The Actin Content of Fibroblasts

By DENNIS BRAY* and CLIVE THOMAS*

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

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Cultures of chick skin fibroblasts were dissolved in solutions of sodium dodecyl sulphate, and their entire protein content was examined by gel electrophoresis. The most abundant species migrated in the same position as muscle actin. It gave a similar pattern of iodinated peptides after reaction with radioactive sodium iodide and digestion with proteinases, and contained comparable amounts of *N*¹-methylhistidine. Its amount was estimated by quantitative densitometry of stained gels with bovine serum albumin as an internal standard, and by radioactive assay of cultures that had been grown in the presence of [³⁵S]methionine. The values obtained ranged from 7 to 14% of the total cellular protein, with an average of 8.5%. A protein band in the position of muscle myosin was also present and accounted for about 2.5% of the total protein. Both this and the actin band increased in relative amount with the age of the cultures.

The evidence that actin is present in cell types other than muscle is overwhelming (Pollard & Weihing, 1974). Almost every tissue of the body has, by one test or the other, been shown to contain actin-like proteins and the similarities which these show to muscle actin are extensive.

Actin was first detected in cultured fibroblasts by electron microscopy. Ishikawa *et al.* (1969) showed that cells treated with heavy meromyosin contained thin filaments with regular arrowhead projections along their lengths resembling those formed between filaments of muscle actin and heavy meromyosin (Huxley, 1963). This observation has now been made in many different types of fibroblastic cell (Goldman & Knipe, 1973; Perdue, 1973; Spooner *et al.*, 1973). Biochemical evidence was obtained by Yang & Perdue (1972), who obtained from chick embryo fibroblasts a protein which has the same electrophoretic mobility, morphology and ability to interact with myosin as muscle actin. From the same source, Bray (1973) identified actin by its mobility on sodium dodecyl sulphate-polyacrylamide gels and its pattern of iodinated peptides, and immunological evidence for actin in fibroblastic cells has been obtained by a number of authors (Lazarides & Weber, 1974; Trenchev *et al.*, 1974).

One of the first questions to ask about this non-muscle actin is how much of it is there in the cell. Is it a major component? Does it exist in the same proportion to myosin (which is also present in these cells) as in striated muscle? Unfortunately there is no specific assay for actin which may be used on crude cellular extracts and no direct answer is possible.

A method which has worked well for the proteins

of muscle is their determination by polyacrylamide-gel electrophoresis (Bullard & Reedy, 1973; Tregear & Squire, 1973). The proteins of the myofibril, or even of the entire muscle, when fractionated in this way show two major components corresponding to actin and myosin, and amounts present may be directly gauged by their degree of staining. Although, in principle, this method can be applied to non-muscle cells it encounters the problem that actin and myosin no longer account for the bulk of the protein. It cannot be assumed, as it may for muscle, that the species that co-migrates with actin on electrophoresis is composed predominantly of actin. This must be shown by fingerprinting or compositional analysis, and allowance made for the inaccuracies inherent in the extraction and staining, before a reliable estimation may be made.

Experimental

Materials

Tissue-culture media were purchased from Bio-Cult Laboratories (Bio-Cult Laboratories Ltd., Paisley, U.K.), and included Minimum Essential Medium with Earle's salts and 2.2 g of NaHCO₃/litre, Earle's Balanced Salt Solution, and Dulbecco's phosphate buffered saline (Paul, 1970).

L-[2,5-³H]Histidine (30 Ci/mmol), L-[³⁵S]methionine (5–25 Ci/mmol) and ¹²⁵I (carrier-free) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Fluorescamine was from Roche Diagnostics, Nutley, N.J., U.S.A.

Muscle actin was prepared as described by Straub (1942), by the modified procedure of Mommaerts & Parrish (1951), and was the gift of Dr. J. Kendrick-Jones of this laboratory. The acetone-dried powder was extracted at 0°C and the actin polymerized with

* Present address: MRC Biophysics Unit, Kings College, 26–29 Drury Lane, London WC2B 5RL, U.K.

