BBA 45731

# STUDIES ON THE EXCHANGE OF G-ACTIN-BOUND CALCIUM WITH BIVALENT CATIONS

HANNA STRZELECKA-GOŁASZEWSKA AND WITOLD DRABIKOWSKI
*Department of Biochemistry, Nencki Institute of Experimental Biology, Warsaw (Poland)*

(Received December 28th, 1967)
(Revised manuscript received May 29th, 1968)

SUMMARY

1. The possibility of the replacement of G-actin-bound calcium by various bivalent cations has been investigated. After the reaction with all cations studied, with the exception of $Cu^{2+}$, actin remains active, *i.e.*, contains bound ATP and polymerizes in 0.1 M KCl.

2. The amount of G-actin-bound calcium, as well as the sum of bivalent cation after replacement, not removable by short-time Dowex-50 treatment, accounts to about 1 mole per 50000 g of G-actin.

3. The rate of exchange is of the same order for bivalent cations studied, including calcium.

4. G-actin-bound $Ca^{2+}$ is fully replaced, besides free $Ca^{2+}$, by free $Mn^{2+}$ and $Cd^{2+}$. The replacement with $Mg^{2+}$, $Co^{2+}$, $Ni^{2+}$ and $Zn^{2+}$ is not complete, and there is practically no reaction with $Ba^{2+}$ and $Sr^{2+}$.

5. Assuming the affinity constant of $Ca^{2+}$ as 1, the following affinity constants for other bivalent cations were obtained: $Mn^{2+}$, 0.90; $Cd^{2+}$, 1.07; $Mg^{2+}$, 0.27; $Zn^{2+}$, 0.22; $Co^{2+}$, 0.18; $Ni^{2+}$, 0.08.

6. The results obtained show that there exists a close correlation between the ionic radius of a particular bivalent cation, and its ability to replace bound $Ca^{2+}$.

## INTRODUCTION

It has been already well established that G-actin preparations contain strongly bound ATP in the amount of about 1 mole per mole protein (*cf.* ref. 1). This bound ATP is easily exchangeable with free ATP (ref. 2) and, to a variable extent, with some other nucleotides[3,4]. In recent years it has been found that actin contains also about 1 mole of strongly bound $Ca^{2+}$ per mole protein[5–9]. The bound $Ca^{2+}$, similarly as the bound ATP, is necessary for maintaining all the specific properties of G-actin and completely exchanges with free $Ca^{2+}$ (ref. 7).

The experiments of STROHMAN AND SAMORODIN[10] gave an indirect evidence for some replacement of G-actin-bound $Ca^{2+}$ by $Mg^{2+}$, $Mn^{2+}$ and $Zn^{2+}$. Similar possibility for $Mg^{2+}$ and $Mn^{2+}$ was suggested by KATZ[11] on the basis of the effect of these cations on the reactivity of actin sulphydryl groups. BÁRÁNY AND CHRAMBACH[12] directly showed that bound $Ca^{2+}$ could partially exchange with free $Mg^{2+}$ and OOSAWA

*et al.*[13] obtained G-actin preparations in which most of the bound $Ca^{2+}$ was replaced by $Mg^{2+}$. All these observations pointed to the possibility of the replacement of $Ca^{2+}$ bound to G-actin by some bivalent cations other than $Ca^{2+}$ and this exchange was demonstrated in our previous work[14]. In the present paper the exchange of G-actin-bound $Ca^{2+}$ with free $Ca^{2+}$ as well as with other bivalent cations was further studied and the relative affinity constants of these cations to actin were calculated. Preliminary reports of some of the results were already presented[14,15].

## MATERIALS AND METHODS

Preparation of G-actin and labelling its bound $Ca^{2+}$ and ATP by $^{45}Ca^{2+}$ and [$^{14}$C]ATP, respectively, was performed according to the routine procedures described previously[16]. Free bivalent cations and free nucleotides were removed from actin solutions by 2–3-min treatment with Dowex 50 or Dowex 1, respectively, as described in the previous paper[16]. Control experiments showed that the presence of an excess of all bivalent cations studied did not interfere with the quantitative removal of free $Ca^{2+}$ at the concentrations used in this work. Also ATP, in concentrations up to 1 mM, had no effect on the removal of free $Ca^{2+}$.

For the measurements of radioactivity samples of actin solutions containing $^{45}Ca^{2+}$ or [$^{14}$C]ATP were dried in aluminium planchets under infrared lamp and counted in a Chicago Nuclear Corporation gas-flow counter with Micromil window. Throughout each experiment the same amounts of the protein, not higher than 2 mg, were used for radioactivity determinations in order to avoid corrections for self-absorption.

The amounts of bivalent cations bound to actin were determined by micro-titration with EDTA at pH 10 using Eriochrome Black T as indicator. The conditions of titration were based essentially on the paper by HILDEBRAND AND REILLEY[17] and the spectrophotometric titration technique according to FLASCHKA[18] was used. Actin solutions were treated with Dowex 50, then deproteinized with 0.6 M $HClO_4$ and, after removal of protein, neutralized with concentrated NaOH using methyl red as an indicator. (The yellow colour of methyl red in alkaline solution did not interfere with the endpoint of EDTA titration.) Samples were titrated at 650 m$\mu$ (absorption peak of free indicator) with 10 mM EDTA in the cuvettes of 1 cm light path in the presence of ammonium buffer (67.5 g of $NH_4Cl$ and 570 ml of concentrated $NH_4OH$ diluted to 1 l with deionized water) and indicator solution (0.2 g of Eriochrome Black T in 50 ml of methanol) against water as a blank. When samples contained $Mn^{2+}$ an excess of hydroxylamine hydrochloride was added to prevent the oxidation of this ion to higher oxidation states by atmospheric $O_2$. The endpoint of the titration was obtained from the plot of $\mu$l EDTA used *versus* absorbance values. The final results were calculated after subtracting the amounts of EDTA used for titration of the control samples, containing 0.6 M $HClO_4$ neutralized with NaOH, buffer, and the indicator.

Protein concentration was determined by the biuret method[19] calibrated by micro-Kjeldahl nitrogen determination.

Viscosity measurements were made at 21° in Ostwald-type viscometers with an outflow time of 50–60 sec for 3 ml water. pH of the solutions was measured with a Radiometer (Copenhagen) pH-meter 22.

To avoid metal ion contaminations throughout all experiments, glassware

soaked in 1 mM EDTA solution for several hours and then washed exhaustively with deionized water, was used. Polyethylene centrifuge tubes and polyethylene bottles were washed with deionized water. All solutions were prepared using deionized water and stored in polyethylene bottles.

