^{2+} release from mitochondria (Reed & Lardy, 1972). The results reported here for pigeon erythrocyte 'ghosts' (Fig. 5*a* and 5*b*), although at a somewhat higher concentration of ionophore A23187, agree with those of other workers (Eimerl *et al.*, 1974; Selinger *et al.*, 1974) in that maximum effects of the ionophore are obtained by preincubation with a low concentration of Mg^{2+} in the absence of Ca^{2+} . There appears to be a competition between the ionophore binding to the membrane and its remaining in solution bound to Ca^{2+} or Mg^{2+} .

The results of the present investigation show that the Ca^{2+} -activated luminescent protein obelin can be inserted into pigeon erythrocyte 'ghosts'. These 'ghosts' can be prepared relatively impermeable to Ca^{2+} and can be used to study the effects of membrane agents on free Ca^{2+} within the 'ghosts'. Before investigating possible effects of hormones on this system, it will be necessary to increase the ATP concentration within the 'ghosts' and to ensure that they contain a reasonably high activity of Ca^{2+} activated ATPase.

We are greatly indebted to the Director and staff of the Marine Biological Association Laboratory, Plymouth, and also Commander Bax of Plymouth Ocean Projects Ltd., the Underwater Centre, Plymouth. Our thanks go particularly to Skipper Knott and the crew of the *Gammarus*. We thank Mr. M. E. T. Ryall for constructing the luminescence detection apparatus. This work was supported by a grant from the Science Research Council and a grant from the British Diabetic Association to Professor Hales, whom we also thank for technical assistance and valuable advice. Finally, we thank Dr. L. H. N. Cooper, F.R.S., for his encouragement.

References

- Ashley, C. C. & Caldwell, P. C. (1974) *Biochem. Soc. Symp.* **39**, 29–50
- Ashley, C. C. & Ridgeway, E. B. (1968) *Nature (London)* **219**, 1168–1169
- Ashley, C. C. & Ridgeway, E. B. (1970) *J. Physiol. (London)* **209**, 105–130
- Ashley, C. C., Ellory, J. C. & Hainaut, K. (1974) *J. Physiol. (London)* **242**, 255–272

- Ashley, C. C., Campbell, A. K. & Moisesescu, D. G. (1975) *J. Physiol. (London)* **245**, 9P-10P
- Baker, P. F. (1972) *Prog. Biophys. Mol. Biol.* **4**, 177-223
- Baker, P. F., Hodgkin, A. L. & Ridgeway, E. B. (1971) *J. Physiol. (London)* **218**, 709-755
- Bergmeyer, H. U. (1965) in *Methods of Enzymatic Analysis*, pp. 997, Academic Press, New York
- Bramley, T. A. & Coleman, R. (1972) *Biochim. Biophys. Acta* **290**, 219-228
- Bramley, T. A., Coleman, R. & Finean, J. B. (1971) *Biochim. Biophys. Acta* **241**, 752-769
- Buckley, J. T. (1974) *Biochem. J.* **142**, 521-526
- Campbell, A. K. (1974) *Biochem. J.* **143**, 411-418
- Davie, J. V. & Lewis, S. M. (1970) *Practical Haematology*, pp. 37-39, J. and A. Churchill, London
- Davoren, P. R. & Sutherland, E. W. (1963) *J. Biol. Chem.* **238**, 3009-3015
- Dodge, J. T., Mitchell, C. & Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* **100**, 119-130
- Eimerl, S., Savion, N., Heichal, O. & Selinger, Z. (1974) *J. Biol. Chem.* **249**, 3991-3993
- Entman, M. L., Allen, J. C., Bornet, E. P., Gillette, P. C., Wallick, E. T. & Schwartz, A. (1972) *J. Mol. Cell. Cardiol.* **4**, 681-687
- Ettienne, E. M. (1970) *J. Gen. Physiol.* **56**, 168-179
- Exton, J. A., Friedman, N., Wong, E. H. A., Brineaux, J. P., Corbin, J. D. & Park, C. R. (1972) *J. Biol. Chem.* **247**, 3579-3588
- Friedmann, N. & Park, C. R. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **61**, 504-508
- Garcia, A. G., Kirpekar, S. M. & Prat, J. C. (1975) *J. Physiol. (London)* **244**, 253-262
- Glynn, I. M. & Hoffman, J. F. (1971) *J. Physiol. (London)* **218**, 239-256
- Hanahan, D. J., Ekholm, J. & Hildenbrandt, G. (1973) *Biochemistry* **12**, 1347-1387
- Harris, J. R. & Brown, J. N. (1971) *Br. Poult. Sci.* **12**, 95-99
- Katocs, A. S., Largis, E. E. & Allen, D. O. (1974) *J. Biol. Chem.* **241**, 2000-2004
- King, C. D. & Mayer, S. E. (1974) *Mol. Pharmacol.* **10**, 941-953
- Kissebah, A. H., Tulloch, B. R., Vydelingum, N., Hope-Gill, H., Clarke, P. & Fraser, T. R. (1974) *Horm. Metab. Res.* **6**, 357-364
- Llinas, R. & Nicholson, C. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 187-190
- Moisesescu, D. G., Ashley, C. C. & Campbell, A. K. (1975) *Biochim. Biophys. Acta* **396**, 133-140
- Morin, J. G. & Hastings, J. W. (1971) *J. Cell. Physiol.* **77**, 305-312
- Rasmussen, H., Goodman, D. B. P. & Tenenhouse, A. (1972) *Crit. Rev. Biochem.* **1**, 95-148
- Reed, P. W. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 635
- Reed, P. W. & Lardy, H. A. (1972) *J. Biol. Chem.* **247**, 6970-6977
- Robison, G. A., Butcher, R. W. & Sutherland, E. W. (1971) *Cyclic AMP*, Academic Press, New York
- Rose, B. & Lowenstein, W. R. (1975) *Nature (London)* **254**, 250-252
- Scarpa, A., Baldassare, J. & Inesi, G. (1972) *J. Gen. Physiol.* **60**, 735-749
- Schatzmann, H. J. (1973) *J. Physiol. (London)* **235**, 551-569
- Schatzmann, H. J. & Vincenzi, F. F. (1969) *J. Physiol. (London)* **201**, 369-395
- Schwoch, G. & Passow, H. (1973) *Mol. Cell. Biochem.* **2**, 197-218
- Selinger, Z., Eimerl, S. & Schramm, M. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 128-131
- Siddle, K. & Hales, C. N. (1974) *Biochem. J.* **142**, 345-351
- Siddle, K., Kane-Maguire, B. & Campbell, A. K. (1973) *Biochem. J.* **132**, 765-773
- Steck, T. L., Weinstein, R. S., Strauss, J. H. & Wallach, D. F. H. (1970) *Science* **168**, 255-257
- Steinhardt, R. A., Epel, D., Carroll, E. J. & Yanagimachi, R. (1974) *Nature (London)* **252**, 41-43
- Weiner, M. L. & Lee, K. S. (1972) *J. Gen. Physiol.* **59**, 462-475
- Werner, S., Alm, B. & Low, A. (1972) *Horm. Metab. Res.* **4**, 195-201
- Wessels, J. M. C. & Veerkamp, J. H. (1973a) *Biochim. Biophys. Acta* **291**, 178-189
- Wessels, J. M. C. & Veerkamp, J. H. (1973b) *Biochim. Biophys. Acta* **291**, 190-196
- Wessels, J. M. C., Pals, D. T. F. & Veerkamp, J. H. (1973) *Biochim. Biophys. Acta* **291**, 165-177
- Whittam, R. (1962) *Biochem. J.* **84**, 110-118