0.7mM-MgCl₂ to lessen contamination with tropomyosin and troponin. Purified myosin from chicken leg muscle was a gift of Dr. A. Weeds of this laboratory. Bovine serum albumin [crystalline; Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.] was dried in a vacuum desiccator to constant weight before being used as a protein standard. Other chemicals were the commercially available products of analytical-grade purity.

Assays

Protein assays were by the Lowry *et al.* (1951) method as modified by Layne (1957) with bovine serum albumin as reference. In most assays 0.1% sodium dodecyl sulphate was present in both specimen and reference samples.

Stained gels were scanned in a 15cm-long glass cuvette on a Chromoscan densitometer (Joyce Loebl and Co. Ltd., Gateshead, Co. Durham, U.K.) by using a 550nm filter. The amounts of stain in each band were assessed by direct measurements of the areas of the recorded peaks. The baseline for actin and myosin peaks were taken as the lowest part of the recorded scan, and that for bovine serum albumin was obtained from gels lacking this additional protein (see Fig. 3).

Radioactive proteins were assayed by sectioning and counting the gels for radioactivity. The gel was fixed, stained and then frozen. It was pressed on to an array of single-edged razor blades so that transverse slices approx. 1.2mm in thickness were produced. Each of these was then dissolved in 0.5ml of H₂O₂ (20vol.) at 37°C overnight, dried on a glass-fibre disc (GF/C, 2.5cm; Whatman Biochemicals Ltd., Maidstone, Kent, U.K.). Then they were shaken with a mixture of 2.7ml of scintillation fluid (Bray, 1960) and 0.3ml of water and counted for radioactivity in a liquid-scintillation counter.

Fibroblast cultures

The dorsal skin of 11-day chick embryos was collected and freed from adhering tissues by dissection. Small pieces of skin were incubated in a solution of 0.25% trypsin (Bio-Cult Laboratories Ltd.) in Earle's Balanced Salt Solution (see above) lacking Ca²⁺ or Mg²⁺ for 20min at 37°C. The trypsin solution was removed and the pieces of tissue were washed once in complete Balanced Salt Solution and then dissociated in the same solution by repeated pipetting. Large pieces of tissue were allowed to settle and the remaining suspension was passed through a double layer of sterile lens paper held in a Swinnex 13mm filter holder (Millipore Co., Bedford, Mass., U.S.A.).

The filtered suspension, which contained mostly single cells but also some small clumps, was

centrifuged at 600g for 3min in a bench-top centrifuge. It was resuspended in Balanced Salt Solution, usually 10ml, and the cells were counted in a haemocytometer. About 300000 cells were inoculated into each 9cm tissue-culture dish (Sterilin Ltd., Richmond, Surrey, U.K.).

The cells were grown in Minimal Essential Medium, supplemented by 10% calf serum, 100 units of penicillin/ml and 100µg of streptomycin/ml. They were incubated at 37°C in a humid atmosphere containing 5% CO₂ with exchange of medium every 2–3 days and were examined daily in an inverted microscope (model M40, Wild Heerbrugg Ltd., Heerbrugg, Switzerland). After 5–7 days they were treated with trypsin in the above solutions for 10min and replated. These secondary cultures were grown until the cells formed a confluent layer over the surface of the dish (about 10⁷ cells/9cm dish).

Preparation of extracts

At 10min before harvesting, the culture medium was removed and the cultures were incubated in Balanced Salt Solution. They were rinsed twice in phosphate buffered saline and then harvested in phosphate buffered saline containing 1% sodium dodecyl sulphate (0.5ml/dish), and immediately heated in boiling water for 2min. The cultures were then sonicated briefly [3min at an intermediate setting of a Soniprobe (Dawe Instruments Ltd., London W.3, U.K.)] and a rough estimate of the protein content was made. The extracts were then centrifuged at 5000g for 15min on a bench-top centrifuge to remove particulate material and the protein content of the supernatant material was assayed by the Lowry *et al.* (1951) procedure.

Gel electrophoresis

Fibroblast extracts were mixed with a solution of bovine serum albumin so that this accounted for 1, 2 or 5% of the total protein, itself usually 1mg/ml. Portions of extract, usually about 50µl, were diluted to 200µl with sample buffer [0.1% sodium dodecyl sulphate, 1% β-mercaptoethanol, 0.12M-Tris-glycine, pH 6.8, 0.001% Bromophenol Blue and 10% (v/v) glycerol], and applied to polyacrylamide gels (0.7cm × 12cm).

Gels were prepared by the method of Laemmli (1970). They were prepared with either 8 or 10% (w/v) acrylamide, with an acrylamide/bisacrylamide ratio of 38:1 (w/w) and prepared in a buffer containing 0.38M-Tris-glycine, pH 8.8, and 1% sodium dodecyl sulphate. The stacking gel contained 3% (w/v) acrylamide and 0.12M-Tris-glycine, pH 6.8. Electrophoresis was carried out overnight at room temperature at 15–20V (about 3mA per tube). The gels were fixed by immersion in 50% (w/v) trichloro-

acetic acid for 1 h or longer, and stained with 0.05% Coomassie Brilliant Blue, or 0.5% Fast Green (Gorovsky *et al.*, 1970), in 50% trichloroacetic acid for 1 h at 37°C. Excess of stain was removed by repeated washes in 7% (v/v) acetic acid at 37°C.

Peptide 'maps'

These were prepared by the iodination procedure of Bray & Brownlee (1973). Fibroblast proteins were resolved on 8% polyacrylamide gels as described above and then stained with Coomassie Brilliant Blue. To avoid hydrolysis of the protein in trichloroacetic acid, the stain for this purpose was made up in an aqueous mixture of methanol (45%, v/v) and acetic acid (9%, v/v) (Weber & Osborn, 1969). After destaining, the presumptive actin band was cut out with a razor blade and chopped into small pieces. The protein and stain were eluted into a solution containing 0.05 M-sodium phosphate, pH 7.5, 0.1% sodium dodecyl sulphate and 1 mM-phenyl methylsulphonyl fluoride, and precipitated by the addition of KCl to a final concentration of 0.2 M. Stain was removed by washing with acetone and the remaining protein iodinated with Na¹²⁵I in the presence of chloramine-T (Hunter & Greenwood, 1962). The reaction was stopped by the addition of sodium metabisulphite and the radioactive protein precipitated with ice-cold 5% trichloroacetic acid. After washing in acetone this material was digested with 0.04 µg of trypsin or chymotrypsin/ml in 0.1 M-NH₄HCO₃ for 15 h at 37°C. Residual radioactive contaminants were removed on a Sephadex G-25 column (1.2 cm × 10 cm) and the final mixture of peptides was electrophoresed on Whatman 3MM paper (Bray & Brownlee, 1973). The radioactive peptides were detected on X-ray film placed in contact with the electrophoretogram.

Assay of N¹-methylhistidine content*

Cultures of fibroblasts were grown in 5 cm dishes in medium supplemented with 40 µCi of histidine/ml for about five generations. They were washed and harvested as described above and the total contents of each dish fractionated on a single 16 cm × 16 cm slab gel prepared with 8% acrylamide and 1% sodium dodecyl sulphate. The gel was stained as usual and dried under vacuum. The presumptive actin band was cut out and broken into small pieces. It was eluted with shaking in 0.05% proteinase (type VI; Sigma) in 0.2 M-NH₄HCO₃ solution at 37°C overnight. The eluted material was collected

and the extraction repeated twice. Control experiments in which these gel pieces were further extracted with 1% sodium dodecyl sulphate and 0.1% mercaptoethanol showed that very little radioactivity remained after the proteinase treatment.

The eluted samples were freeze-dried several times to remove the NH₄HCO₃ and then taken up in 0.5 ml of 6 M-HCl and heated for 15 h at 110°C in a sealed evacuated tube. The acid was then removed under vacuum and the samples were dissolved in water. They were electrophoresed on Whatman no. 54 paper at pH 6.5 for 2 h at 3 kV, dried and stained with 0.025 mg of fluorescamine/ml in acetone containing 0.5% pyridine (Udenfriend *et al.*, 1972). The carrier amino acids were detected by u.v. illumination and their positions were recorded. The radioactive amino acids were detected by cutting out strips 1.5 cm wide, soaking these in 1 ml of water for 1 h and then determining their radioactivity in a scintillation counter.

In other experiments the material which co-migrated with N¹-methylhistidine after electrophoresis was eluted and run together with standards in a descending-chromatography apparatus on Whatman no. 1 paper (see Fig. 2).

Results

As a first step it was necessary to determine how much of the fibroblast protein remained undissolved by the detergent treatment. This was estimated in cultures which had been radioactively labelled. The cells were grown for 5 days in methionine-free Minimal Essential Medium which had been supplemented with 30 µCi of [³⁵S]methionine/ml. They were extensively washed before harvesting, heated in sodium dodecyl sulphate as usual, and centrifuged at about 5000g for 15 min in a bench-top centrifuge. The sedimented residue was dissolved in 5 M-NaOH (100°C for 20 min followed by 37°C for 3 h), and then neutralized with HCl. The radioactivity of both dissolved and residual material was measured and corrections were made for quenching. The fraction of the radioactivity that by this test did not dissolve in the detergent was, in four determinations, 2.0, 2.1, 2.3 and 4.1%. Together with the gel patterns, which showed that only minor amounts of the protein failed to penetrate the gels (Plate 1a), this was taken as evidence that the correction necessary to account for undissolved protein was negligible.

Actin was measured in cultures in which the cells had just formed a confluent layer over the surface of the dish. In a representative dish (9 cm diameter) cell counts gave a value of 1.0 × 10⁷ cells, and an amount of protein determined by the Lowry *et al.* (1951) method, after removal of insoluble debris,

* The IUPAC-IUB Commission on Biochemical Nomenclature recommends that the imidazole N of histidine nearer to the alanine residue be designated *pros* (symbol π) and the one farther away *tele* (symbol τ) [*Biochem. J.* (1972) 126, 775].

of 1.54 mg per dish. Therefore the protein content of each cell was approx. 150 pg.

The dissolved protein was fractionated on polyacrylamide gels and a typical result is shown in Plate 1(a). Up to 80 bands could be distinguished, and the pattern was essentially the same in all of the confluent cultures examined. Patterns of radioactivity from cultures which had been grown in the presence of [35 S]methionine showed differences in the relative intensity of some bands, but otherwise were very similar. Calibration of the gels was carried out by adding small amounts of certain proteins to the extracts just before electrophoresis, usually 2–5 μ g of the protein standards to 25–50 μ g of the fibroblast extract. Comparison with control gels then showed the precise positions of the additional proteins which are indicated in Fig. 1. A major component of the fibroblast extract migrated in the same position as muscle actin. To make a positive identification, however, it was necessary to obtain other evidence, and tests of two kinds were made. One was a procedure developed in this laboratory by which stained protein bands may be eluted from gels, radioactively labelled with [125 I]iodide and then digested with proteinases (Bray & Brownlee, 1973). This was found to give patterns of radioactive peptides from the presumptive actin band which were very similar to those from muscle actin (Plate 1b). The comparison shown is that of peptides produced by chymotrypsin digestion and resolved by electrophoresis at pH 3.5; others were made by trypsin digestion and by electrophoresis at pH 6.5. The patterns were in all cases extremely similar in their major feature.

The second direct test of the presumptive actin was an analysis of its N^{ϵ} -methylhistidine content. This unusual amino acid is present in both muscle actin (Asatoor & Armstrong, 1967; Johnson *et al.*, 1967) and in a number of cytoplasmic actins (Pollard & Weihing, 1974). The analysis was carried out on cultures of fibroblasts which had been radioactively labelled with [3 H]histidine. These were harvested and fractionated on slab gels, which were then dried down and the major bands cut out. The radioactive proteins that they contained were broken down to amino acids by digestion with a non-specific proteinase, and this was completed by acid hydrolysis. The radioactive material was dried under vacuum and mixed with an aqueous solution containing histidine, N^{ϵ} -methylhistidine and N^{π} -methylhistidine. This mixture was electrophoresed on paper and the amino acid standards were detected by fluorescamine staining. The 3 H-labelled compounds were then located by scintillation counting of strips cut from the electrophoretogram.

The results of this analysis showed, in each of the protein bands tested, a major radioactive component

Fig. 1. Electrophoresis of hydrolysed fibroblast actin from cultures labelled with [3 H]histidine

Cultures of chick fibroblasts were grown for about five generations in medium containing L-[2,5- 3 H]histidine. They were washed and harvested and their proteins fractionated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. A number of protein bands were cut out and hydrolysed by treatment first with proteinases, and then with HCl. The mixtures of amino acid were then applied to Whatman no. 54 paper and electrophoresed at 3 V for 2 h in pH 6.5 buffer (100 ml of pyridine, 3 ml of acetic acid, 897 ml of water), together with standard amino acids. The electrophoretograms were stained with fluorescamine to detect the standards and then cut into strips 1.5 cm wide. They were eluted with water and the radioactivity they contained (given on the ordinate in c.p.m.) was measured by scintillation counting. 3 MeHis, N^{ϵ} -methylhistidine; O, origin; X, unidentified material from Pronase.

which migrated in the position of histidine (Fig. 1). For the presumptive fibroblast actin, however, and only in this one, a minor component was also present, which migrated with N^{ϵ} -methylhistidine. The identity of this radioactive species was confirmed by eluting it from the electrophoretogram and comparing its behaviour in a descending chromatography system with that of authentic N^{ϵ} -methylhistidine (Fig. 2). The relative amount of radioactivity in N^{ϵ} -methylhistidine compared with histidine found in the experiments was about 1:6, and a little higher than that expected for muscle actin (1:8; Elzinga *et al.*, 1973). Together with the iodinated 'fingerprints' described above, and the analysis by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, this provided strong evidence that the band in question was composed largely of a protein very similar to muscle actin.

The distribution of protein in the stained gels was examined by densitometry (Fig. 3). Almost all of the visible bands could be resolved in this way and the intensity of their staining could be estimated from the areas of the peaks in the recorded scans. The principal difficulty in making these measurements was in the assignment of a baseline. For actin and myosin this was taken as the background value

EXPLANATION OF PLATE I

(a) Proteins of cultured chick fibroblasts resolved by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis

Secondary cultures of skin fibroblasts were dissolved in 1% sodium dodecyl sulphate and a portion containing 50 μ g of protein was fractionated on an 8% sodium dodecyl sulphate-polyacrylamide gel as described in the text. Duplicate gels containing the fibroblast extract together with a small amount of a protein standard were also run. The positions of the standards are indicated (this designation does not imply the identity of the fibroblast proteins). The protein standards, and their approximate subunit molecular weights, were: myosin (chicken breast muscle), 200000; RNA polymerase (*Escherichia coli*), 145000 and 155000; bovine serum albumin (BSA), 66000; tubulin (pig brain), 55000; glutamate dehydrogenase (cow), 53000; actin (chicken breast muscle), 42000; tropomyosin (rabbit back muscle), 35000.

(b) