ic observation and histochemical straining via Von Kossa's method[9] to demonstrate calcium deposition.

*Results and discussion.* Based on histochemical straining and observation of whole mounts, marked calcium deposition in *T. spiralis* cysts was observed in rats with a 14-week infection (figure 1) as opposed to infected control rats not administered vitamin $D_3$ (figure 2). The enhancement of cyst calcification was very slight in rats with a 4-week-old infection.

The main scope of this investigation was an attempt to inhibit the calcification process of the *T. spiralis* cyst. Although a somewhat artifical system was employed in that vitamin $D_3$ was used to accelerate cyst calcification, the end result of vitamin $D_3$ treatment was similar to an infection of long duration. In rats given EHDP while under vitamin $D_3$ treatment, cyst calcification was not evident (figure 3) and the cysts appeared similar to infected rats not receiving vitamin $D_3$ (figure 2). The table lists the relative amount of calcium deposition in the experimental groups. Thus, the ability of EHDP to block the calcification of *T. spiralis* cysts demonstrated for the first time the inhibition of this mechanism. This demonstrates that cyst calcification is not an irreversible process and is subject to drug therapy.

1 Present address: Dept of Biology, Memphis State University, Memphis (Tenn. 38152, USA).
2 Supported in part by EKU faculty research grant No. 03-05.
3 Much appreciation goes to Dr Gordon S. Hassings and the Proctor and Gamble Company, Miami Valley Laboratories, Cincinnati, Ohio USA for providing the EHDP used in this study.
4 S.E. Gould, in: *Trichinosis in Man and Animals*, p. 101. Ch. C. Thomas, Springfield Ill. 1970.
5 D. Thienpoint, O. Vanparijs and R. Vandesteen, 3rd Int. Conf. Trichinellosis. Ed. C.W. Kim. Educational Publ., New York 1974.
6 S. DeNollin, M. Borgers, O. Vanparijs and H. van Der Bossche, Parasitology *69*, 55 (1974).
7 V.D. van Someren, Br. Med. J. *1*, 376 (1939).
8 M.D. Francis and R.G.G. Russel, Science *156*, 1264 (1970).
9 F.A. Putt, Manual of Histopathological Staining. John Wiley & Sons, New York 1972.

## Anti-myosin stains chromaffin cells[1]

J.M. Trifaró, C. Ulpian and H. Preiksaitis

*Department of Pharmacology and Therapeutics, McGill University, 3655 Drummond Street, Montreal (Quebec H3G 1Y6, Canada), 2 May 1978*

*Summary.* The presence in fixed chromaffin cells of antigenic sites for a myosin antibody was demonstrated using immunofluorescence techniques. Tests on viable cells showed that at least some of the antigenic sites seem to be localized on or close to the cell surface and explained the cell agglutination that occurred with the addition of the myosin antibody to cells isolated by a method described in this paper.

Actin, myosin, tropomyosin and troponin have been isolated from a variety of nonmuscle cells[2,3]. We have recently isolated from the chromaffin cell a protein with electrophoretic mobility, enzymatic activity, amino acid composition and electron microscopic appearance closely resembling those myosins from muscle and nonmuscle cells[4]. Although all major vessels were dissected out of the intact adrenal medulla before myosin was extracted, it remained possible that adrenal myosin originated in the smooth muscle of the small vessels of the medulla rather than in the chromaffin cells. However, myosin has been identified in extracts from cultures of sympathetic neurons[5]; such neurons have the same embryological origin as the chromaffin cells. To determine more directly whether myosin is a component of the chromaffin cells, the present experiments used immunohistochemical techniques to demonstrate the presence in the chromaffin cell of antigenic sites for a myosin antibody.

