The binding of calmodulin to myelin basic protein and histone H2B

Roger J. A. GRAND and S. Victor PERRY

Department of Biochemistry, University of Birmingham, P.O.Box 363, Birmingham B15 2TT, U.K.

(Received 10 January 1980)

1. A calmodulin-binding protein of apparent mol.wt. 19 000 has been purified from chicken gizzard. Similar proteins have been isolated from bovine uterus, rabbit skeletal muscle and rabbit liver. 2. These proteins migrated as an equimolar complex with bovine brain calmodulin on electrophoresis on polyacrylamide gels in the presence of Ca^{2+} and 6 M-urea. The complex was dissociated in the presence of EGTA. 3. The chicken gizzard calmodulin-binding protein has been shown to be identical with chicken erythrocyte histone H2B on the basis of partial amino acid sequence determination. 4. The calmodulin-binding proteins of apparent mol.wt. 22 000 isolated previously from bovine brain [Grand & Perry (1979) *Biochem. J.* 183, 285–295] has been shown, on the basis of partial amino-acid-sequence determination, to be identical with myelin basic protein. 5. The activation of bovine brain phosphodiesterase by calmodulin is inhibited by excess bovine uterus calmodulin-binding protein (histone H2B). 6. The phosphorylation of myelin basic protein by phosphorylase kinase is partially inhibited, whereas the phosphorylation of uterus calmodulin-binding protein (histone H2B) is unaffected by calmodulin or troponin C. 7. The subcellular distribution of myelin basic protein and calmodulin suggests that the two proteins do not exist as a complex *in vivo*.

Calmodulin was originally identified as a Ca^{2+} -sensitive activator of phosphodiesterase in brain (Cheung, 1970; Kakiuchi & Yamazaki, 1970). Since that time its role in conferring calcium sensitivity on a number of different enzymes has been firmly established. These include brain adenylyl cyclase (Brostrom *et al.*, 1975), phosphorylase kinase (Cohen *et al.*, 1978), myosin light-chain kinase from skeletal muscle (Yagi *et al.*, 1978; Nairn & Perry, 1979) and smooth muscle (Sherry *et al.*, 1978) and the membrane-bound (Ca^{2+} + Mg^{2+})-sensitive ATPase (Gopinath & Vincenzi, 1977). Although calmodulin has been shown to be closely associated with all of these enzymes most mammalian tissues appear to contain very much more calmodulin than would be required if 1 mol of each enzyme needed 1 mol of calmodulin for full activity (Grand & Perry, 1979). When mammalian tissues are homogenized in 9 M-urea and then subjected to polyacrylamide-gel electrophoresis at pH 8.3, calmodulin can be detected as a fast-running band in those samples from which calcium has been removed with EGTA. If the samples contain excess Ca^{2+} no calmodulin is visible (Head *et al.*, 1977; Grand *et al.*, 1979). It has therefore been concluded that calmodulin exists in tissue homogenates and possibly *in vivo* in the form of a urea-stable Ca^{2+} -sensitive complex with one or more 'calmodulin binding proteins'. Wang & Desai

(1977) and Klee & Krinks (1978) have reported the isolation of proteins of mol.wts. 80 000 and 61 000 respectively that form Ca^{2+} -dependent complexes with calmodulin. More recently, Grand & Perry (1979) have reported the presence of two calmodulin-binding proteins of mol.wts. 77 000 and 60 000 that presumably correspond to those described by Wang & Desai (1977) and Klee & Krinks (1978), as well as one of mol.wt. about 140 000 in a number of mammalian tissues. In addition a protein of apparent mol.wt. about 22 000 was obtained in high yield from bovine brain.

In the present paper we report evidence that the 22 000-mol.wt. calmodulin-binding protein of bovine brain is identical with myelin basic protein (for review see Carnegie & Dunkley, 1975). A further calmodulin-binding protein of apparent mol.wt. 19 000 has been purified from a number of other vertebrate tissues and this protein has been identified as histone H2B (for review see Elgin & Weintraub, 1975).

Materials and methods

Materials

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and iodo $[\text{C}^{14}]\text{acetic acid}$ were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Histones and cyclic AMP-dependent

protein kinase were obtained from the Sigma Chemical Co., Poole, Dorset BH17 7NH, U.K. Trypsin (treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one) was obtained from Cambrian Chemicals, Croydon CR0 4XB, Surrey, U.K. Thermolysin was obtained from Calbiochem, San Diego, California, U.S.A.

Tissues

Bovine brains and uteri and chicken gizzards were obtained from a local abattoir and poultry slaughterer respectively and either homogenized in 9M-urea/75 mM-Tris adjusted to pH 8.0 with 1M-HCl/1 mM-CaCl₂/15 mM- β -mercaptoethanol (buffer A) as soon as possible after death of the animal or stored frozen at -20°C until required. Rabbit liver and skeletal muscle were excised from the animal and used immediately after death.

Preparation of calmodulin-binding proteins

Calmodulin-binding proteins were obtained by the method described by Grand & Perry (1979) for the preparation of the calmodulin-binding protein 22K from bovine brain. An additional chromatographic step was included for the purification of the calmodulin-binding protein 19K. In this the partially purified calmodulin-binding protein, after elution from the CM-cellulose column (Fig. 1) was dialysed against water and freeze-dried. The protein was redissolved in 2 ml of 0.01M-HCl and applied to a column (2.2 cm $\times$ 120 cm) of Sephadex G-100 equilibrated and eluted with 0.01M-HCl; 5 ml fractions were collected. A typical elution profile is shown in Fig. 2. Calmodulin-binding proteins were detected by the method described by Grand & Perry (1979) in which ^{125}I -labelled calmodulin was added to portions from the fractions to be tested and electrophoresis was then carried out at pH 8.3 in the presence of 6M-urea and 1 mM-CaCl₂. The absence of a fast-running radioactive band of calmodulin was taken to indicate the presence of a calmodulin-binding protein.

Preparation of muscle proteins

Rabbit skeletal-muscle troponin was prepared by the method of Ebashi *et al.* (1971); troponin C and troponin I were isolated from it by the method of Perry & Cole (1974). Rabbit skeletal-muscle phosphorylase kinase was prepared by the method of Cohen (1973) and was a gift from Dr. A. Raggi, Department of Biochemistry, University of Birmingham.

Preparation of calmodulin

Calmodulin was prepared from an acetone-dried powder of bovine brain by the organic-solvent method of Grand *et al.* (1979).