Dowex 50 (W-X4 or W-X2, 200–400 mesh) and Dowex 1 (X-8, 200–400 mesh) were products of J. T. Baker Chemical Co., Phillipsburg, U.S.A. Both resins were purified as described previously[16]. Crystalline ATP (disodium salt) was purchased from Boehringer and Soehne GmbH, Mannheim, Germany, [$^{14}$C]ATP (disodium salt) from Schwarz Bioresearch, Orangeburg, N.Y., U.S.A. and $^{45}CaCl_2$ from Institute of Nuclear Research, Swierk, Poland. For standardization of EDTA solutions analytical grade calcium carbonate, the product of B.D.H., Laboratory Chemicals Group, Poole, England, was used.

## RESULTS

Preliminary experiments[14] have shown that during incubation of G-actin containing bound $^{45}Ca^{2+}$ with several bivalent cations, the decrease of radioactivity bound to actin took place; the extent depending on the kind of cation. Only for $Ba^{2+}$ no decrease of bound radioactivity was found, and for $Sr^{2+}$ a negligible one. The observed decrease of the amount of initially bound $^{45}Ca$ could be the result either of exchange with the added cation or release due to inactivation of G-actin. In order to check the latter possibility the content of bound nucleotide as well as viscosity of actin in 0.1 M KCl after incubation with bivalent cations were measured. Only during incubation with $Cu^{2+}$, even in the presence of free ATP, the release of the bound ATP and loss of polymerizability of actin were observed. Therefore this cation was not used for further investigations. After incubation with the other cations in the presence of 0.2 mM free ATP the viscosity of actin in 0.1 M KCl, measured after removal of free bivalent cations, indicated the presence of F-actin.

### *The content of bound nucleotide after the reaction of G-actin with bivalent cations*

Fig. 1 shows the results of experiments in which the amount of nucleotide remaining bound to actin was determined after incubation with various bivalent cations at 0.1 mM or 0.5 mM concentration. The experiments were performed both in the absence and in the presence of 0.1 or 1.0 mM free ATP. All the results were related to the amount of nucleotide remaining bound after incubation with $Ca^{2+}$, which was shown to stabilize the G-actin–ATP complex even in the absence of free ATP (ref. 16). In the absence of free ATP a more or less pronounced release of actin-bound nucleotide took place during incubation at the higher concentration of most of the cations studied (0.5 mM). 0.1 mM ATP in some cases and 1.0 mM ATP in all cases prevented any liberation of the bound nucleotide. These results showed that no inactivation of G-actin took place if ATP was present in a sufficiently high concentration.

### *Amount of bivalent cations bound to actin*

The amount of $Ca^{2+}$ in actin preparations remaining bound after short Dowex-50 treatment was found as the average from several experiments to be about 2 moles

per 100000 g of actin*. It can be seen from Table I that the amount of bound $Ca^{2+}$ remained practically the same after equilibration with 0.05–1.0 mM free $Ca^{2+}$ in the presence of various concentrations of ATP. The total amount of bivalent cations

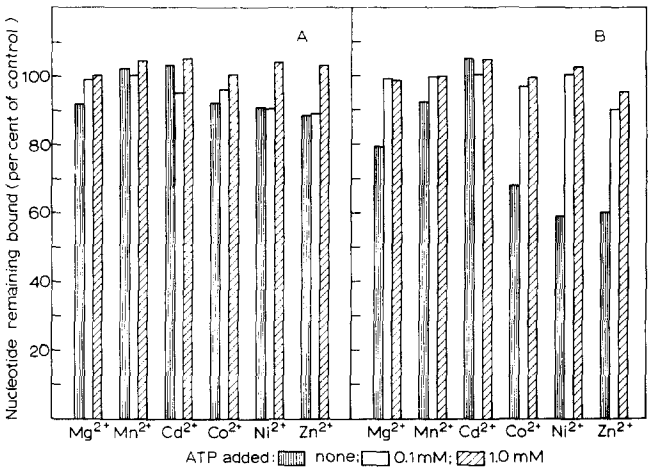


Fig. 1. Effect of bivalent cations on the amount of actin-bound nucleotide. G-actin solutions (about 3 mg protein/ml) in 4 mM Tris–HCl (pH 7.5) containing bound [$^{14}$C]ATP and various concentrations of free [$^{14}$C]ATP of the same specific activity, were treated with Dowex 50 and then incubated for 20 min at room temperature with 0.1 mM (A) or 0.5 mM (B) bivalent cations. The incubation was followed by successive Dowex-50 and Dowex-1 treatments. After centrifugation of the resins, radioactivity and protein concentration in the supernatants were measured. Each bar is the average of the results of two independent experiments.

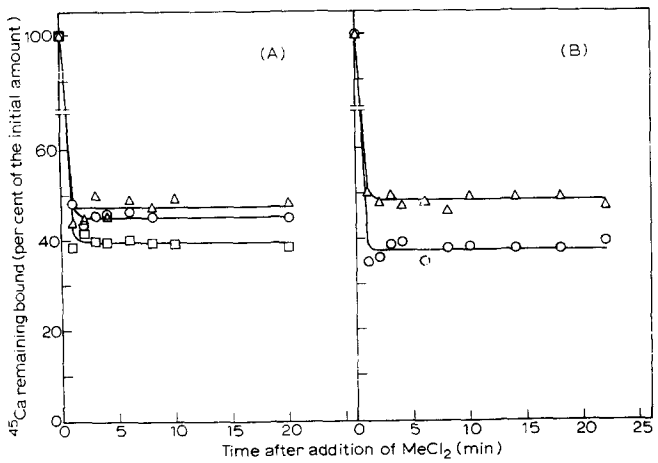


Fig. 2. Rate of exchange of G-actin-bound $Ca^{2+}$ with free $Ca^{2+}$ (A) and free $Mn^{2+}$ (B). G-actin–$^{45}Ca^{2+}$ solutions (about 3 mg protein/ml) in 4 mM Tris–HCl (pH 7.5) were treated successively with Dowex 50 and Dowex 1 to remove free bivalent cations and free nucleotides. After addition of ATP at the concentrations indicated below, $CaCl_2$ or $MnCl_2$ at 0.1 mM final concentration were added at zero time. At the time intervals indicated on the abscissa samples of the solutions were treated with Dowex 50 and after removal of the resin radioactivity was determined. Temperature, 0° throughout the whole experiment. □, no ATP added; △, 0.1 mM ATP added; ○, 1.0 mM ATP added.

* The amount of $Mg^{2+}$ in actin preparations determined with Titan yellow was very small and did not exceeded 0.15 mole per 100000 g.

tightly bound to actin after incubation with free $Mn^{2+}$, $Cd^{2+}$, $Mg^{2+}$ and $Zn^{2+}$ was also approximately the same. The amount bound did not depend on the concentration of free cations, indicating that Dowex 50 applied after incubation removed bivalent cations loosely bound to actin. Besides, the results obtained showed that $Ca^{2+}$ released from its complex with G-actin during incubation was fully replaced by the equivalent amounts of the other cations studied. The same was supposed to be the case also for $Co^{2+}$ and $Ni^{2+}$ in the presence of sufficient concentration of free ATP.