*Material and methods.* Bovine muscle myosin was prepared and its purity established by polyacrylamide gel electrophoresis as described previously[4]. Rabbit antiserum was prepared against purified myosin, following a procedure previously published[6], and the IgG fraction of this antiserum was isolated as described elsewhere[7]. A normal IgG fraction was also prepared from serum of pre-immunized rabbits. The anti-muscle myosin (AMM) IgG fraction when tested in an Ochterlony plate against either purified bovine muscle myosin or adrenal actomyosin[4,8] gave single precipitin lines which fused completely demonstrating immunological identity (figure 1). No precipitin lines were observed when the antibody was tested against 4 different concentrations of bovine muscle actin (figure 1). Furthermore, the AMM-IgG fraction when tested against bovine aortic muscle myosin gave a precipitin line which fused completely with that obtained against adrenal actomyosin. The AMM-IgG was then tested on isolated chromaffin cells by the standard (indirect) immunofluorescence test[9]. The isolated cells were prepared as follows: a bovine adrenal gland obtained from a slaughterhouse was freed from its cortex and perfused in vitro for 30 min (37 °C) with $Ca^{2+}$-free, $Mg^{2+}$-free Locke's solution as described previously[10]. Perfusion was then continued for 40 min with fresh solution to which 0.05% collagenase (Sigma Chemical Co.) had been

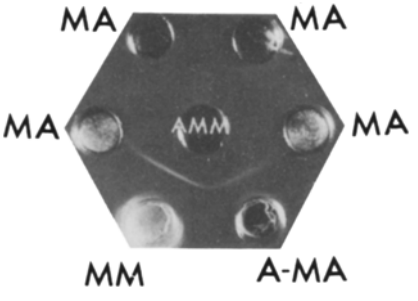


Fig. 1. Double immunodiffusion test between AMM-IgG (center well, 5 µg) against bovine adrenal medullary actomyosin (A-MA, 40 µg), bovine muscle myosin (MM, 40 µg) and 4 concentrations of bovine muscle actin (MA, 160, 80, 40 and 20 µg from left to right). Muscle actin was isolated as described elsewhere[26]. The capacity of each well was 30 µl and the plate contained the following mixture: 1% agar, 0.1% sodium azide, 0.4 M KCl, 0.04 M sodium pyrophosphate and 0.5 M Tris-HCl buffer, pH 8.0. The plate was left at room temperature for 48 h before the photograph was taken.

added. At the end of this perfusion period, the medulla was minced in small pieces and incubated with collagenase-containing Locke's solution in a Dubnoff incubator (37 °C) for an additional 40 min. The preparation was filtered through a 200–400 nylon mesh and centrifuged at 120×g for 5 min. The sediment thus obtained was suspended in Locke's solution and centrifuged again at the same speed for the same period of time. The final sediment was resuspended in Locke's solution and contained isolated viable chromaffin cells which released catecholamines in response to either carbamylcholine ($10^{-4}$ M) or depolarizing concentrations of $K^+$ (56 mM). Most (90–95%) of these cells were not penetrated by trypan blue when suspended in Locke's solution containing the dye (2 mg/ml).

*Results and discussion.* Smears of fixed isolated chromaffin cells were treated with either normal IgG or AMM-IgG followed by exposure to fluorescein-isothiocyanate conjugated (goat) anti-rabbit IgG. The cells treated with normal IgG did not exhibit fluorescence, but the cells treated with AMM-IgG showed an intense bright fluorescence of granular appearance localized to the cytosol. No fluorescence was detected in the cell nucleus (figure 2). The results from these experiments clearly indicate that an antigen which reacts with AMM-IgG is present in chromaffin cells, and that this antigen is likely to be myosin. Because the fixation procedure used disrupts cell membranes, the experiments indicate little about the subcellular localization of myosin except that antigenic sites appear absent from the cell's nucleus. Therefore, another approach was used based on recent evidence showing that, in cultured fibroblasts, one of the localization sites for myosin is on the cell surface[11]. Isolated viable chromaffin cells were incubated in Locke's solution in the presence of either normal IgG (2.5 mg/ml) or AMM-IgG (2.5 mg/ml). Immediately upon the addition of AMM-IgG, agglutination of chromaffin cells was observed (figure 3). This agglutination response, which has previously been noted when cultured fibroblasts were treated with antiserum prepared against L-cell myosin[12], suggested the presence of myosin on the chromaffin cell surface. This suggestion was confirmed by the indirect immunohistochemical test. The pattern of immunofluorescent reactivity in live chromaffin cells was quite distinctive and differed from that observed in fixed cells. There was surface fluorescence that showed in some cases a uniform 'ring' distribution, and in others a 'patchy' distribution of the fluorescent staining (figure 4). Focussing the incident light beam to progressively deeper planes of a single cell showed that the fluorescence was confined to the cell surface. 4 experiments on 756 normal IgG-treated and 898 AMM-IgG-treated cells indicated that most of the anti-

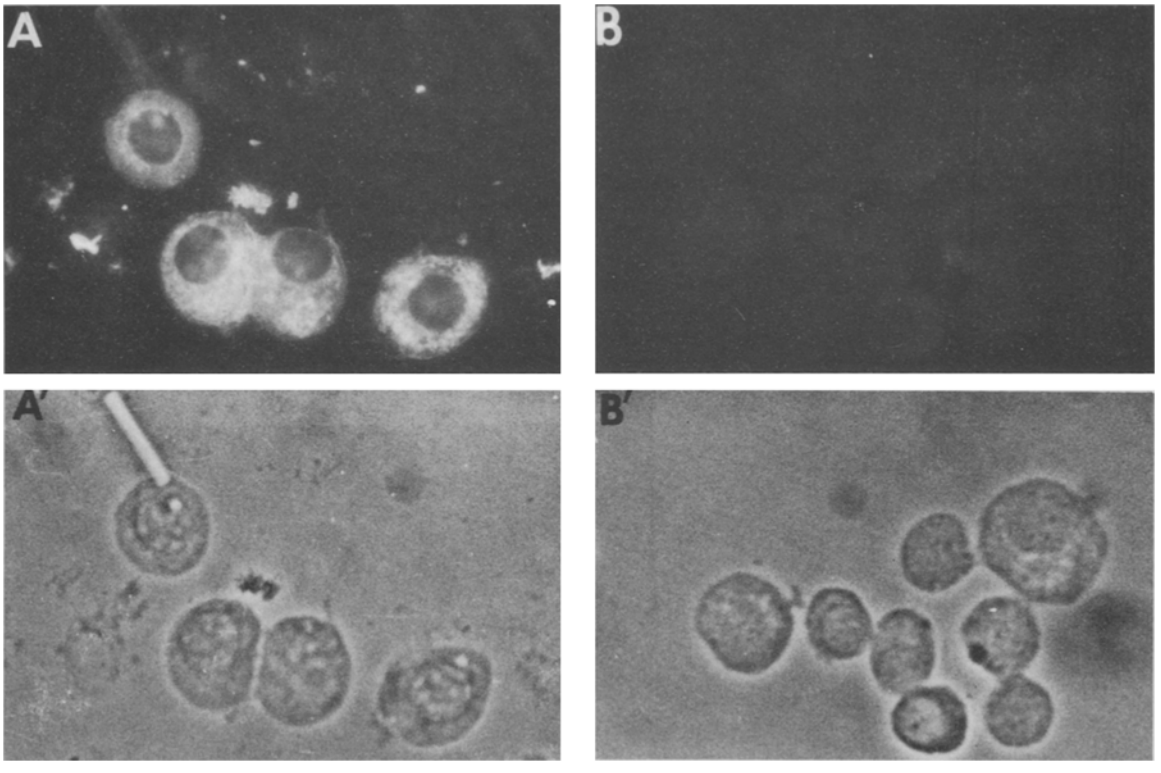


Fig. 2. Indirect immunofluorescence staining of adrenal chromaffin cells by a bovine muscle myosin antibody. Chromaffin cell suspensions were smeared on glass slides and air-dried for 30 min. The cells were fixed in chloroform:methanol (2:1 by volume) for 30 min at 4 °C[27] before they were stained by the standard indirect immunofluorescence test[9]. Each slide containing fixed chromaffin cells was divided in 2 halves by the application of nail polish. 1 half was covered with normal IgG (5 mg/ml of PBS buffer: 0.16 M NaCl and 0.02 M sodium phosphate, pH 7.2) and the other half with AMM-IgG (5 mg/ml PBS). The slides were incubated at 30 °C for 1 h in a 100% humidity chamber. They were then removed and washed 10 times with PBS, and once with distilled water to remove the salt residue. The washed slides were covered with fluorescein-isothiocyanate (FITC, 0.5 mg protein/ml PBS) conjugated (goat) anti-rabbit IgG with fluorescein-to-protein molar ratio of 2.3 (Miles Laboratories) and again incubated for 1 h. After this, the slides were washed as before and mounted in a mixture of 9:1 glycerol:PBS, pH 7.2. The preparations were observed with a Leitz ortholux II fluorescence microscope equipped with a 200 W/4 high pressure mercury lamp and Ploemopack incident light illuminator with 3 exciting filters, 1 K445 and 2 KP490, dichroic beam splitting mirror TK510 and supression filter TK515. Photographs were taken with a 35-mm Kodak Tri X pan film (ASA 400). *A* and *B show cells treated with AMM-IgG and normal IgG respectively* (×63/1.30 oil immersion objective; ×760). The exposure times during photography, printing and developing of pictures was the same in *A* and *B*. The same cell preparations are shown by phase contrast microscopy (×760) in *A'* and *B'* respectively.

body-treated cells showed bright fluorescence, whereas only a few control cells showed a faint patchy fluorescence (table). As a further control for specificity, the AMM-IgG solution was absorbed with either isolated chromaffin cells or muscle myosin; this inhibited the staining of the surface of viable chromaffin cells (table).