Fig. 1. Chromatography on CM-cellulose of partially purified chicken gizzard calmodulin-binding protein 19K. Protein not bound to a column of DEAE-cellulose (Grand & Perry, 1979) was applied to a column (10 cm $\times$ 3 cm) of CM-cellulose equilibrated and developed with 7M-urea/32.5 mM-sodium citrate/25 mM-citric acid (pH 5.1)/1 mM-CaCl₂/7.5 mM- β -mercaptoethanol. Bound protein was eluted with a gradient of 0–0.35 M-NaCl and 10 ml fractions were collected. Fractions under the bar were pooled. —, A_{280} ; ----, conductivity.

Fig. 2. Chromatography on Sephadex G-100 of the partially purified chicken gizzard calmodulin-binding protein 19K.

Protein (30 mg in 2 ml of 0.01M-HCl) after elution from CM-cellulose (see the text) was applied to a column (2.2 cm $\times$ 120 cm) of Sephadex G-100 equilibrated and eluted with 0.01 M-HCl. Fractions (5 ml) were collected. —, A_{215} ; ----, A_{280} . Fractions under the bar were pooled.

Preparation of myelin

Myelin was prepared from whole rabbit brain by the method described by Norton (1974). The brain (6 g) was removed from a rabbit immediately after death, rinsed briefly in cold tap water and then

homogenized for 2×30 s in ice-cold 0.32 M-sucrose (40 ml). The homogenate was diluted to 100 ml and one-sixth was layered on 10 ml of 0.85 M-sucrose in each of 6×25 ml centrifuge tubes. These were centrifuged at 75 000 g for 30 min in a 'swing-out' head. The crude myelin, deposited at the interface of the two sucrose solutions, was removed and homogenized in 180 ml of ice-cold water. This homogenate was centrifuged at 75 000 g for 15 min, and the supernatant was discarded. The precipitate was again dispersed in 180 ml of water and the suspension was centrifuged at 12 000 g for 10 min. The supernatant was discarded and the step was repeated. The myelin pellets were then collected, suspended in 0.32 M-sucrose (100 ml), layered on 0.85 M-sucrose and centrifuged at 75 000 g for 30 min. The pure myelin, again deposited at the interface, was removed and dissolved in buffer A (20 ml).

Fractionation of bovine brain homogenates

Bovine brain was fractionated by the method of Rodriguez de Lores Arnaiz *et al.* (1967). Cerebral cortex (5 g) was homogenized in ice-cold 0.32 M-sucrose (20 ml) adjusted to pH 7.0 with NaHCO_3 . The homogenate was centrifuged at 900 g for 10 min to sediment the nuclear fraction, which was washed twice with 0.32 M-sucrose. The mitochondrial fraction was obtained by centrifuging the combined supernatants at 11 500 g for 20 min. A microsomal fraction was obtained by centrifuging the supernatant obtained after removal of the mitochondria at 100 000 g for 60 min. The supernatant from this separation was the soluble fraction.

Phosphorylation of bovine brain and uterus calmodulin-binding proteins

Procedures used were based, with slight modifications, on those described by Cole & Perry (1975).

(a) *Cyclic AMP-dependent protein kinase*. Assays were carried out in a total volume of 1 ml containing 50 mM-Tris, 50 mM- α -glycerophosphate, adjusted to pH 7.2 with 1 M-HCl/5 mM-magnesium acetate/0.1 mM- CaCl_2 /2.5 mM-NaF/1 mM-dithiothreitol/0.1 mM-cyclic AMP/1 mM-AMP (containing $[\gamma\text{-}^{32}\text{P}]$ -ATP, sp. radioactivity 2 Ci/mol)/0.7 mg of cyclic AMP-dependent protein kinase and troponin I or calmodulin-binding protein with bovine brain calmodulin or troponin C (100 μg of each protein). After incubation at 30°C the reaction was stopped by the addition of an equal volume of 20% (w/v) trichloroacetic acid containing 1 mM-ATP. Bovine serum albumin (0.1 ml, 20 mg/ml) was added and the precipitate was washed three times with 5 ml portions of ice-cold 5% (w/v) trichloroacetic acid by centrifugation and resuspension. The washed protein

was dissolved in 10 ml of 0.1 M-NaOH for determination of radioactivity.

(b) *Phosphorylase kinase*. Assays were carried out in a total volume of 1 ml containing 50 mM Tris, 50 mM- α -glycerophosphate, adjusted to pH 8.6 with 1 M-HCl/5 mM-magnesium acetate/1 mM-dithiothreitol/5 mM-NaF/1 mM-ATP (containing $[\gamma\text{-}^{32}\text{P}]$ -ATP, sp. radioactivity 2 Ci/mol)/phosphorylase kinase (25 μg) and troponin I or bovine brain calmodulin-binding protein or bovine uterus calmodulin-binding protein and troponin C or bovine brain calmodulin (100 μg of each protein) as was appropriate. After incubation at 30°C, the reaction was stopped by the addition of 20% (w/v) trichloroacetic acid. Samples were prepared for the determination of ^{32}P incorporation by the procedure used in the protein kinase experiments.

Isolation of the phosphorylated peptide from bovine brain 22K calmodulin-binding protein

Bovine brain calmodulin-binding protein (9 mg) was phosphorylated by incubation with phosphorylase kinase (100 μg) at 30°C. Conditions for the incubation were as described by Cole & Perry (1975). After incubation for 3 h the reaction was stopped by the addition of sufficient 1 M-HCl to decrease the pH to approx. 2; solid guanidine hydrochloride was added to a concentration of 4 M and the phosphorylated protein was desalted on a column (2 cm $\times$ 110 cm) of Sephadex G-25 equilibrated and eluted with 10 mM-HCl and freeze-dried. A portion of the protein was hydrolysed for 2 h at 110°C in 6 M-HCl and the hydrolysate was subjected to high-voltage electrophoresis at pH 2.0. Under these conditions over 90% of the radioactivity migrated with a mobility identical with that of a known sample of serine phosphate.

The remainder of the protein was dissolved in 2 ml of 50 mM- NH_4HCO_3 , pH 7.9, and digested with trypsin (1:50, w/w) overnight at 25°C. The radioactive peptides were purified by high-voltage electrophoresis on Whatman 3 MM chromatography paper at pH 6.5, 3.5 and 2.0.

Phosphodiesterase assays

Adenosine 3':5'-cyclic monophosphate phosphodiesterase, deficient in calmodulin, was prepared from bovine brain by the method of Wang & Desai (1977). Enzymic activity was assayed by a modification of the procedure of Teo *et al.* (1973) as described by Watterson *et al.* (1976). Enough calmodulin (400 ng) was added to the enzyme to give almost maximal activation.

Polyacrylamide-gel electrophoresis

Electrophoresis was carried out on 10% polyacrylamide gels run in 14 mM-Tris/90 mM-glycine,

pH 8.3, either in the presence or absence of 6 M-urea. Polyacrylamide gels (10, 15 and 20%) were also run in the presence of 0.1% sodium dodecyl sulphate/0.1 M-Tris/0.1 M-Bicine [*NN*-bis-(2-hydroxyethyl)-glycine], pH 8.3.

Tryptic digestion of proteins and preparation of peptides for sequence determination

Bovine brain calmodulin-binding protein 22K (30mg) and chicken gizzard calmodulin-binding protein 19K (15mg) were labelled at the methionine residues with iodo[¹⁴C]acetic acid by the method of Wilkinson (1969). The radioactive proteins were desalted on a column (2cm × 120cm) of Sephadex G-25 equilibrated and eluted with 10mM-HCl and freeze-dried. The proteins were dissolved in water (3ml) and 0.3ml of 10% NH₄HCO₃ was added followed by 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one-treated trypsin at an enzyme/substrate ratio of 1:50 (by weight). After incubation at 37°C for 4h the digest of brain calmodulin-binding protein was chromatographed on a column (2.2cm × 120cm) of Sephadex G-50 equilibrated and eluted with 10mM-HCl; the digest of chicken gizzard calmodulin-binding protein was chromatographed on a column (2.2cm × 120cm) of Sephadex G-25 equilibrated and eluted with 10mM-HCl.

Peptides were further purified by high-voltage electrophoresis at pH 6.5, 3.5 or 2.0 on Whatman 3MM or no. 1 chromatography paper. Radioactive peptides were detected with Kodak Blue Brand X-ray film. Tryptophan-containing peptides were detected by the method of Easley (1965).

Amino acid analysis

Amino acid analyses of proteins were carried out as described by Wilkinson *et al.* (1972) on duplicate samples after 24 and 72h hydrolysis.

Sequencing methods

Purified peptides were sequenced either by the dansyl (5-dimethylaminonaphthalene-1-sulphonyl)-Edman procedure described by Gray (1967) or by the rapid method of Gray & Smith (1970). The *N*-terminal amino acids of the whole proteins were determined by the modified dansyl method described by Wilkinson (1974). Most tryptic peptides were sequenced directly but in some cases they were further digested with thermolysin. The resultant peptides were purified by high-voltage electrophoresis and then sequenced.

Digestion with thermolysin was carried out at an enzyme/substrate ratio of 1:50 (by weight) in 50mM-NH₄HCO₃, pH 7.9, for 4h at 37°C.

Determination of protein

Protein was determined by the procedure involving Nesslerization (Strauch, 1965). A nitrogen

content of 16% was assumed. In some cases protein was determined by the method of Mejbaum-Katzenellenbogen & Dobryszczyka (1959) or by amino acid analysis.

Determination of phosphorous

Phosphorus covalently bound to protein was determined by the method of Bartlett (1959) on protein precipitates that had been washed with 5% (w/v) trichloroacetic acid.

Results

Preparation of calmodulin-binding proteins

When the procedure used for the preparation of the calmodulin-binding protein 22K from brain (Grand & Perry, 1979) was applied to tissues such as chicken gizzard, rabbit skeletal muscle, rabbit liver and bovine uterus, a low-molecular-weight basic protein that bound calmodulin was eluted from the CM-cellulose column (Fig. 1) at a conductivity of 6.5–8 mmho. This protein fraction was somewhat impure and was therefore further fractionated by chromatography on Sephadex G-100 (see the Materials and methods section) (Fig. 2). The major peak obtained consisted of a single protein that had an apparent mol.wt. of 19000 on electrophoresis in the presence of sodium dodecyl sulphate and was therefore designated calmodulin-binding protein 19K. Calmodulin-binding protein 22K was not isolated from these tissues. The proteins obtained from all tissues studied, other than brain, migrated

Fig. 3. Polyacrylamide-gel electrophoresis of calmodulin-binding proteins

Polyacrylamide gels (10%) run in the presence of 0.1% sodium dodecyl sulphate/0.1 M-Bicine/0.1 M-Tris, pH 8.3. In each case about 50 µg applied to gel. Calmodulin-binding proteins 19K (histone H2B) from: (a) chicken gizzard; (b) bovine uterus; (c) rabbit liver; (d) rabbit skeletal muscle; (e) bovine brain; (f) standard proteins: transferrin (mol.wt. 77000), glutamate dehydrogenase (mol.wt. 55500), carbonic anhydrase (mol.wt. 29200) and haemoglobin (mol.wt. 15100). O, origin.

Fig. 4. Polyacrylamide-gel electrophoresis of calmodulin-binding proteins in the presence of calmodulin. Polyacrylamide gels (10%) run in the presence of 90 mM-glycine/14 mM-Tris/6 M-urea (pH 8.3). Samples (a), (c), (e), (g) and (i) were run with 5 mM-CaCl₂ and (b), (d), (f), (h) and (j) with 5 mM-EGTA in the samples. Bovine brain calmodulin (5 μg) was added in all cases. Gels (a) and (b), bovine brain myelin basic protein (10 μg); gels (c) and (d), rabbit skeletal-muscle calmodulin-binding protein 19K (10 μg); gels (e) and (f), rabbit liver calmodulin-binding protein 19K (10 μg); gels (g) and (h), bovine uterus calmodulin-binding protein 19K (10 μg); gels (i) and (j), chicken gizzard histone H2B (10 μg). O, origin.

Fig. 5. Sequence of bovine brain myelin basic protein

Data were taken from Eylar *et al.* (1971). —, Peptides purified from bovine brain calmodulin-binding protein 22K. → represents one step of the dansyl-Edman procedure and indicates that the residue determined is identical with the corresponding residue of myelin basic protein. ↓ shows the position of digestion with thermolysin after tryptic cleavage. Abbreviation used: Ac, acetyl; *, Phosphorylated residues.

with similar mobilities towards the anode on polyacrylamide gels run in the presence of 0.1% sodium dodecyl sulphate at pH 8.3 (Fig. 3), and towards the cathode when electrophoresed in the presence of 6 M-urea at pH 8.3. It was concluded that a similar calmodulin-binding protein 19K was present in rabbit skeletal muscle, rabbit liver, chicken gizzard and bovine uterus. Yields of the protein from all these tissues were approx. 100 mg/kg wet wt. of tissue.