*Rate of exchange of G-actin-bound $Ca^{2+}$ with bivalent cations*

The rate of exchange was examined at various concentrations of free cations from 0.05 to 1.0 mM, and in the presence of several concentrations of free ATP. As an example of the results obtained, the time course of the exchange with free $Ca^{2+}$ and free $Mn^{2+}$ is presented in Fig. 2. In all cases the reaction was found to be very fast; being finished within 1 min even at 0°, both at low concentrations of the added cations, which did not change the physico-chemical state of G-actin, and at the higher concentrations, which, depending on the cation, induced polymerization or even precipitation of actin. It was also found that the rate of reaction did not depend on the concentration of free ATP.

*Extent of exchange of bound $Ca^{2+}$ with free $Ca^{2+}$*

In order to examine the extent of exchange, G-actin solutions containing bound $^{45}Ca^{2+}$ were treated first with Dowex 1 and preincubated for several minutes with ATP added at a required concentration. Subsequently free $^{45}Ca^{2+}$ ions were removed by Dowex-50 treatment. The solutions were then equilibrated with various concentrations of nonradioactive $CaCl_2$ and, after the exchange, treated again with Dowex 50 in order to remove all the free calcium ions*.

Per cent of exchange was defined as the ratio of the determined specific activity of free $Ca^{2+}$ after equilibration $S_F = \frac{a_0 - a_x}{A}$ to the theoretical one $S_T = \frac{a_0}{A + B}$ calculated from the isotopic dilution.

$$\% \text{ exchange} = \frac{S_F}{S_T} = \frac{(a_0 - a_x)(A + B)}{a_0 A} \times 100 \qquad (1)$$

where $a_0$ and $a_x$ are radioactivities bound to actin (in counts/min per mg protein) before and after exchange respectively, A is the concentration of the added free $Ca^{2+}$, and B is the concentration of actin-bound $Ca^{2+}$. The later was taken in accordance with the results from Table I as 1 mole per 50000 g of actin.

The results presented in Table II show that the exchange of G-actin-bound $Ca^{2+}$ with free $Ca^{2+}$ was practically complete over the whole range of the concentrations of this cation studied. The extent of exchange of G-actin-bound $Ca^{2+}$ with free $Ca^{2+}$ did not also depend on free ATP concentration at least up to a concentration of 1.0 mM.

* In such a procedure impurities of $Ca^{2+}$ in ATP preparations might not be taken into account since the addition of ATP was followed by Dowex-50 treatment prior to the exchange with nonradioactive $Ca^{2+}$.

TABLE I

TOTAL AMOUNT OF BIVALENT CATIONS BOUND TO ACTIN AFTER EXCHANGE

G-actin solutions (about 3 mg protein/ml) in 4 mM Tris–HCl (pH 7.5) and 0.08–0.15 mM ATP were treated with Dowex 50 to remove free $Ca^{2+}$ and subsequently incubated for 20 min at room temperature with chlorides of bivalent cations at the concentrations indicated in the table. After the next treatment with Dowex 50 the amounts of cations remaining bound were determined by chelatometric titration as described in MATERIALS AND METHODS.

| *Incubation with* | *Sum of $Me^{2+}$ bound to actin (moles per 100 000 g protein)* | | | | |
|---|---|---|---|---|---|
| *Concn. of free $Me^{2+}$ added (mM)* | *0.05* | *0.1* | *0.2* | *0.5* | *1.0* |
| $CaCl_2$ | 2.32 | 2.17 | — | 1.70 | 1.66 |
| | 1.48 | 1.77 | 1.88 | 1.57 | 1.54 |
| | 1.77 | 1.68 | 2.35 | 2.12 | 2.30 |
| | 1.82 | — | 1.50 | 1.88 | 1.59 |
| $MgCl_2$ | — | 1.55 | — | 1.83 | — |
| | 1.66 | 1.65 | 1.85 | 2.20 | — |
| | 1.87 | 2.00 | 1.98 | 2.04 | 1.78 |
| | 1.82 | 1.84 | 1.77 | 1.57 | 1.82 |
| $MnCl_2$ | — | 1.92 | — | 2.20 | — |
| | 1.46 | 1.51 | 1.67 | — | 1.67 |
| | — | 1.46 | — | 2.20 | 2.32 |
| | 1.90 | 1.67 | — | 2.00 | 1.80 |
| | 2.17 | 2.07 | 2.05 | 2.15 | 1.99 |
| | 2.17 | 2.20 | 1.95 | 2.10 | — |
| $ZnCl_2$ | 2.14 | 2.22 | 2.25 | 2.27 | 2.18 |
| | 1.59 | 1.87 | 1.87 | 1.99 | 2.22 |
| $CdCl_2$ | — | — | — | 1.52 | — |
| | 1.81 | 1.47 | 1.44 | 1.78 | — |
| | 1.51 | 1.38 | — | 1.67 | 1.94 |
| | 2.00 | 1.90 | 1.90 | 1.84 | — |
| | 1.84 | 1.53 | 1.68 | 1.74 | 1.85 |
| Average | 1.84 | 1.78 | 1.86 | 1.92 | 1.90 |

*Replacement of actin-bound $Ca^{2+}$ by other bivalent cations*

In this type of experiment G-actin–$^{45}Ca^{2+}$, after removal of free $Ca^{2+}$ by treatment with Dowex 50, was incubated with various bivalent cations. After that the solutions were treated again with Dowex 50 and the radioactivity remaining bound was determined.

The extent of exchange of bound $Ca^{2+}$ by other bivalent cations depends on the relative affinity of the particular cation to actin. In order to distinguish from the true exchange with free $Ca^{2+}$ the term 'replacement' will be used throughout this work when other cations were investigated. Consequently, for the calculation of the extent of the replacement of actin-bound $Ca^{2+}$ by other bivalent cations instead of Eqn. 1 the following one was used

$$\% \text{ replacement} = \frac{a_0 - a_x}{a_0} \times 100 \qquad (2)$$

where $a_0$ and $a_x$ were radioactivities bound to actin before and after incubation with bivalent cations.

