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PARTIALLY POLYMERIZED ERYTHROCYTE ACTIN OBTAINED BY AFFINITY CHROMATOGRAPHY ON DNAse SEPHAROSE. COMPARISON WITH RABBIT SKELETAL MUSCLE ACTIN AND ROLE OF CALCIUM

Didier Dhermy, Odile Bournier and Pierre Boivin

Laboratoire d'Enzymologie des cellules sanguines,
INSERM U.160, CNRS ERA 573, Hôpital Beaujon,
92118 Clichy Cédex, France

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SUMMARY : A new procedure, consisting of affinity chromatography on DNAse sepharose, is worked out for the purification of human erythrocyte actin from an extract of acetone powder. Comparison of skeletal muscle and erythrocyte actin purified either by reversible polymerization or affinity chromatography on DNAse Sepharose led us to infer that the erythrocyte actin isolated by affinity chromatography was pure, devoid of spectrin, and was obtained in part under polymerized (di and tetrameric) forms. This partial polymerization is related to a loss of calcium bound to actin.

INTRODUCTION : Actin is a protein found in most eucaryotic cells, including erythrocyte ghosts (1, 2). All actins, both muscle and non muscle, polymerize at an appropriate ionic strengh to form F-actin and native actin can be recovered in very high purity from many sources by this procedure. However, the erythrocyte actin obtained by polymerization-depolymerization (3) is contaminated by spectrin.

We have previously described that, like muscle actin, erythrocyte actin inhibits the activity of deoxyribonuclease I (DNAse I) (4). In this paper, we propose affinity chromatography on DNAse sepharose as a new procedure of erythrocyte actin purification. This erythrocyte actin obtained by affinity chromatography is pure but partially polymerized because of loss of calcium bound to actin.

MATERIALS AND METHODS

Human erythrocytes are obtained from phlebotomies (treatment of polycythemia vera). CNBr activated Sepharose 4B is purchased from Pharmacia Fine Chemicals. Deoxyribonuclease I from bovine pancreas is obtained from Sigma Chemical Company. Guanidine hydro-chloride (for biochemistry) is purchased from Merck.

Purification of erythrocyte actin by reversible polymerization

Actin was prepared from acetone powder of red cell ghosts as described by Sheetz (3) including one cycle of polymerization-depolymerization.

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The acetone powder (2 g) was extracted twice with 40 ml of buffer A ($2x10^{-3}$M Tris HCl, $2x10^{-4}$M ATP, $5x10^{-4}$M βmercaptoethanol, $2x10^{-4}$M $CaCl_2$, pH 8.0) at 4°C for 30 minutes. These crude extracts were pooled and concentrated by vacuum dialysis to 1 mg/ml protein.

KCl (to $5x10^{-2}$M) and $MgCl_2$ (to $2x10^{-3}$M) were added and the actin was allowed to polymerize for 2 hours at room temperature and subsequently at 4°C for 20 hours. F-actin was pelleted by centrifugation at 105 000 g for 3 hours. After removing the supernatant, the pellet was washed with 5 ml of buffer A and centrifuged at 105 000 g for 3 hours. The final pellet was resuspended in 3 ml of buffer A and dialyzed against this buffer A for 3 days, changing to fresh buffer every 24 hours. The G-actin was clarified by centrifugation at 105 000 g for 3 hours.

Purification of rabbit skeletal muscle actin

This muscle actin was purified according to Spudich and Watt (5) with two buffer systems for extraction and depolymerization. One buffer (A_1) contained 2 mM Tris HCl pH 8.0, 0.2 mM ATP, 0.5 mM βmercaptoethanol and 0.2 mM $CaCl_2$; the other (A_2) had the same composition except for the absence of $CaCl_2$.

Preparation of DNAse sepharose

CNBr-activated sepharose 4B (3g) was swollen with 10^{-3}M HCl solution (75 ml) and washed three time with the same volume of HCl solution.

DNAse I (30 mg), dissolved in 15 ml of 0.1 M $NaHCO_3$, 0.5 M NaCl, was mixed with the activated sepharose and the mixture was rotated end over end for overnight at 4°C. The newly made DNAse-sepharose was washed three times with coupling buffer and any remaining active groups were reacted with 1M ethanolamine at pH 8.0 for 1 hour. Two washing cycles were then used to remove non-covalently adsorbed DNAse, each cycle consisting of a wash at pH 4.0 (0.1 M acetate buffer containing 1 M NaCl) followed by a wash at pH 8.0 (0.1 M borate buffer containing 1 M NaCl). At last, the DNAse sepharose was packed into a column (inside diameter : 15 mm) and washed with 10^{-2} M Tris HCl, pH 7.5 containing 10^{-3} M $CaCl_2$.

Affinity chromatography on DNAse sepharose

The amount of protein applied to DNAse sepharose varied according to the percentage of actin (between 15 and 20 mg of protein) and the protein solvent was always the same i.e. buffer A plus 30% glycerol. After the application of the proteins, the column was washed with 10^{-2} M Tris HCl pH 8.0 containing 10^{-3} M $CaCl_2$ and 30% glycerol and the adsorbed proteins were eluted stepwise with the following buffers : a) 0.75 M guanidine HCl (pH 6.5) in 0.5 M sodium acetate, 10^{-3} M $CaCl_2$ and 30% glycerol (peak A). b) 3M guanidine HCl (pH 6.5) in 0.5 M sodium acetate, 10^{-3} M $CaCl_2$ and 30% glycerol (peak B). Two ml fractions were analyzed for adsorbance at 280 nm and the fraction eluted with 3M guanidine HCl (peak B) was collected in tubes containing 3 ml of buffer A. The two fractions (peaks A and B) were then pooled and concentrated by vacuum dialysis with a dialysis against buffer A. The affinity chromatography may be carried out at 4°C or 20°C without any modification of the results.

Dodecyl sulfate polyacrylamide gel electrophoresis (P.A.G.E.)

P.A.G.E. in the presence of SDS were carried out according to Fairbanks (6) on 5.6% acrylamide gels using 1% SDS, 10^{-4}M ATP and $2x10^{-4}$ M ascorbic acid in the gel and electrode buffers.

The gels were scanned using a Joyce Loebl densitometer.

Apparent molecular weights were estimated by measuring relative mobility and comparing it with a calibration plot of log. of molecular weights versus relative mobility established with the following proteins :
- cytochrome C (12 500) ; - chymotrypsinogen (25 000) ; - egg albumin (45 000); - catalase monomer (60 000); - bovine serum albumin (67 000) ; - human spectrin (220 and 240 000). With this technique, the accuracy of the molecular weight determination was ±10%.

All protein concentrations were estimated by micromethod of Lowry (7).

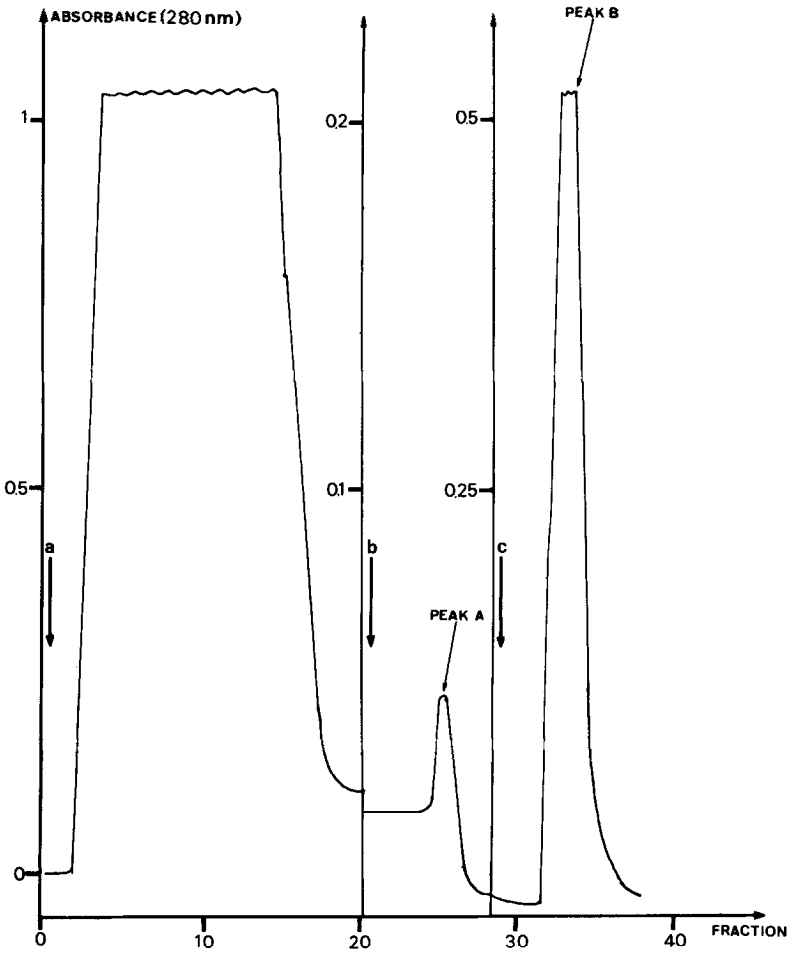


Figure 1

Elution profile of the extrait of acetone powder of red cell ghosts from a DNAse sepharose column.
After the application of the extract (20 mg), the column is washed (a) with 10^{-2} M Tris HCl pH 8.0 containing 10^{-3} $CaCl_2$ and 30% glycerol and adsorbed protein is eluted with 0.75 M guanidine HCl (b) and 3.0 M guanidine HCl (c). See materials and methods for details.

RESULTS

Extraction of the acetone powder of the red cell ghosts removes primarily actin with traces of spectrin (fig. 2 gel 1). The elution profile of the affinity chromatography of this extract on DNAse sepharose is shown in fig.1. All contaminants (as spectrin) were eluted with washing buffer (fig. 2 gel 2). The peak A could not be analyzed by polyacrylamide gel

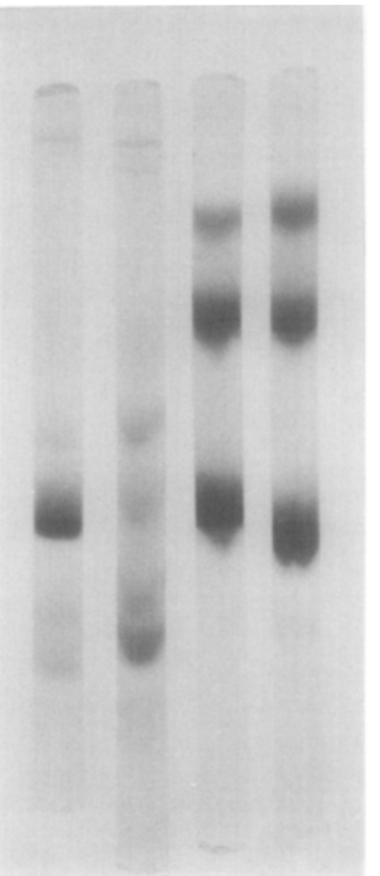


Figure 2

Analysis by SDS polyacrylamide gel electrophoresis of the affinity chromatography of the extract from acetone powder of red cell ghosts.

gel 1 : crude extract of the acetone powder
gel 2 : proteins eluted with washing buffer
gel 3 : peak B (elution with 3M guanidine HCl)
gel 4 : for comparison, peak B (elution with 3M guanidine HCl) of the affinity chromatography of purified muscle actin (see figures 3 and 4).

electrophoresis because of too small quantity of protein. The sodium dodecyl sulfate polyacrylamide gel of the peak B (figure 2 gel 3) shown the presence of actin (apparent molecular weight of 50 000 daltons) and two others proteins with apparent molecular weight of 127 000 and 205 000 daltons estimated by dodecyl sulfate gel electrophoresis. As suggested by their apparent molecular weight, the two supplementary bands probably represent

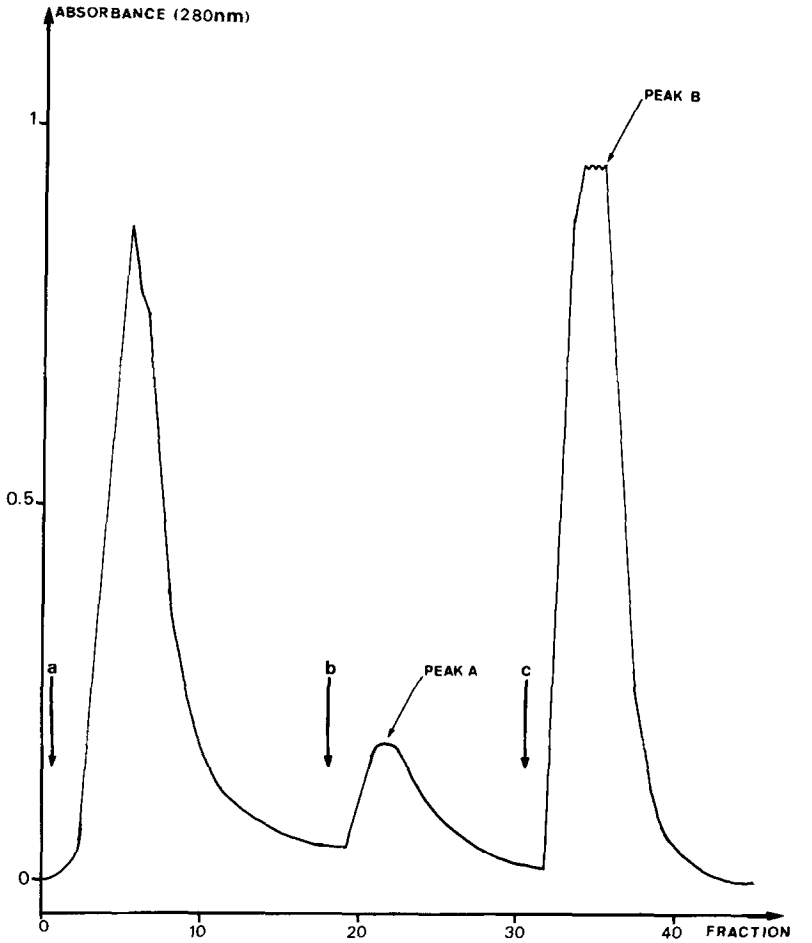


Figure 3

Elution profile of purified skeletal muscle actin from a DNAse sepharose column.
Skeletal muscle actin (20 mg) is applied to the DNAse sepharose column ; washing (a) and elution (b and c) are performed as described in figure 1.

a dimeric and tetrameric form of actin. Indeed, these two bands are absent in the extraction of the acetone powder (fig. 2 gel 1). Furthermore, the elution profile of the affinity chromatography on DNAse sepharose of skeletal muscle actin purified with buffer A_1 (fig. 3) was the same as the elution profile of erythrocyte actin and dodecyl sulfate polyacrylamide gels (fig. 4) showed the same phenomenon : the peak A (elution with 0.75 M guanidine HCl) contained the actin monomer and the peak B (elution with 3M guanidine HCl) the actin monomer, dimer and tetramer.

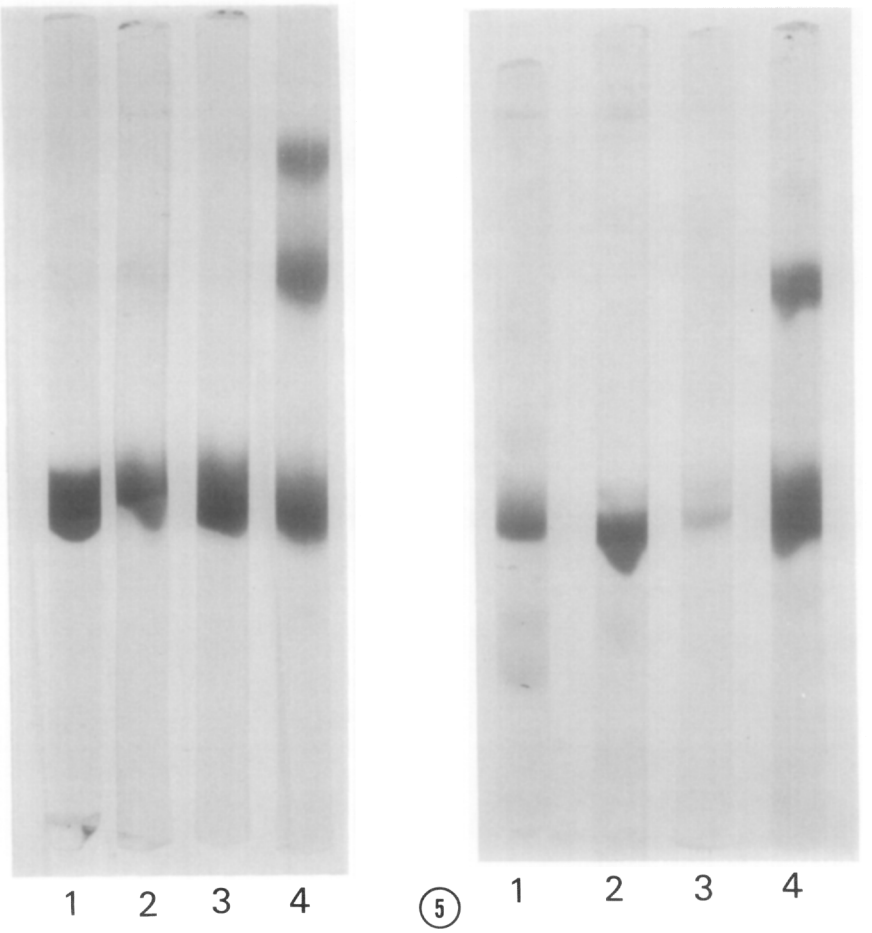


Figure 4

Analysis by SDS polyacrylamide gel electrophoresis of the affinity chromatography of purified skeletal muscle actin.

gel 1 : purified skeletal muscle actin
gel 2 : proteins eluted with washing buffer
gel 3 : peak A (elution with 0.75 M guanidine HCl)
gel 4 : peak B (elution with 3.0 M guanidine HCl)

Figure 5

Analysis by SDS polyacrylamide gel electrophoresis of the affinity chromatography of the erythrocyte actin obtained by polymerization-depolymerization procedure.

gel 1 : crude extract of the acetone powder of erythrocyte ghosts
gel 2 : erythrocyte actin obtained by polymerization-depolymerization procedure.
gel 3 : proteins eluted with washing buffer.
gel 4 : actin eluted with 3.0 M guanidine HCl.

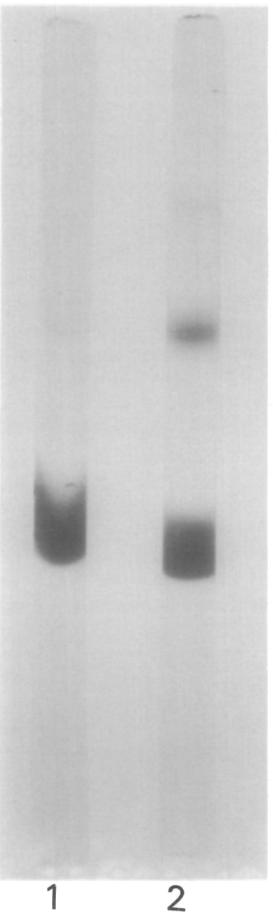


Figure 6

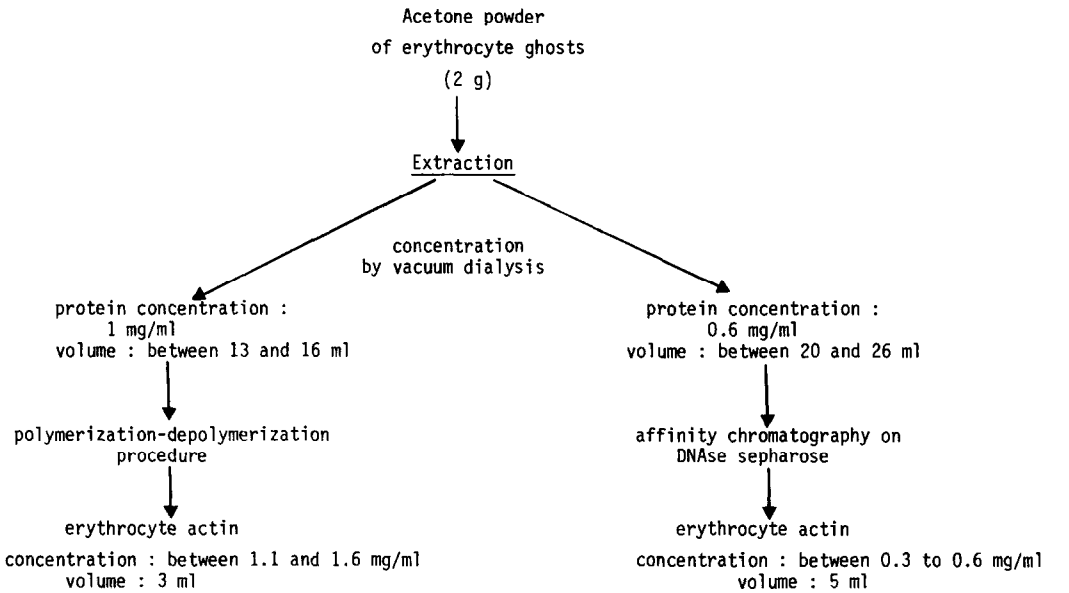
SDS polyacrylamide gel electrophoresis of skeletal muscle actin purified with buffer A_1(gel 1) and with buffer A_2 (gel 2).

Affinity chromatography of erythrocyte actin obtained by polymerization-depolymerization procedure was also performed and the dodecyl sulfate gel electrophoresis of fractions are shown in fig. 5. The peak B (elution with 3 M guanidine HCl) consists of actin monomer and dimer with traces of tetrameric form. The dodecyl sulfate gel electrophoresis of rabbit skeletal muscle actin purified by polymerization-depolymerization procedure without Ca^{2+} (buffer A_2) is shown in fig. 6. The absence of $CaCl_2$ let to a partially polymerization with di and tetrameric forms.

The final yield of the purification of erythrocyte actin according to Sheetz (3) compared with that of purification performed with affinity

TABLE I

Final yield of the purification of erythrocyte actin by polymerization-depolymerization procedure and by affinity chromatography on DNAse sepharose.



chromatography as above described is shown in table I. Extracts of the acetone powder must be concentrated to 1 mg/ml in order to get a complete polymerization of actin.

The final concentrations of erythrocyte actin obtained by the polymerization procedure were thus higher but traces of spectrin were always persisting (between 5.4 and 6.4 %).

DISCUSSION

The affinity chromatography procedure presented here is adequate to produce a preparation that was >95% pure (monomer : 49%, dimer 34%, tetramer 13%) We have not yet been able to obtain a rigourous proof that the higher molecular weight bands represent partially polymerized actin. Many attemps were made to purify the dimeric and tetrameric forms of erythrocyte actin by gel filtration, but without success in our experiment. The concentrations of erythrocyte actin purified by affinity chromatography are indeed too low and after application to a column of gel filtration, no any peak containing actin polymers is photometrically revealed. These actin polymers may be purified

through dodecyl sulfate gel electrophoresis but their characterization by the use of inhibition effect on DNAse I activity is impossible : indeed, DNAse activity is inhibited by dodecyl sulfate.

Nevertheless, we believe that our present data demonstrate that the complete isolation by affinity chromatography of the erythrocyte actin is possible but a partial actin polymerization is likely induced by loss of calcium during the affinity chromatography. Indeed, the preparation of muscle actin without Ca^{2+} in extraction and depolymerization buffer reproduced the same partial polymerization.

This partial polymerization after affinity chromatography seems to have not been previously reported : Lazarides and Lindberg (8) described the affinity chromatography of actin from chicken skeletal muscle on DNAse agarose. The elution peak with 3.0 M guanidine HCl contained only actin monomer but the quantity of actin applied to the column was very small (0.5 mg). Schachat et al. (9) have prepared actin from a nematode by affinity chromatography. Electrophoresis analysis did not show actin polymers but the high concentration of acrylamide (15%) did not lead to a long enough migration to resolve the actin polymers. Multiple forms of actin have been characterized by Garrels and Gibson (10) by the use of two dimensional gel electrophoresis (in the first dimension by isoelectric point and in the second one by electrophoretic mobility in 10% SDS). In addition to the three major actins (α, β and γ actin) two other proteins, designated as δ and ε actin, were identified as probable forms of actin by affinity for DNAse I agarose. However, these δ and ε actin, had the same mobility in the second dimension and so do not represent the actin polymers we have observed.

On the other hand, a partial polymerization of actin induced by lack of calcium in the medium has been well described. In 1963 Lewis et al (11) demonstrated that muscle G actin inactivated by EDTA treatment had molecular weight of 125 000 (by the means of ultracentrifugation). For these authors, the inactivation is a denaturation and "a change in internal organization is provided by the changes in specific rotation and λc". Likewise two forms of chicken gizzard F-actin, 83 S and 36 S, were obtained by Suzuki (12) depending on whether or not calcium was present in the medium. 83 S F-actin was similar to skeletal F-actin and when gizzard G-actin was dialyzed against a buffer without calcium, it was transformed to 36 S forming G-actin with concomitant loss of a considerable amount of calcium.

We think that these di and tetrameric forms do not represent a physiological polymerization state of erythrocyte actin. On the contrary, since calcium ions are essential for maintaining the conformation of actin a pathological lack of calcium in the erythrocyte would modify the properties of actin.

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REFERENCES

1. Ohnishi, T. (1962) J. Biochem. 52, 307-308.
2. Tilney, L.G. and Detmers, P. (1975) J. Cell. Biol. 66, 508-520.
3. Sheetz, M.P., Painter, R.G. and Singer, S.J. (1976) Biochemistry 15, 4486-4492.
4. Dhermy, D., Bournier, O. and Boivin, P. (1977) Biomed. 27, 1316-1318.
5. Spudich, J.A. and Watt, S. (1971) J. Biol. Chem. 246, 4866-4871.
6. Fairbanks, G., Steck, T.L. and Wallach, Q.F.H. (1971) Biochemistry 1, 2606-2616.
7. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 139, 265-275.
8. Lazarides, E. and Lindberg, U. (1974) Proc. Nat. Acad. Sci. USA 71, 4742-4746.
9. Schachat, F.H., Harris, H.E. and Epstein, H.F. (1977) Biochim. Biophys. Acta 493, 304-309.
10. Garrels, J.I. and Gibson, W. (1976) Cell 9, 793-805.
11. Lewis, M.S., Maruyama, K., Carroll, W.R., Kominz, D.R. and Laki, K. (1963) Biochemistry 2, 34-39.
12. Suzuki, K., Yamaguchi, M. and Sekine, T. (1978) J. Biochem. 83, 869-873.