Interactions of calmodulin-binding proteins with calmodulin and troponin C

Calmodulin-binding proteins of 19 000 mol.wt. from all the tissues listed were shown to interact in a calcium-sensitive manner with bovine brain calmodulin on 8% polyacrylamide gels at pH 8.3 in the presence of 6 M-urea (Fig. 4). When equal amounts of calmodulin and calmodulin-binding protein were applied to the gel in the presence of 1 mM- CaCl_2 , little or no free calmodulin was visible on the gel. In the presence of 5 mM EGTA, however, the calmodulin migrated as a fast-moving band with its normal mobility. Removal of Ca^{2+} by addition of EGTA to calmodulin produces only a slight decrease in its electrophoretic mobility under these conditions (Grand *et al.*, 1980). Similar results have been reported with the calmodulin-binding protein 22K from bovine brain (Grand & Perry, 1979). Troponin C from rabbit fast skeletal muscle formed similar complexes to calmodulin with the calmodulin-binding proteins of mol.wt. 19 000.

Identification of bovine brain calmodulin-binding protein 22K with myelin basic protein

The fact that the calmodulin-binding protein 22K was present in brain in large amounts, estimated at greater than 1 g/kg of tissue (Grand & Perry, 1979) on the basis of the yields obtained by the preparative procedure described, suggested that it was unlikely that it had not been characterized before. Myelin basic protein, which is a major component of myelinated nerve, had a similar amino acid composition and molecular weight to the calmodulin-binding protein 22K and the possibility of their identity was therefore investigated by partial amino-acid-sequence studies.

Sequence studies

After tryptic digestion of the carbamoylmethylated protein 18 peptides representing about 80% of the total sequence were separated and purified for amino acid analysis by gel filtration and electrophoresis. These results are presented in Table 1. A number of the lysine and arginine residues were not cleaved due to the proximity of proline residues. No *N*-terminal amino acid could be detected after dansylation of the whole protein.

With the exception of peptides T1a, T2a and T2c2 (Table 1), the tryptic peptides were sequenced directly. The other peptides were first digested with thermolysin and the smaller fragments purified by high-voltage electrophoresis and then sequenced. The results are summarized in Fig. 5 from which it can be seen that about half of the total sequence of the myelin basic protein was determined. The sequences obtained in every case corresponded with regions of the primary sequence of myelin basic protein reported by Eylar *et al.* (1971) (Fig. 5).

Distribution of calmodulin-binding protein 22K in subcellular fractions of bovine brain

When equivalent portions of fractions obtained from bovine brain homogenates were examined by electrophoresis in sodium dodecyl sulphate, a band corresponding to the calmodulin-binding protein 22K was found in the nuclear fraction where much of the myelin present also precipitates. A much fainter corresponding band was also present in portions from the mitochondrial and microsomal fractions, but was absent from the supernatant fraction. If electrophoresis was carried out in 6 M-urea/5 mM-EGTA (pH 8.3), a strong band corresponding to calmodulin was apparent in all fractions. Under these conditions the myelin basic protein migrated to the cathode.

When a sample of bovine brain myelin was electrophoresed at pH 8.3 in the presence of sodium dodecyl sulphate, a very strong band of mol.wt. 22 000 was visible; when a sample was run on a gel at pH 8.3 in the presence of 6 M-urea and 5 mM-EGTA, only an extremely faint band due to calmodulin was visible. It was therefore considered that the amount of myelin basic protein in the myelin preparation was far in excess of the calmodulin and most was not present in a complex with this protein.

In view of these findings it was therefore concluded that calmodulin-binding protein 22K and myelin basic protein were identical. This conclusion was confirmed by the observation that an antibody prepared by injecting calmodulin-binding protein 22K into the guinea pig by Dr. G. K. Dhoot of the Department of Immunology, University of Birmingham, specifically stained myelinated nerve in brain sections studied by the immunoperoxidase procedure (Dhoot *et al.*, 1978).

Identification of calmodulin-binding protein 19K with histone H2B

It is well established that all tissues contain several classes of histones with mol.wts. in the region 11 000–22 000. The possibility that calmodulin-binding protein 19K might be a histone was investigated, in view of its general basic properties and the similarity of its amino acid composition to that of calf thymus histone (Table 2). Most of the histones

Table 1. *Amino acid analyses of the tryptic peptides from bovine brain calmodulin-binding protein 22 K*
The presence of Trp in peptide T3b was confirmed by the specific colorimetric spray described by Easley (1965). Mobilities were measured relative to aspartic acid (−1) and the net charge was calculated by the method of Offord (1966). Abbreviation used: N, neutral.

Peptide	...	Amino acid content (mol/mol of peptide)																T3b	
		T1a	T1b	T2a	T2b4	T2c1a	T2c2	T2d2a	T2e1b	T2e2b	T2f1	T2f2	T2g2a	T2g2b	T2h2	T2h3	T3a5		T3a3a3
Asp	2.1	1.2	2.0	1.1	1.1	1.0	—	—	—	—	—	—	—	—	—	—	0.9	1.1	—
Thr	—	—	1.0	—	0.9	0.9	1.0	—	1.6	—	—	—	—	—	—	—	—	—	—
Ser	—	1.0	1.0	1.0	1.9	1.0	1.0	—	1.1	—	—	0.9	1.0	1.0	1.0	—	0.9	0.9	1.0
Glu	2.8	—	—	—	—	1.1	0.9	—	1.0	—	—	—	1.0	1.0	—	—	—	—	1.8
Pro	2.4	—	—	—	—	—	2.3	2.2	1.0	1.1	1.0	—	1.2	—	—	—	—	—	0.9
Gly	1.2	3.2	2.1	—	—	2.2	1.0	—	1.3	1.1	1.1	—	—	—	—	—	1.2	—	5.3
Ala	1.1	2.0	—	1.0	2.9	1.1	—	—	—	—	1.0	—	—	1.0	—	—	—	—	1.1
Val	1.2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Met	—	—	—	—	0.4	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Ile	—	—	1.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Leu	—	—	2.0	—	1.1	0.9	—	1.0	1.1	1.0	—	—	—	—	—	—	—	—	—
Tyr	—	—	—	1.0	1.1	—	—	—	1.1	—	—	—	—	—	—	—	—	—	1.2
Phe	1.7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.3	—	1.9
His	1.7	1.9	—	—	1.2	0.8	—	—	1.0	—	—	—	—	1.1	1.0	—	—	—	—
Lys	0.9	1.0	—	1.0	—	1.0	0.9	—	0.9	0.9	0.9	1.1	—	1.0	—	—	—	—	0.9
Arg	0.8	1.7	0.9	—	0.9	—	—	0.9	—	—	—	—	1.8	—	1.0	0.6	1.0	1.0	0.9
Trp	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+
Mobility	0.28	0.66	−0.23	N	0.24	0.24	0.38	0.55	0.55	0.63	0.63	0.75	0.75	0.86	0.86	N	N	N	0.13
Net charge	+2	+3	−1	0	+1	+1	+1	+1	+2	+1	+1	+1	+2	+2	+2	0	0	0	+1

Table 2. *Amino acid analyses of calmodulin-binding proteins*

Abbreviations used: n.d., not determined; C.B.P., calmodulin-binding protein. 22 K C.B.P. and 19 K C.B.P. are the calmodulin-binding proteins of apparent mol.wt. 22000 isolated from bovine brain and of apparent mol.wt. 19000 isolated from chicken gizzard and other tissues respectively.

Amino acid content (mol/mol of peptide)

	Bovine brain 22 K C.B.P.*	Bovine brain myelin basic protein†	Bovine uterus 19 K C.B.P.‡	Chicken gizzard 19 K C.B.P.‡	Rabbit liver 19 K C.B.P.‡	Rabbit skeletal- muscle 19 K C.B.P.‡	Calf-histone H2B§
Asp	11.7	11	7.8	7.7	8.0	7.9	6
Thr	7.5	8	7.1	7.8	7.1	7.3	8
Ser	17.1	17	10.6	10.3	9.2	8.3	14
Glu	11.7	10	11.6	11.7	11.3	12.6	10
Pro	11.8	13	6.6	5.1	4.9	4.8	6
Gly	25.3	25	10.0	10.0	9.8	11.2	7
Ala	14.1	14	12.6	11.9	12.5	11.7	13
Val	4.3	3	8.2	7.7	8.8	7.7	9
Met	2.2	2	1.7	1.9	1.6	1.0	2
Ile	3.6	4	5.9	6.2	6.1	6.1	6
Leu	11.5	9	8.9	9.0	10.2	10.2	6
Tyr	4.3	4	4.1	4.0	4.0	4.0	5
Phe	7.2	8	2.6	2.7	2.4	2.6	2
His	8.7	10	3.4	3.5	3.1	3.2	3
Lys	13.4	13	15.9	15.5	15.7	14.1	20
Arg	16.6	18	8.9	9.8	10.2	11.0	8
Trp	1.0	1	—	—	—	—	—
Cys	0.7	—	n.d.	n.d.	n.d.	n.d.	—

* Re-calculated from Grand & Perry (1979) adjusted to a mol.wt. of 183 000.

† Calculated from the sequence taken from Eylar *et al.* (1971).

‡ Calculated on the basis of a mol.wt. of 13 700.

§ Calculated from the sequence taken from Iwai *et al.* (1972).

Table 3. *Complex formation on electrophoresis of calf thymus histones with calmodulin*

Electrophoresis was on 10% polyacrylamide gel, pH 8.3, containing 6 M-urea. CaCl_2 (1 mM) and EGTA (5 mM) were added to each sample. Complex formation occurred in all cases in the presence of CaCl_2 . If the complex was not dissociated in the presence of EGTA the interaction was considered to be calcium-insensitive.

Histone	Sigma nomenclature	Complex formation in the presence of EGTA
Mixture of histones	Type IIIS + Type II	Not dissociated
H1	Type V-S	Not dissociated
H2A	Type VI-S	Partially dissociated
H2B	Type VII	Fully dissociated
H3	Type VIII-S	Not dissociated

studied formed a complex that migrated on electrophoresis in 6 M-urea, whether Ca^{2+} or EGTA were present (Table 3). The only clear exception was histone H2B, which behaved on electrophoresis in 6 M-urea exactly like bovine brain myelin basic protein and calmodulin-binding protein 19K. Histone H2A was partially dissociated in the presence of EGTA.

Sequence studies

Chicken gizzard calmodulin-binding protein 19K

was digested with trypsin and 14 peptides were isolated from the digest by chromatography and electrophoresis. Amino acid analyses are given in Table 4. The sequences or partial sequences determined on 13 of these peptides and representing 71% of the total sequence all matched up with regions of calf thymus histone H2B as determined by Iwai *et al.* (1972) or chicken erythrocyte histone H2B as determined by Van Helden *et al.* (1978) (Fig. 6).

Although most of the peptides purified were clearly derived from histone H2B a small amount of

Table 4. *Amino acid analyses of the tryptic peptides isolated from the calmodulin-binding protein 19 K from chicken gizzard*
Mobilities were measured relative to aspartic acid (−1) and the net charge was calculated by the method of Offord (1966). Free arginine and lysine were also isolated in high yield. Abbreviation used: N, neutral.

Peptide	...	Amino acid content (mol/mol of peptide)														Tr3a
		Tr2e3	Tr2f2a	Tr2f2b	Tr2f4	Tr2g	Tr2h3	Tr2j	Tr2k1	Tr2k2	Tr2o	Tr3b	Tr3d2	Tr3e2		
Asp	3.2	—	—	—	—	—	1.2	—	—	1.1	—	—	—	—	2.9	
Thr	—	—	1.0	—	—	1.0	1.0	—	0.8	—	—	—	—	1.4	—	
Ser	1.2	—	—	—	—	1.0	1.8	0.8	—	—	0.9	—	0.9	2.0	—	
Glu	1.6	1.2	1.8	—	1.0	1.0	1.1	—	—	—	—	1.1	1.2	—	2.2	
Pro	—	1.1	—	—	2.0	—	1.1	2.1	—	—	—	2.2	—	—	—	
Gly	1.6	1.1	—	—	—	1.2	1.4	—	—	1.1	1.2	—	1.2	—	—	
Ala	1.1	1.1	1.2	1.0	1.0	1.1	—	2.0	0.9	—	—	0.9	1.8	—	—	
Val	1.1	—	1.0	—	—	1.0	1.0	—	0.8	—	—	—	—	—	—	
Met	0.3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Ile	1.8	—	1.0	—	—	—	1.1	—	—	—	—	—	—	—	—	
Leu	—	3.6	—	—	—	—	—	—	—	—	—	—	0.9	—	1.0	
Tyr	—	—	—	—	—	—	—	—	—	—	—	—	—	0.7	—	
Phe	1.7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
His	—	—	—	—	—	0.7	0.6	—	—	—	—	—	—	—	—	
Lys	—	0.8	—	—	1.0	1.0	0.7	1.1	1.4	1.8	1.9	0.7	—	0.9	0.9	
Arg	0.9	—	0.9	—	—	—	—	—	—	—	—	—	1.1	—	—	
Mobility	−0.07	N	N	N	N	+0.12	+0.21	+0.39	+0.48	+0.48	+0.88	−0.38	N	+0.4	−0.56	
Net charge	−1	0	0	0	0	+ $\frac{1}{2}$	+ $\frac{1}{2}$	+1	+1	+1	+2	−1	0	+1	−2	

a peptide sequence Asx-Asx-Glx-Leu-Asx-Lys (peptide T3a, Table 4) was obtained. It seems likely that this peptide originates from histone H2A (residues 89–95 of the sequence given by Yeoman *et al.*, 1972). The rather high values obtained for leucine and the low values obtained for serine and lysine after amino acid analysis of the calmodulin-binding proteins (Table 2) support the view that a certain amount of other protein, quite possibly histone H2A, was present as an impurity. No particular difficulties were encountered in the sequencing, although one point of interest did emerge. Two peptides of similar analysis (T2f4 and T3b in Table 4 and Fig. 6) but different mobilities were purified. Peptide T3b was acidic, suggesting that it had a blocked *N*-terminus. This view was supported by the inability to obtain any sequence data for the peptide. Peptide T2f4 was neutral and could be sequenced quite normally, corresponding to residues 1–5 of the histone. Determination of the *N*-terminal residue of the protein gave a faint proline spot on chromatography after dansylation.

In view of the sequence data it was concluded that calmodulin-binding protein 19K of chicken gizzard and probably the proteins of similar molecular weight obtained from other tissues were identical with histone H2B.

Comparison of interactions of calcium-binding proteins with myelin basic protein, histone H2B and fast-skeletal-muscle troponin I

As the electrophoretic studies indicated that the interaction of calmodulin with the calmodulin-binding proteins exhibited many similarities to the interaction of troponin C with troponin I, it was of interest to compare other aspects of the interaction.

Effect of phosphodiesterase activity

When increasing amounts of bovine brain myelin basic protein or bovine uterus histone H2B were added to bovine brain phosphodiesterase activated by calmodulin to slightly below its maximum activity, progressive inhibition of the enzyme occurred (Fig. 7). The two calmodulin-binding proteins were equally effective and their inhibitory action on the phosphodiesterase was almost identical with that obtained with rabbit skeletal-muscle troponin I (Grand & Perry, 1979) with complete inhibition occurring at a molar ratio of calmodulin-binding protein to calmodulin of approx. 10 : 1.

Effect of calmodulin and troponin C on the phosphorylation of bovine myelin basic protein and chicken gizzard histone H2B

The phosphorylation of myelin basic protein has

Fig. 6. Sequence of calf thymus and chicken erythrocyte histone H2B

Data were taken from Iwai *et al.* (1972) and Van Helden *et al.* (1978). —→, Peptides purified from chicken gizzard calmodulin-binding protein 19K. — represents one step of the dansyl–Edman procedure and indicates that the residue determined is identical with the corresponding residue of histone H2B.

previously been reported by several groups of workers (reviewed by Weller, 1979). The protein isolated by the method described in the present paper was more readily phosphorylated than troponin I by phosphorylase kinase and cyclic AMP-dependent protein kinase.

The phosphorylation of the myelin basic protein by phosphorylase kinase (Fig. 8) was only partially inhibited by troponin C and calmodulin, whereas the phosphorylation of troponin I under similar conditions is inhibited almost completely by equimolar amounts of troponin C (Perry & Cole, 1974) and partly by excess calmodulin (Vanaman & Perry, 1978). The phosphorylation of the brain protein by cyclic AMP-dependent protein kinase was virtually

unaffected by the presence of slightly greater than equimolar amounts of troponin C or calmodulin, whereas the phosphorylation of troponin I is completely blocked in the presence of these proteins.

Bovine uterus histone H2B was also phosphorylated by phosphorylase kinase, but the total incor-

Fig. 7. Inhibition of bovine brain phosphodiesterase by myelin basic protein and chicken gizzard histone H2B. Assays (0.5 ml) contained 10 μ l of phosphodiesterase solution/40 mM-Tris, adjusted to pH 8.0 with 1 M-HCl/1 mM-CaCl₂/0.5 mM-MnCl₂/2 mM-cyclic AMP. Calmodulin was added to give slightly less than maximal activity (0.4 μ g). Results are expressed as a percentage of the activity in the absence of calmodulin-binding protein. O, Bovine uterus calmodulin-binding protein 19K (histone H2B); ●, bovine brain myelin basic protein.

Fig. 8. Time course of phosphorylation of bovine brain myelin basic protein and rabbit fast-skeletal-muscle troponin I by phosphorylase kinase. For incubation conditions see the Materials and Methods section. O, Troponin I; ●, troponin I + troponin C; □, bovine brain myelin basic protein; ■, bovine brain myelin basic protein 22K + troponin C; ▲, bovine brain myelin basic protein + calmodulin; 100 μ g of each protein was added.

Table 5. Phosphate contents of calmodulin-binding proteins

Calmodulin-binding protein	Phosphate content after isolation (mol/mol of binding protein)	Increase in phosphate content after incubation with:	
		Phosphorylase kinase (mol/mol of binding protein)	Protein kinase (mol/mol of binding protein)
Bovine brain myelin basic protein†	0.86*	1.53	0.17
Bovine uterus histone H2B‡	0.61	0.28	Not determined
Rabbit fast-skeletal-muscle troponin I	Not determined	0.27	0.08

* Mean of three preparations.
† Calculated on the basis of a mol.wt. of 18 300.
‡ Calculated on the basis of a mol.wt. of 13 700.

poration of phosphate was considerably less than that obtained with the bovine brain protein. No significant difference could be seen in the phosphorylation of the uterus protein either in the presence or absence of troponin C or calmodulin. In Table 5 data on the phosphate contents of bovine brain myelin basic protein and bovine uterus H2B after isolation and subsequent incubation with enzymes is presented.

Phosphorylated peptides isolated from myelin basic protein

Two major radioactive peptides were isolated by high-voltage electrophoresis in a single experiment when myelin basic protein was phosphorylated with phosphorylase kinase and subsequently digested with trypsin. These had the amino acid analyses (residues/mol): Ser (1.8), Gly (2.4), Leu (1.7), Arg (1.6); and Ser (2.0), Gly (2.1), Leu (1.9), Arg (0.7). Both peptides contained slightly less than 1 mol of covalently bound radioactive phosphate/mol. It was concluded that they corresponded to the tryptic peptide residues 107–113 and 108–113 respectively (Fig. 5).