Studies of myosin localized on the cell surface of fibroblasts have suggested that myosin, a large asymmetric molecule (1400 Å), might be a transmembranal protein with an antigenic rod portion exposed on the outside and, presumably, with a head portion residing in the cell interior where it can interact with actin[11]. The adrenal myosin is also a large asymmetric molecule[4] which, as demonstrated here, seems to be, at least in part, localized on or close to the cell surface. However, the possibility exists that the surface myosin was derived from the cytosol of dead cells. That is to say, that the staining patterns seen in figure 4 could have arisen as a result of myosin being liberated from dead cells which sticks to the plasma membrane of viable cells. To exclude this possibility, myosin was labelled with $^{125}I$ according to the method described by Bolton and Hunter[13]. The purity of the labelled myosin preparation was determined by SDS polyacrylamide gel electrophoresis as shown in figure 5. Only the heavy and light chains of myosin were labelled. 4 aliquots, each containing $2.5 \times 10^6$ chromaffin cells were incubated with 2 ng of $^{125}I$-myosin ($8 \times 10^5$ DPM/ng protein) for 60 min at 30 °C. These preparations were then washed 16 times as described in the legend of the table. After the last wash, the cells were collected by centrifugation (100,000 × g for 30 min) and the radioactivity measured. The radioactivity present in the cell sediments represented only 0.069±0.009% (n = 4) of the original radioactivity. Therefore, these results make unlikely the possibility that the surface myosin is derived from the cytosol of dead cells. Moreover, a paper on the subcellular distribution of the ($K^+$, EDTA)-ATPase in the adrenal medulla has been published recently[14]. In this study, the distribution in sucrose density gradients of acetylcholinesterase, adenylate cyclase, guanylate cyclase and ($K^+$, EDTA)-ATPase was similar. The authors concluded from these studies that the adrenal medullary myosin, evaluated by a ($K^+$, EDTA)-ATPase activity, was localized mainly in plasma membranes. Although these investigators did not discard the possibility that, as before, and due to the use of solutions of low ionic strength, myosin had precipitated on plasma membranes, the results with the $^{125}I$-myosin presented here make also this possibility improbable. There is still another possibility to be discarded, and that is, that the collagenase treatment used during the isolation of the cells might expose on the cell surface myosin which is normally covered by surface components of the cell (components which have been removed by the treatment with collagenase). This latter possibility should be considered since it has been shown recently that the surface fluorescence of live cells in indirect fluorescence studies with antimyosin

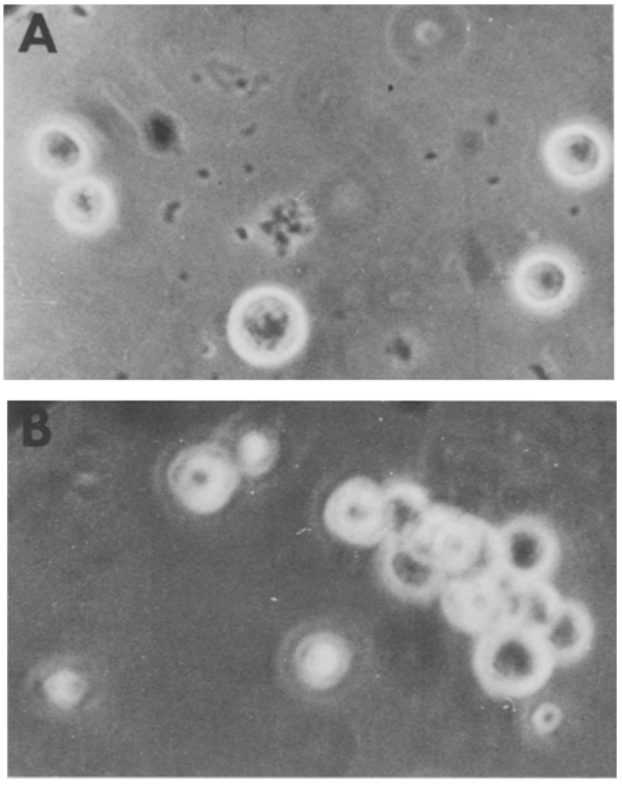


Fig. 3. Agglutination of chromaffin cells by bovine muscle myosin antibody. Viable cells were prepared as indicated in the text; they were suspended in Locke's solution in the presence of either normal IgG (A) or AMM-IgG (B) and observed by phase contrast microscopy (× 510).

Immunofluorescence staining of chromaffin cells by antimyosin antibody

| Experiment No. | Condition | Number of cells observed | Positive fluorescence No. of cells | % |
|---|---|---|---|---|
| 1 | Normal IgG | 102 | 0 | 0 |
| | Anti-myosin IgG | 84 | 81 | 96 |
| 2 | Normal IgG | 450 | 9 | 2 |
| | Anti-myosin IgG | 438 | 419 | 96 |
| 3 | Normal IgG | 204 | 9 | 4 |
| | Anti-myosin IgG | 220 | 208 | 95 |
| | CC absorbed anti-myosin IgG | 225 | 15 | 7 |
| 4 | Anti-myosin IgG | 156 | 150 | 96 |
| | MM absorbed anti-myosin IgG | 204 | 29 | 14 |

Viable cells were suspended in Locke's solution containing either normal IgG (2.5 mg/ml) or AMM-IgG (2.5 mg/ml) and then incubated for 1 h at 30 °C. The cells were washed 10 times with Locke's solution and then exposed to FITC for 1 h, washed 6 more times, smeared on glass slides, mounted and observed with incident blue light as described in the legend to figure 2. In experiments 3 and 4, samples containing AMM-IgG were incubated at 30 °C for 1 h with either isolated chromaffin cells (CC) or bovine muscle myosin (MM; 1 mg/ml). The incubations were continued at 4 °C for 18 h and the preparations centrifuged at 100,000 × g for 60 min. The supernatants thus obtained were used as a primary step in the indirect immunofluorescence test[9]. The entire procedure was also followed with samples of normal IgG and AMM-IgG but devoid of either chromaffin cells or muscle myosin.

depends on trypsination, and that the cells lose their fluorescence during recovery from protease treatment after plating[15]. Because in our case the cells were used shortly after collagenase treatment, this possibility cannot be excluded at the present time. Nevertheless, whatever the subcellular localization of the myosin might be, the present results indicate that the antigen demonstrated in chromaffin cells is a myosin-like protein, and they strongly support the suggestion that the myosin isolated from the adrenal medulla[4] has its origin in the chromaffin cell.