TABLE II

EXTENT OF EXCHANGE OF G-ACTIN-BOUND $Ca^{2+}$ WITH FREE $Ca^{2+}$

G-actin-$^{45}Ca^{2+}$ solutions (about 3 mg protein/ml) in 4 mM Tris-HCl (pH 7.5) containing free ATP at the concentrations indicated, were treated with Dowex 50 and then incubated for 20 min at room temperature with $CaCl_2$ at the concentrations indicated in the table. After the next Dowex-50 treatment protein concentration and radioactivity remaining bound were measured. For calculation of per cent exchange see text.

| *Expt. No.* | *Free ATP (mM)* | *Final concn. of the added $CaCl_2$ (mM)* | *Per cent exchange of G-actin-bound $Ca^{2+}$ with free $Ca^{2+}$* | | | | |
|---|---|---|---|---|---|---|---|
| | | | *0.05* | *0.1* | *0.2* | *0.5* | *1.0* |
| 6 | 0 | | — | 106.2 | — | 99.0 | — |
| 8 | 0 | | — | — | — | 101.2 | — |
| 10 | 0 | | — | — | — | 98.2 | — |
| 7 | 0.08 | | 94.0 | 97.0 | 99.5 | 97.4 | 96.2 |
| 9 | 0.1 | | 106.5 | 106.0 | 102.2 | 98.1 | 90.2 |
| 6 | 0.1 | | — | 104.0 | — | 97.3 | — |
| 8 | 0.1 | | — | 103.6 | — | 98.9 | — |
| 10 | 0.1 | | — | — | — | 97.6 | — |
| 2 | 0.1 | | — | — | — | 97.3 | — |
| 4 | 0.1 | | — | 106.2 | — | — | — |
| 1 | 0.15 | | 108.8 | 104.2 | 104.0 | 94.2 | 89.2 |
| 3 | 0.15 | | 105.0 | 102.8 | 100.1 | 97.7 | 96.1 |
| 5 | 0.15 | | 101.0 | 100.0 | 99.0 | 97.1 | 96.8 |
| 6 | 1.0 | | — | 103.2 | — | 96.5 | — |
| 8 | 1.0 | | — | — | — | 98.6 | — |
| 10 | 1.0 | | — | — | — | 97.3 | — |
| 2 | 1.0 | | — | — | — | 92.4 | — |
| 4 | 1.0 | | — | 106.0 | — | — | — |
| 11 | 1.0 | | 97.0 | 100.2 | 99.2 | 92.4 | 82.6 |
| 12 | 1.0 | | 101.2 | 101.0 | 98.2 | 88.5 | 80.7 |
| Average | | | 101.9 | 103.0 | 100.3 | 96.5 | 90.3 |

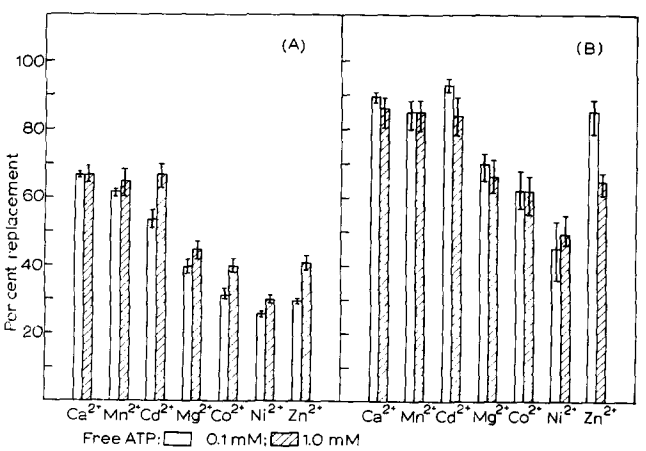


Fig. 3. Influence of free ATP on the extent of replacement of G-actin-bound $Ca^{2+}$ with bivalent cations. For procedure see legend to Table II. The cations were used at concentration of 0.1 mM (A) or 0.5 mM (B). Calculation of per cent replacement as described in text. The means of the results of 3–7 experiments ± S.D. are given.

Fig. 3 shows the results of experiments in which the extent of replacement of bound $Ca^{2+}$ by 0.1 and 0.5 mM bivalent cations was determined at two different concentrations of free ATP, *i.e.*, 0.1 and 1.0 mM. For comparison, the results for the exchange with free $Ca^{2+}$ calculated according to Eqn. 2 are also presented. One can see that the extent of replacement depended on the kind of cation. $Mn^{2+}$ and $Cd^{2+}$ replaced the bound $Ca^{2+}$ to about the same extent as free $Ca^{2+}$, whereas the replacement by $Mg^{2+}$, $Co^{2+}$, $Ni^{2+}$ and $Zn^{2+}$ was much smaller. The amount replaced during incubation with 0.1 mM bivalent cations other than $Ca^{2+}$ was somewhat enhanced in the presence of 1 mM free ATP. At higher, 0.5 mM, concentration of the cations no significant influence of free ATP concentration was observed except for $Zn^{2+}$ and to a smaller extent for $Cd^{2+}$. In the case of the two latter cations the per cent of replacement was found higher in the presence of 0.1 mM ATP, probably due to some inactivation of actin.

Fig. 4 shows that the per cent of replacement of bound $Ca^{2+}$ increased with the increase of concentration of the free cation up to about 0.5 mM. In the presence of 1 mM ATP the increase was much more regular. At higher, 1.0 mM, concentration of all cations except $Zn^{2+}$ and $Cd^{2+}$ some lowering of the replacement was observed, probably as a result of fast polymerization or precipitation of G-actin under these conditions. In the experiments performed at 0°, when the polymerization proceeded much more slowly than at room temperature, this small decrease of the extent of exchange was not observed. Since F-actin-bound $Ca^{2+}$ is essentially non-exchangeable[7], the rate of polymerization of G-actin is an important factor determining the extent of replacement of bound $Ca^{2+}$ by free cations. However, even at room temperature the replacement by all bivalent cations studied, essentially preceded the formation of F-actin and the observed decrease at 1.0 mM concentration of bivalent cations was rather small.

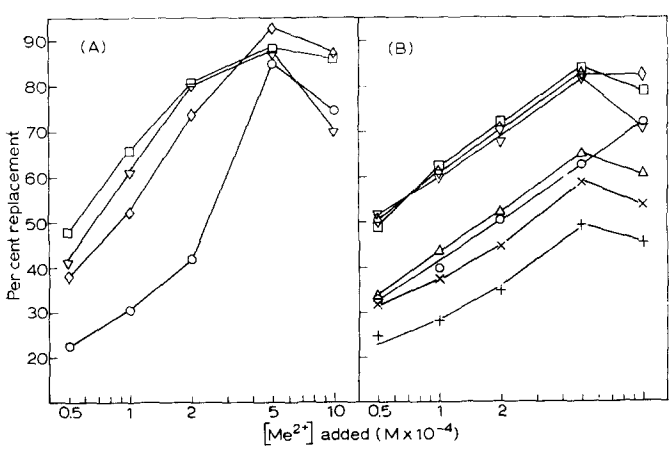


Fig. 4. Effect of concentration of bivalent cations on replacement of G-actin-bound $Ca^{2+}$. A, 0.1 mM free ATP; B, 1.0 mM free ATP present. G-actin-$^{45}Ca^{2+}$ solutions (3.1 mg in the series A and 2.9 mg/ml in the series B) in 4 mM Tris–HCl (pH 7.5) were treated with Dowex 50 and than incubated for 20 min at room temperature with bivalent cations at the concentrations indicated on the abscissa. After the next Dowex-50 treatment protein concentration and radioactivity remaining bound were measured. For calculation of per cent of replacement of bound calcium see text. □, $Ca^{2+}$; ▽, $Mn^{2+}$; ◇, $Cd^{2+}$; ○, $Zn^{2+}$; △, $Mg^{2+}$; ×, $Co^{2+}$; +, $Ni^{2+}$.

*Effect of ATP on the polymerization of actin induced by bivalent cations*

The observed effect of free ATP on the extent of the replacement of G-actin-bound $Ca^{2+}$ by bivalent cations seems to be related to the influence of ATP on the changes in the physico-chemical state of actin induced by these cations.

Bivalent cations differ from each other in their effect on G-actin. Among the cations studied $Cd^{2+}$, $Zn^{2+}$ and $Ni^{2+}$ have the highest tendency to induce polymerization and, at higher concentrations, precipitation of actin. In the presence of 0.1–0.2 mM free ATP, at pH 7.5, maximum viscosity was reached at 0.7–0.8 mM concentration of $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$ or $Co^{2+}$. The same value of viscosity was reached at as low as 0.2–0.3 mM concentration of $Cd^{2+}$ and 0.4–0.5 mM of $Zn^{2+}$ or $Ni^{2+}$ and

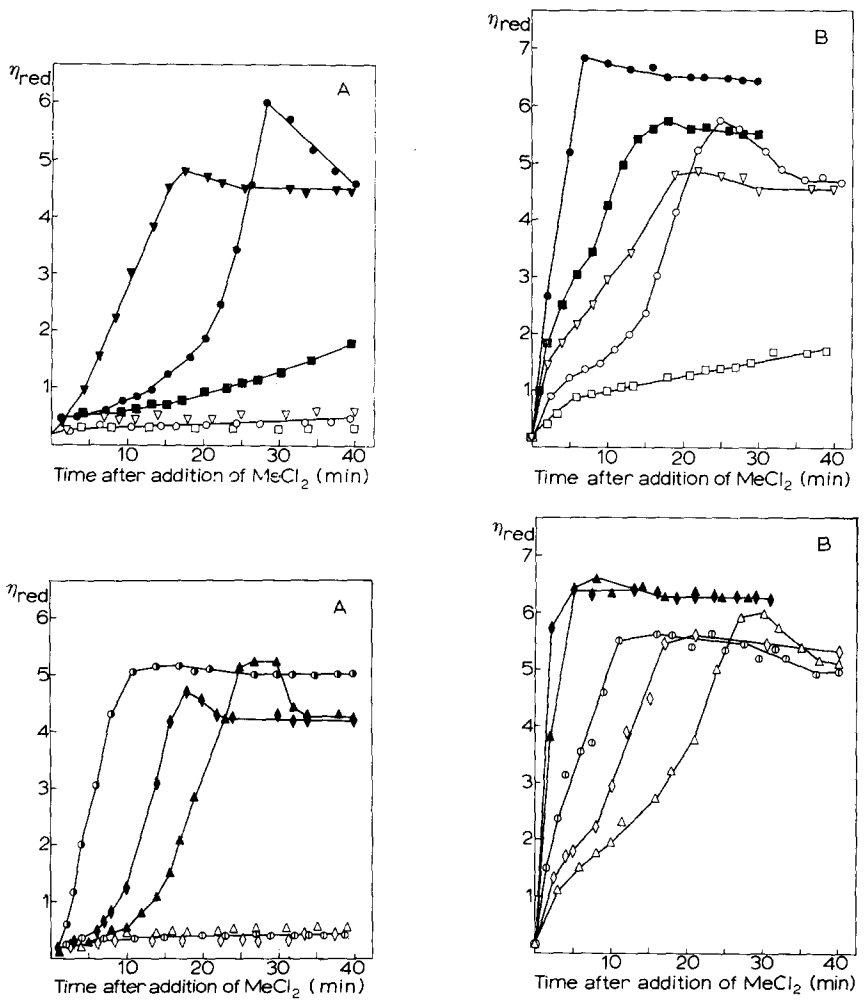


Fig. 5. Effect of free ATP on the polymerization of G-actin induced by bivalent cations. To G-actin solution (3.3 mg protein/ml) in 4 mM Tris–HCl (pH 7.5) treated with Dowex 1, ATP (0.1 mM, full symbols or, 1.0 mM, empty symbols) was added. After 20 min the solutions were treated with Dowex 50; bivalent cations at 0.5 mM final concentration in (A) and 1 mM in (B) were added at zero time and viscosity was measured at 21°. □, ■ – $CaCl_2$; ○, ● – $MgCl_2$; ▽, ▼ – $NiCl_2$; △, ▲ – $MnCl_2$; ◇, ◆ – $CoCl_2$; ⓘ, ◑ – $ZnCl_2$.

an increase of the concentration of the three latter cations caused first the appearance of turbidity and, subsequently, at the concentration of 0.8–1.0 mM full precipitation of actin. When, however, 1.0 mM ATP was present actin did not polymerize at least up to 0.5 mM concentration of all the cations studied (Fig. 5A). Also the precipitation of actin induced by 1 mM $Cd^{2+}$, $Zn^{2+}$ and $Ni^{2+}$ was completely prevented by increasing the concentration of free ATP to 1.0 mM; instead, under these conditions polymerization took place (Fig. 5B).

*Relative affinities of bivalent cations to actin*

In order to gain a better insight into the relationship between the bivalent cations studied and their ability to replace actin-bound $Ca^{2+}$ an attempt was made to calculate the affinity of particular bivalent cations to actin in relation to the affinity of $Ca^{2+}$. These relative affinities can be expressed by the formula:

$$\frac{K_{Me}}{K_{Ca}} = \frac{[Ca^{2+}]\cdot[\text{actin-}Me^{2+}]}{[Me^{2+}]\cdot[\text{actin-}Ca^{2+}]} \tag{3}$$

where $K_{Me}/K_{Ca}$ is the ratio of the respective affinity constants, $[Ca^{2+}]$ and $[\text{actin-}Ca^{2+}]$ are the concentrations of free and actin-bound $Ca^{2+}$, whereas $[Me^{2+}]$ and $[\text{actin-}Me^{2+}]$ are the concentrations of the investigated cation, free and bound, respectively.

On the basis of the results presented above the total amount of bivalent cations bound to actin after reaction was accepted to be equal to the amount of initially bound $Ca^{2+}$. The incubation of G-actin with the added $Me^{2+}$ was in our experiments always preceded by the removal of free $Ca^{2+}$ from the solution. Thus, the concentration of free $Ca^{2+}$ after equilibration was equal to the concentration of $Me^{2+}$ bound by actin and they both were given by the difference between the concentration of actin-bound $Ca^{2+}$ before and after reaction:

$$[Ca^{2+}] = [\text{actin-}Me^{2+}] = [\text{actin-}Ca_0^{2+}] - [\text{actin-}Ca^{2+}] \tag{4}$$

where $[\text{actin-}Ca_0^{2+}]$ represents the concentration of $Ca^{2+}$ initially bound to actin.