Phosphorylation of tissue homogenates and slices

The relatively rapid rates of phosphorylation of the purified calmodulin binding proteins *in vitro* and the fact that the proteins were isolated containing approx. 1 mol of phosphate/mol suggested that kinases were present in the tissue to phosphorylate these proteins and that the process may be of some biological significance. ^{32}P -labelled basic myelin protein was isolated from the following systems after the treatment indicated. (1) Bovine brain (6g) homogenized in 20ml of 25 mM-Tris adjusted to pH 7.3 with 1 M-HCl/1 mM-magnesium acetate/1 mM- CaCl_2 /1 mM-ATP, containing 100 μCi of [γ - ^{32}P]ATP was incubated for 1.5 h at 30°C. The reaction was stopped by addition of buffer A and the myelin basic protein was isolated by the method described by Grand & Perry (1979). (2) Thin slices of bovine brain (4g) were incubated at 37°C in Krebs–Henseleit solution (20ml) (Krebs & Henseleit, 1932), containing 1 mM-ATP and 100 μCi of [γ - ^{32}P]ATP. After 1 h the slices were centrifuged down, blotted with tissue paper, homogenized in 20ml of buffer A and myelin basic protein was isolated.

No ^{32}P was incorporated in histone H2B isolated from homogenates and slices of chicken gizzard treated in exactly the same way as the bovine brain preparations.

Discussion

Myelin basic protein and histone H2B have both been shown to bind calmodulin in a calcium-dependent manner under denaturing and non-dena-

turing conditions. On balance the evidence suggests that the bulk of these proteins are probably not associated with calmodulin *in vivo*. Further, it is unlikely that these proteins are present in complexes in homogenates of tissue in high urea concentrations, for no significant amounts of calmodulin-binding protein–calmodulin complexes with mol.wts. of 35 000–40 000 could be detected in non-muscle tissues under these conditions (Grand & Perry, 1979). In this respect they differ from the calmodulin-binding proteins of mol.wts. 140 000, 77 000 and 61 000 that have been isolated as their complexes from bovine brain and then further purified after dissociation of the complex (Grand & Perry, 1979). The latter two lower-molecular-weight proteins are probably similar to the calmodulin-binding proteins described by other investigators. Three groups of workers have purified a calmodulin-binding protein consisting of subunits of mol.wts. approx. 60 000 and 15 000 that will inhibit calmodulin-activated phosphodiesterase (Wang & Desai, 1977; Klee & Krinks, 1978; Sharma *et al.*, 1979) and adenyl cyclase (Wallace *et al.*, 1978).

In the present work calmodulin-binding proteins have been detected by their ability to form a stable complex with calmodulin on polyacrylamide gels in the presence of 6 M-urea and Ca^{2+} . It was considered that this criterion of calmodulin-binding ability was rather more selective than the inhibition of calmodulin-activated phosphodiesterase, which has been widely used by other workers. Non-specific interactions that could produce inhibition in the presence of a large molar excess of the calmodulin-binding proteins are much less likely to occur under denaturing conditions. It should be noted that all the calmodulin-binding proteins described formed a 1:1 molar complex in urea, but usually a large molar excess was required for inhibition of the phosphodiesterase. The calcium-sensitive interaction of two proteins in high urea concentrations was first described for troponin I and troponin C (Perry *et al.*, 1972; Head & Perry, 1974). In view of the marked similarities between calmodulin and troponin C it is tempting to suppose that the special features of the interaction imply that there are strongly conserved regions on the calmodulin and calmodulin-binding proteins similar to those identified on troponin C (Weeks & Perry, 1978; Leavis *et al.*, 1978) and troponin I (Syska *et al.*, 1976).

With histone H2B and myelin basic protein, however, there may be dangers in taking the analogy too far. There is little obvious similarity in the primary sequences of troponin I on the one hand and myelin basic protein and histone H2B on the other, apart from their generally basic nature. All three proteins can be phosphorylated by phosphorylase kinase and protein kinase. The phosphorylation of myelin basic protein by phosphorylase kinase is

partially inhibited by calmodulin and troponin C, suggesting that the former protein contains at least two phosphorylation sites, one of which is in an area blocked by the specific interaction with the calcium-binding proteins. One of the phosphorylation sites (serine-110) appears to be identical with that reported by Carnegie *et al.* (1973) as the site of phosphorylation by cyclic AMP-dependent protein kinase. Miyamoto & Kakiuchi (1974) have reported the amount of phosphate on myelin basic protein as approx. 0.2 mol/mol. It is possible that the higher values reported by us are due to the use of denaturing conditions throughout the preparative procedure. In consequence the activity of the phosphatase that is normally associated with myelin (Miyamoto & Kakiuchi, 1974) will be inhibited and the protein isolated will have a phosphate content closer to that existing *in vivo*.

The sequence data presented here for chicken gizzard histone H2B are in agreement with the sequence of the calf thymus protein (Iwai *et al.*, 1972), except for differences at residues 25 and 26. These two substitutions are, however, identical with those reported by van Helden *et al.* (1978) for the chicken erythrocyte histone H2B. The studies on the sequences of the tryptic peptides suggest that when isolated by the procedure described histone H2B exists with and without a blocked N-terminus.

Although calmodulin was bound strongly by all the calf thymus histones the complex formed with histone H2B was unique in that it required Ca^{2+} for its formation in high urea concentrations. The significance of this feature of the interaction is far from clear. Smoake *et al.* (1974) have found that considerable amounts of calmodulin are associated with the nuclear fraction in platelets and the results obtained in our study of brain support their findings. As yet there is no evidence to suggest that it forms part of chromatin (Elgin & Weintraub, 1975), but by analogy with other membrane systems calmodulin may be an integral part of the nuclear membrane.

The results of the studies *in vitro* indicate that a complex consisting of equimolar amounts of the two proteins can be formed by interaction of calmodulin with a defined site on myelin basic protein and probably on histone H2B. Nevertheless *in vivo*, calmodulin may not be bound to these proteins due to the fact that it is probably sequestered elsewhere in the cell. It can be concluded that basic proteins generally will bind calmodulin and for this reason it is unlikely that much of the calmodulin will exist in the free form. This type of complex formation does not necessarily imply that calmodulin will be inactive, for little inhibition of calmodulin in activating phosphodiesterase is obtained when equimolar proportions of calmodulin-binding proteins are present (Klee & Krinks, 1978; Grand & Perry, 1979).

The properties of the myelin basic proteins and histone H2B are very similar to those that have previously been used to characterize calmodulin-binding proteins, yet the interaction with calmodulin does not appear to occur *in vivo* and is therefore of doubtful physiological significance. Clearly care must be taken in future in drawing conclusions about the physiological role of a number of proteins that have been shown to bind to calmodulin unless there is also evidence of effects on biological activity when the two proteins are present in similar concentrations.

We thank Dr. A. J. G. Moir for help in the purification of the phosphorylated peptide from myelin basic protein, Dr. G. K. Dhoot for the preparation of antibodies to myelin basic protein, Dr. A. C. Nairn for help in the preparation of phosphodiesterase and Mr. C. M. Wall for advice on the phosphorylation experiments. This work was supported by a programme grant from the Medical Research Council.