Although the functional significance of these contractile proteins in nonmuscle cells is not known, it has been suggested that they might be involved in such functions as cell motility[2], cell division[16], cell viscosity[17], cell shape[18], cell secretion[3,19,20], phagocytosis[21], membrane motility and mobility of membrane receptors[3,22]. It is possible that, in chromaffin cells, the contractile proteins are involved in one or more of the above functions. Considering the migration of chromaffin cells from the neural crest to their final anatomical position in the adult animal, it may be surmised that chromaffin cells possess some kind of motility, at least in early stages of development. Previous studies have shown, moreover, that cultured pheochromocytoma and adrenomedullary cells do display signs of motility[23]. However, the similarities between 2 apparently different processes, contraction and secretion[24,25], together with the hypothesis that a true contractile event might be involved in the molecular mechanism of exocytosis[19], suggest that the study of these contractile proteins may be of crucial importance in the understanding of the secretory process at cellular and molecular levels.

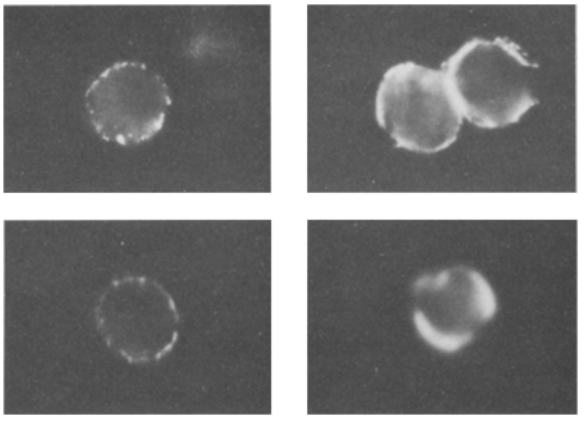

Fig. 4. Fluorescent antibody localization on chromaffin cells with antibody to bovine muscle myosin without fixation. Viable cells were suspended in Locke's solution containing AMM-IgG and the procedure described in the legend to the table was followed. The incident blue light was focussed at the equatorial plane of the cells. The left panels show the typical 'patchy' distribution of fluorescence, whereas the right panels show 'ring' fluorescence (× 63/1.30 oil immersion objective; × 650).

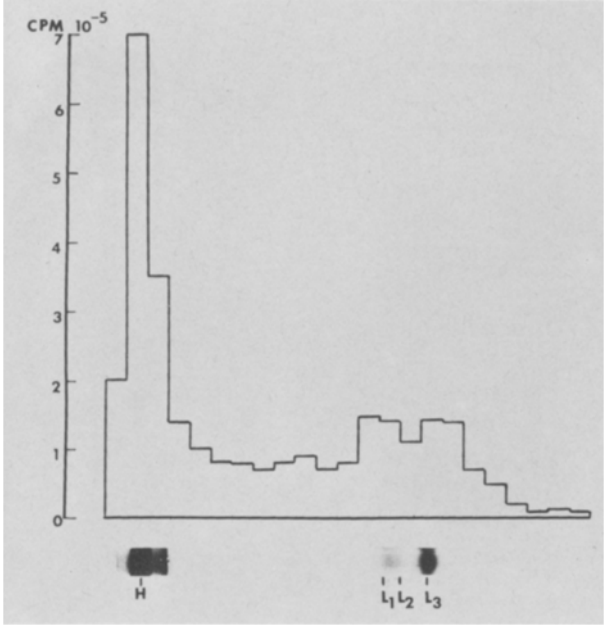


Fig. 5. Polyacrylamide gel electrophoresis of $^{125}$I-myosin. An aliquot of $^{125}$I-labelled myosin ($4 \times 10^5$ dpm) was mixed with 40 µg of myosin and incubated at 60 °C for 30 min in the presence of 1% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol. The mixture was applied to 6% acrylamide gels containing 0.1% SDS and the electrophoresis was carried out as described by Weber and Osborn[28]. After electrophoresis, the gel was cut in 4-mm sections and the radioactivity measured in a gamma spectrometer, $L_1$, $L_2$ and $L_3$ indicate the heavy and light chains of myosin respectively.

1 Acknowledgments. This work was supported by grants from the Medical Research Council of Canada. We thank Dr B.G. Livett for advice in the preparation of isolated cells, Drs J. Lowenthal and B. Collier for comments and Mrs J. Ritchie for technical assistance.

2 T.D. Pollard and R.R. Weihing, CRC Crit. Rev. Biochem. *2*, 1 (1974).
3 J.M. Trifaró, Neuroscience *3*, 1 (1978).
4 J.M. Trifaró and C. Ulpian, Neuroscience *1*, 483 (1976).
5 K. Burridge and D. Bray, J. molec. Biol. *99*, 1 (1975).
6 J.M. Trifaró, A.C. Duerr and J.E.B. Pinto, Molec. Pharmac. *12*, 536 (1976).
7 R.C. Roberts, D.G. Makey and U.S. Seal, J. biol. Chem. *241*, 4907 (1966).
8 J.M. Trifaró and C. Ulpian, FEBS Lett. *57*, 198 (1975).
9 M. Goldman, in: Fluorescent Antibody Methods. New York, Academic Press 1968.
10 J.M. Trifaró, A.M. Poisner and W.W. Douglas, Biochem. Pharmac. *16*, 2095 (1967).
11 K. Olden, M. Willinghan and I. Pastan, Cell *8*, 383 (1976).
12 M. Willinghan, R.E. Ostlund and I. Pastan, Proc. natl Acad. Sci., USA *71*, 4144 (1974).
13 A.E. Bolton and W.M. Hunter, Biochem. J. *133*, 529 (1973).
14 J.E. Hesketh, D. Aunis, M. Pescheloque and P. Mandel, FEBS Lett. *80*, 324 (1977).
15 U. Gröschel-Stewart, J.H. Chamley, J.D. McConnell and G. Burnstock, Histochemistry *47*, 285 (1976).
16 J.W. Sanger, Proc. natl Acad. Sci., USA *72*, 1913 (1975).
17 T.D. Pollard, J. Cell Biol. *67*, 72 (1976).
18 E. Lazarides and K. Weber, Proc. natl Acad. Sci., USA *71*, 4561 (1974).
19 A.M. Poisner and J.M. Trifaró, Molec. Pharmac. *3*, 561 (1967).
20 S. Berl, S. Puszkin and W.J. Nicklas, Science *179*, 441 (1973).
21 J.H. Hartwig and T.P. Stossel, J. biol. Chem. *250*, 5696 (1975).
22 J.F. Ash and S.J. Singer, Proc. natl Acad. Sci., USA *73*, 4575 (1976).
23 I. Costero, A. Chávez, L. Peralta, E. Monroy and F. Ramón, Texas Rep. Biol. Med. *23*, suppl. 1, 213 (1965).
24 W.W. Douglas, in: Handbook of Physiology, Endocrinology, vol. 6, p. 367. Ed. H. Blaschko, G. Sayers and A.D. Smith. American Physiological Society, 1975.
25 J.M. Trifaró, A. Rev. Pharmac. Tox. *17*, 27 (1977).
26 J.A. Spudich and S. Watt, J. biol. Chem. *246*, 4866 (1971).
27 B.K. Hartman and S. Udenfriend, Molec. Pharmac. *6*, 85 (1970).
28 K. Weber and M. Osborn, J. biol. Chem. *244*, 4406 (1969).