The concentration of free $Me^{2+}$ after replacement was equal to the difference between the known concentration of the added $Me^{2+}$, $[S_{Me}]$, and the concentration of actin-bound $Me^{2+}$ after reaction, $[\text{actin-}Me^{2+}]$.

$$[Me^{2+}] = [S_{Me}] - [\text{actin-}Me^{2+}] = [S_{Me}] - [\text{actin-}Ca_0^{2+}] + [\text{actin-}Ca^{2+}] \tag{5}$$

Consequently:

$$\frac{K_{Me}}{K_{Ca}} = \frac{([\text{actin-}Ca_0^{2+}] - [\text{actin-}Ca^{2+}])^2}{([S_{Me}] - [\text{actin-}Ca_0^{2+}] + [\text{actin-}Ca^{2+}])\cdot[\text{actin-}Ca^{2+}]} \tag{6}$$

Since in the experiments G-actin containing bound $^{45}Ca^{2+}$ was used, the term $[\text{actin-}Ca^{2+}]$ was obtained from the proportion:

$$\frac{[\text{actin-}Ca^{2+}]}{[\text{actin-}Ca_0^{2+}]} = \frac{a_{Me}}{a_0} \tag{7}$$

where $a_0$ and $a_{Me}$ are radioactivities of actin-bound $^{45}Ca^{2+}$ before and after equilibration with the added $Me^{2+}$, respectively, in counts/min per mg protein.

The concentration of initially bound $^{45}Ca^{2+}$, $[\text{actin-}Ca_0^{2+}]$, was determined in every experiment in the control samples on the basis of its exchange with free non-

radioactive $Ca^{2+}$ assuming 100% exchange (*cf.* Table II). Thus, [actin-$Ca_0^{2+}$] was obtained from the following proportion:

$$\frac{[\text{actin-Ca}_0^{2+}]}{[\text{actin-Ca}_0^{2+}] + [S_{Ca}]} = \frac{a_{Ca}}{a_0} \tag{8}$$

where $[S_{Ca}]$ denotes the known concentration of the free $Ca^{2+}$ added and $a_{Ca}$ is radioactivity remaining bound after exchange with free $Ca^{2+}$.

Substituting [actin-$Ca^{2+}$] and [actin-$Ca_0^{2+}$] defined by the Eqns. 7 and 8, respectively, into Eqn. 6 one obtains the final equation for the relative affinity constants expressed by radioactivities of actin-bound $^{45}Ca^{2+}$ before, $a_0$, and after reaction with $Me^{2+}$, $a_{Me}$, or with $Ca^{2+}$, $a_{Ca}$.

In all experiments free $Me^{2+}$ and free $Ca^{2+}$ were always used at the same concentrations. This allows the elimination of both $[S_{Me}]$ and $[S_{Ca}]$ from the final equation:

$$\frac{K_{Me}}{K_{Ca}} = \frac{X(1 - Y)^2}{Y(1 - 2X + XY)} \tag{9}$$

where $X = a_{Ca}/a_0$ and $Y = a_{Me}/a_0$.

Taking into account the results of the experiments presented in the preceding sections for the calculation of the relative affinity constants of bivalent cations to actin, only the results of the reaction with 0.05–0.5 mM free bivalent cations in the presence of 1.0 mM free ATP were used. Under these conditions actin was well protected from inactivation, its polymerization was inhibited, and the extent of the replacement was proportional to the concentration of free cation.

As can be seen from Table III, the affinity constants of $Cd^{2+}$ and $Mn^{2+}$ to actin

TABLE III

RELATIVE AFFINITIES OF BIVALENT CATIONS TO G-ACTIN

For calculation of the relative affinity constants see text.

| *Expt. No.* | *Concn. of the added cation (mM)* | $K_{Mn}/K_{Ca}$ | $K_{Cd}/K_{Ca}$ | $K_{Mg}/K_{Ca}$ | $K_{Zn}/K_{Ca}$ | $K_{Co}/K_{Ca}$ | $K_{Ni}/K_{Ca}$ |
|---|---|---|---|---|---|---|---|
| 11 | 0.05 | — | 1.04 | 0.39 | 0.24 | 0.19 | 0.09 |
| 12 | 0.05 | 1.28 | 1.06 | 0.29 | 0.29 | 0.23 | 0.13 |
| 4 | 0.1 | 1.12 | 1.12 | 0.19 | — | 0.16 | — |
| 6 | 0.1 | 1.09 | 1.31 | 0.26 | 0.23 | 0.19 | 0.08 |
| 8 | 0.1 | 0.91 | 1.10 | 0.22 | 0.15 | 0.14 | 0.07 |
| 11 | 0.1 | 1.01 | 1.02 | 0.28 | 0.19 | 0.19 | 0.09 |
| 12 | 0.1 | 0.69 | 0.83 | 0.23 | 0.17 | 0.15 | 0.07 |
| 11 | 0.2 | 0.84 | 0.87 | — | 0.17 | 0.16 | 0.07 |
| 12 | 0.2 | 0.62 | 0.77 | 0.21 | 0.21 | 0.14 | 0.07 |
| 2 | 0.5 | 0.88 | 0.76 | 0.38 | 0.28 | — | — |
| 6 | 0.5 | 0.88 | 1.24 | 0.20 | 0.20 | 0.17 | 0.07 |
| 8 | 0.5 | 1.06 | 1.39 | 0.23 | 0.17 | 0.16 | 0.07 |
| 10 | 0.5 | 0.95 | 1.10 | — | — | 0.18 | 0.06 |
| 11 | 0.5 | 0.67 | 0.76 | 0.28 | 0.22 | 0.20 | 0.09 |
| 12 | 0.5 | 0.58 | 0.84 | 0.28 | 0.27 | 0.18 | 0.10 |
| Average: | | 0.90 | 1.07 | 0.27 | 0.22 | 0.18 | 0.08 |

are similar to that of $Ca^{2+}$ while the affinities of other bivalent cations are much lower and follow the order:

$$Mg^{2+} < Zn^{2+} < Co^{2+} < Ni^{2+}$$

The affinity of $Mg^{2+}$ appears to be 4 times, those of $Zn^{2+}$ and $Co^{2+}$ 5–6 times and that of $Ni^{2+}$ about 12 times lower than that of $Ca^{2+}$.