References

- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
Brostrom, C. O., Huang, Y. C., Breckenridge, B. & Wolff, D. J. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 64–68
Carnegie, P. R. & Dunkley, P. R. (1975) *Advances in Neurochemistry* (Agranoff, B. W. & Aprison, M. H., eds.) vol. 1., pp. 95–135, Plenum Press, New York
Carnegie, P. R., Kemp, B. E., Dunkley, P. R. & Murray, A. W. (1973) *Biochem. J.* **135**, 569–572
Cheung, W. Y. (1970) *Biochem. Biophys. Res. Commun.* **38**, 533–538
Cohen, P. (1973) *Eur. J. Biochem.* **34**, 1–14
Cohen, P., Burchell, J., Foulkes, G., Cohen, P. T. W., Vanaman, T. C. & Nairn, A. C. (1978) *FEBS Lett.* **92**, 287–293
Cole, H. A. & Perry, S. V. (1975) *Biochem. J.* **149**, 525–533
Dhoot, G. K., Gell, P. G. H. & Perry, S. V. (1978) *Exp. Cell Res.* **117**, 357–370
Easley, C. W. (1965) *Biochim. Biophys. Acta* **107**, 386–388
Ebashi, S., Wakabayashi, T. & Ebashi, I. F. (1971) *J. Biochem. (Tokyo)* **69**, 441–445
Elgin, S. C. R. & Weintraub, H. (1975) *Annu. Rev. Biochem.* **44**, 725–774
Eylar, E. H., Brostoff, S., Hashim, G., Caccam, J. & Burnett, P. (1971) *J. Biol. Chem.* **246**, 5770–5784
Gopinath, R. M. & Vincenzi, F. F. (1977) *Biochem. Biophys. Res. Commun.* **77**, 1203–1209
Grand, R. J. A. & Perry, S. V. (1979) *Biochem. J.* **183**, 285–295
Grand, R. J. A., Perry, S. V. & Weeks, R. A. (1979) *Biochem. J.* **177**, 521–529
Grand, R. J. A., Nairn, A. C. & Perry, S. V. (1980) *Biochem. J.* **185**, 755–760

- Gray, W. R. (1967) *Methods Enzymol.* **11**, 469–475
- Gray, W. R. & Smith, J. F. (1970) *Anal. Biochem.* **33**, 36–42
- Head, J. F. & Perry, S. V. (1974) *Biochem. J.* **137**, 145–154
- Head, J. F., Weeks, R. A. & Perry, S. V. (1977) *Biochem. J.* **161**, 465–471
- Iwai, K., Hayashi, H., Ishikawa, K. (1972) *J. Biochem. (Tokyo)* **72**, 357–367
- Kakiuchi, S. & Yamazaki, R. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1104–1110
- Klee, C. B. & Krinks, M. H. (1978) *Biochemistry* **17**, 120–126
- Krebs, H. A. & Henseleit, K. (1932) *Z. Physiol. Chem.* **210**, 33–66
- Leavis, P. C., Rosenfeld, S. S., Gergely, J., Grabarek, Z. & Drabikowski, W. (1978) *J. Biol. Chem.* **253**, 5452–5459
- Mejbaum-Katzenellenbogen, W. & Dobrzychycka, W. H. (1959) *Clin. Chim. Acta* **4**, 515–522
- Miyamoto, E. & Kakiuchi, S. (1974) *J. Biol. Chem.* **249**, 2769–2777
- Nairn, A. C. & Perry, S. V. (1979) *Biochem. J.* **179**, 89–97
- Norton, W. T. (1974) *Methods Enzymol.* **31**, 435–444
- Offord, R. E. (1966) *Nature (London)* **211**, 591–593
- Perry, S. V. & Cole, H. A. (1974) *Biochem. J.* **141**, 733–743
- Perry, S. V., Cole, H. A., Head, J. F. & Wilson, F. J. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 251–262
- Rodriguez de Lores Arnaiz, G., Alberici, M. & De Robertis, E. (1967) *J. Neurochem.* **14**, 215–225
- Sharma, R. K., Desai, R., Waisman, D. M. & Wang, J. H. (1979) *J. Biol. Chem.* **254**, 4276–4282
- Sherry, J. M. F., Gorecka, A., Aksoy, M. O., Dabrowska, R. & Hartshorne, D. J. (1978) *Biochemistry* **17**, 4411–4418
- Smoake, J. A., Song, S-Y & Cheung, W. Y. (1974) *Biochim. Biophys. Acta* **341**, 402–411
- Strauch, L. (1965) *Z. Klin. Chem.* **3**, 165–167
- Syska, H., Wilkinson, J. M., Grand, R. J. A. & Perry, S. V. (1976) *Biochem. J.* **153**, 375–387
- Teo, T. S., Wang, T. H. & Wang, J. H. (1973) *J. Biol. Chem.* **248**, 588–595
- Vanaman, T. C. & Perry, S. V. (1978) *Abstr. Proc. Eur. Conf. Muscle Motility 7th* 37
- Van Helden, P., Strickland, W. N., Brandt, W. F. & Von Holt, C. (1978) *Biochim. Biophys. Acta* **533**, 278–281
- Wallace, R. W., Lynch, R. J., Tallant, E. A. & Cheung, W. Y. (1978) *J. Biol. Chem.* **254**, 377–382
- Wang, J. H. & Desai, R. (1977) *J. Biol. Chem.* **252**, 4175–4184
- Watterson, D. M., Harrelson, W. G., Keller, P. M., Sharief, F. & Vanaman, T. C. (1976) *J. Biol. Chem.* **251**, 4501–4513
- Weeks, R. A. & Perry, S. V. (1978) *Biochem. J.* **173**, 449–457
- Weller, P. (1979) *Protein Phosphorylation*, pp. 344–346, Pion, London
- Wilkinson, J. M. (1969) *FEBS Lett.* **4**, 170–172
- Wilkinson, J. M. (1974) *Biochim. Biophys. Acta* **359**, 379–388
- Wilkinson, J. M., Perry, S. V., Cole, H. A. & Trayer, I. P. (1972) *Biochem. J.* **127**, 215–228
- Yagi, K., Yazawa, M., Kakiuchi, S., Ohshima, M. & Uenishi, K. (1978) *J. Biol. Chem.* **253**, 1338–1340
- Yeoman, L. C., Olson, M. O. J., Sugano, N., Jordan, J. J., Taylor, C. W., Starbuck, W. C. & Busch, H. (1972) *J. Biol. Chem.* **247**, 6018–6023