## DISCUSSION

The amount of $Ca^{2+}$ tightly bound to G-actin, *i.e.*, not removable by short-time treatment with Dowex 50, has been found in this work to correspond to about 2.0 moles per 100000 g of actin. This value does not depend on the concentration of free $Ca^{2+}$ in the initial mixture, indicating that all $Ca^{2+}$ losely bound to actin[20] is completely removed by the resin. This amount of bound $Ca^{2+}$ corresponds to about 1.2 moles per 60000 g of actin, usually accepted as the molecular weight of actin monomer[1]. Similarly, the content of the bound $Ca^{2+}$ reported previously[5–9], was also somewhat above 1.0 mole when calculated per 60000 g. There have recently appeared some reports (refs. 21–23; K. TSUBOI, personal communication) presenting more or less direct evidence that the molecular weight of G-actin is 45000–48000. Taking into account the latter value the amount of bound $Ca^{2+}$ found in this work would correspond very closely to 1 mole per mole of actin monomer.

BÁRÁNY and coworkers[7,12] have shown that $Ca^{2+}$ bound to G-actin exchanges completely with free $Ca^{2+}$, whereas $Ca^{2+}$ bound to F-actin is nonexchangeable. The results of this work are in accordance with that finding. The exchange with free $Ca^{2+}$ is complete not only at low concentrations of this cation, as was already shown by BÁRÁNY, FINKELMAN AND THORATTIL-ANTONY[7], but also at its higher concentrations, causing partial or even full polymerization of G-actin. The extent of exchange does not depend on the concentration of free ATP, at least up to 1 mM.

Present studies have extended our previous observations[14,15] which showed that actin-bound $Ca^{2+}$ could be replaced by various bivalent cations. After reaction with all the cations studied, with the exception of $Cu^{2+}$, actin remains active, *i.e.*, it contains bound nucleotide and preserves the ability to polymerize.

The extent of the replacement of the actin-bound $Ca^{2+}$ depends substantially on the bivalent cation involved. $Ca^{2+}$, $Mn^{2+}$ and $Cd^{2+}$ form one group; the second, cations replacing bound $Ca^{2+}$ to a much smaller extent; and the third, such cations as $Ba^{2+}$ and $Sr^{2+}$, which are not able to replace bound $Ca^{2+}$. Recently DOLP[24], studying properties of bullfrog actin, also found no exchange with $Ba^{2+}$, but quite unexpectedly, approximately the same exchange with free $Ca^{2+}$, $Mn^{2+}$ and $Mg^{2+}$; this exchange, however, was only partial.

For cations other than $Ca^{2+}$ the replacement is slightly influenced by free ATP (*cf.* RESULTS). The effect of this compound, however, seems to depend not only on the kind of bivalent cation but also on the mutual ratios of the concentration of the latter and those of actin and ATP. In this work for all calculations the total concentrations of added bivalent cations were used. In fact, however, part of them was in the form of the complex with ATP (for review see ref. 25). Taking into account the stability constants of the ATP–metal complexes on one hand and a relatively small effect of free ATP on the extent of replacement on the other, one might assume

that not only free, ionized, bivalent cations can replace bound $Ca^{2+}$ but also those in the form of the complexes with ATP. The comparison of the rate of replacement at different ATP concentration was expected to help in the elucidation of this question. However, we have not been able to detect any differences in the rate of replacement among the cations studied, nor were we able to observe the possible effect of free ATP on this process. On the other hand, these results cannot be conclusive since the reaction was very fast and it was too difficult to follow it in sufficiently short time intervals.

Another cause for the decrease in the concentration of free cations might be their additional binding to the actin molecule[20]. It is usually accepted that alkaline-earth metals, especially calcium, are bound primarily to the carboxyl side chains of proteins[26,27]. In contrast $Zn^{2+}$, $Ni^{2+}$, $Co^{2+}$ and $Cd^{2+}$ with the relatively high affinities to ligands in which nitrogen is the donor atom sharing electron pairs through the vacant orbitals of the metal ion[26–28] might be bound to proteins preferentially at other sites than alkaline-earth metals. There is no information, however, so far, about the additional, nonspecific binding of these cations, namely $Zn^{2+}$, $Ni^{2+}$, $Co^{2+}$ and $Cd^{2+}$, to the actin molecule. If, however, the same cations have the highest tendency to induce polymerization and precipitation of actin (the phenomena regarded as the result of a stepwise decreasing of a net negative charge of the molecule by the bound cations[29,30]), one may assume that the affinity of these cations to the additional binding sites in G-actin molecule is much higher. Consequently, one cannot exclude that under certain conditions the additional binding of some cations to actin, may interfere with their ability to replace the G-actin-bound $Ca^{2+}$ by decreasing the concentration of free cations. It is also possible that ATP may to some extent inhibit the additional binding to actin by formation of the complexes with the bivalent cations. This possibility is consistent with the fact that ATP at higher concentration inhibits the polymerization (*cf.* Fig. 5) and the precipitation of actin.

There is no data concerning the extent and strength of the additional binding of most of cations to actin and the data concerning the affinity constants of the ATP–metal complexes differ considerably depending on the author and method used[25]. Hence, it seems rather too difficult at present to calculate for the given concentration of actin, ATP and bivalent cation which part of the cation remains free, how much is additionally bound to actin and how much is in the form of the complex with ATP. Therefore, it was decided in the present work to use for the calculation of the relative affinities of tightly bound cations the total concentration of the added cations. The results show that the order of the relative affinities of bivalent cations to G-actin calculated in this way, does not parallel the order of affinities of these cations either to the carboxyl group of proteins[28], or to ATP[25]. The former could be taken into account on the basis of the postulated binding of $Ca^{2+}$ to actin carboxyl group[7,11]; the latter, in view of the recently suggested participation of $Ca^{2+}$ in the binding of ATP by G-actin[7,10,16]. Instead, the results of the present work fully support our previous observations[15] that there is a direct correlation between the ionic radius of the particular cations and their ability to replace G-actin-bound $Ca^{2+}$. Thus, $Ba^{2+}$ and $Sr^{2+}$, whose ionic radius is much greater than that of $Ca^{2+}$, are practically not able to replace the latter. $Cd^{2+}$ and $Mn^{2+}$, exchanging completely with the bound $Ca^{2+}$, have ionic radii fairly close to that of $Ca^{2+}$, whereas other investigated cations, with less ability to replace G-actin-bound $Ca^{2+}$, have a smaller ionic radius than $Ca^{2+}$.

Such a correlation suggests that the configuration of the binding site in the actin molecule could be one of the main factors determining the ability of cations to replace the bound $Ca^{2+}$.

STROHMAN AND SAMORODIN[10] have shown that free $Ca^{2+}$ but no $Mg^{2+}$ inhibits the rate of exchange of actin-bound ATP with the free nucleotide, and have suggested that $Ca^{2+}$ forms a bridge between the actin moiety and ATP through all three phosphate groups, while $Mg^{2+}$ combines with only two phosphate groups of ATP. This scheme would explain the lower affinity of $Mg^{2+}$ to actin. However, it was shown recently that both $Mg^{2+}$ and $Ca^{2+}$ bind in their complexes with ATP with only $\beta$- and $\gamma$-phosphate groups of ATP[25]. On the other hand, it has been suggested[31] that the complex formation of metals with nucleotides could lead to a considerable conformational change in the phosphate chain of the nucleotide. The chain could be expected to remain more or less linear in the case of binding of larger cations, and to fold in the case of binding of smaller ones. Hence, one may assume that the conformation of the phosphate chain of ATP in its complexes with various bivalent cations is also one of the factors influencing the ability of the cations to replace G-actin-bound $Ca^{2+}$.

Recently IYENGAR AND WEBER[4] have extensively studied the ability of G-actin-bound ATP to exchange with other nucleotides. Contrary to nucleotides, among which none has an affinity similar to that of ATP, some bivalent cations, as $Cd^{2+}$ and $Mn^{2+}$, appear to have an affinity to actin very close to that of $Ca^{2+}$. In spite of this, actin preparations containing metals other than $Ca^{2+}$ seem to be less stable than $Ca^{2+}$-actin[32]. Hence, all the results available so far indicate that the 'physiological' components of G-actin, namely ATP and $Ca^{2+}$, favour the best and most stable configuration of this protein.

NOTE ADDED IN PROOF (Received September 30th, 1968)

After this manuscript was submitted an article[33] appeared in which the authors confirmed our results reported previously[14] and extended the investigations to F-actin. On the basis of their own results as well as ours they come to the same conclusion that there is a close relationship between the ionic radius and the ability of particular cation to replace actin-bound $Ca^{2+}$.

ACKNOWLEDGEMENTS

The authors wish to thank Professor W. NIEMIERKO for his interest during the course of this work, and Dr. J. GERGELY for many valuable discussions during preparation of the manuscript. The chemical determination of the content of bivalent cations was carried out with the participation of Mrs. S. BARYLKO. The technical assistance of Mrs. U. KOPERSKA is gratefully appreciated. This investigation was supported in part by the Foreign Research Agreement No. 6x7715 of National Institutes of Health under P.L.480.

REFERENCES

1 S. SEIFTER AND P. M. GALLOP, in H. NEURATH, *The Proteins*, Vol. IV, Academic Press, New York and London, 2nd Ed., 1966, p. 155.
2 A. MARTONOSI, M. A. GOUVEA AND J. GERGELY, *J. Biol. Chem.*, 235 (1960) 1700.

3 A. MARTONOSI, *Biochim. Biophys. Acta*, 57 (1962) 163.
4 M. R. IYENGAR AND H. H. WEBER, *Biochim. Biophys. Acta*, 86 (1964) 543.
5 A. CHRAMBACH, M. BÁRÁNY AND F. FINKELMAN, *Arch. Biochem. Biophys.*, 93 (1961) 456.
6 K. MARUYAMA AND J. GERGELY, *Biochem. Biophys. Res. Commun.*, 6 (1961) 245.
7 M. BÁRÁNY, F. FINKELMAN AND T. THERATTIL-ANTONY, *Arch. Biochem. Biophys.*, 98 (1962) 28.
8 K. MARUYAMA, *Sci. Papers Coll. Gen. Educ. Univ. Tokyo*, 12 (1962) 73.
9 Y. TONOMURA AND J. YOSHIMURA, *J. Biochem. Tokyo*, 51 (1962) 259.
10 R. C. STROHMAN AND A. J. SAMORODIN, *J. Biol. Chem.*, 237 (1962) 363.
11 A. M. KATZ, *Biochim. Biophys. Acta*, 71 (1963) 397.
12 M. BÁRÁNY AND A. CHRAMBACH, *J. Gen. Physiol.*, 45 (1962) 589A.
13 F. OOSAWA, S. ASAKURA, H. ASAI, M. KASAI, S. KOBAYASHI, K. MIHASHI, T. OOI, M. TANIGUCHI AND E. NAKANO, in J. GERGELY, *Biochemistry of Muscle Contraction*, Little, Brown and Co., Boston, 1964, p. 158.
14 W. DRABIKOWSKI AND H. STRZELECKA-GOŁASZEWSKA, *Biochim. Biophys. Acta*, 71 (1963) 486.
15 H. STRZELECKA-GOŁASZEWSKA, B. BARYŁKO AND W. DRABIKOWSKI, *Abstr. 4th Meeting Federation European Biochem. Soc., Oslo 1967*, p. 83.
16 H. STRZELECKA-GOŁASZEWSKA AND W. DRABIKOWSKI, *Acta Biochim. Polon.*, 14 (1967) 195.
17 G. P. HILDEBRAND AND C. N. REILLEY, *Anal. Chem.*, 29 (1957) 258.
18 H. A. FLASHKA, *EDTA Titrations*, Pergamon Press, Oxford, 2nd Ed., 1964, p. 125.
19 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
20 A. MARTONOSI, C. M. MOLINO AND J. GERGELY, *J. Biol. Chem.*, 239 (1964) 1057.
21 R. S. ADELSTEIN, J. S. GODFREY AND W. W. KIELLEY, *Biochem. Biophys. Res. Commun.*, 12 (1963) 34.
22 P. JOHNSON, C. I. HARRIS AND S. V. PERRY, *Biochem. J.*, 105 (1967) 361.
23 M. K. REES AND M. YOUNG, *J. Biol. Chem.*, 242 (1967) 4449.
24 R. N. DOLP, *Arch. Biochem. Biophys.*, 113 (1966) 20.
25 R. PHILLIPS, *Chem. Rev.*, 66 (1966) 501.
26 R. J. P. WILLIAMS, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. I, Academic Press, New York, 1959, p. 391.
27 J. STEINHARDT AND S. BEYCHOK, in H. NEURATH, *The Proteins*, Vol. II, Academic Press, New York and London, 2nd Ed., 1964, p. 139.
28 F. R. GURD AND P. E. WILCOX, in N. L. ANSON, K. BAILEY AND J. T. EDSALL, *Advances in Protein Chemistry*, Vol. XI, Academic Press, New York, 1956, p. 311.
29 W. F. H. M. MOMMAERTS, *J. Biol. Chem.*, 198 (1952) 459.
30 M. BÁRÁNY, N. A. BIRÓ AND J. MOLNÁR, *Acta Physiol. Acad. Sci. Hung.*, 5 (1954) 63.
31 N. C. MELCHIOR, *J. Biol. Chem.*, 208 (1954) 615.
32 W. DRABIKOWSKI AND H. STRZELECKA-GOŁASZEWSKA, *Abstr. VI Intern. Congr. Biochem. 6th, New York, 1964*, p. 649.
33 M. KASAI AND F. OSAWA, *Biochim. Biophys. Acta*, 154 (1968) 520.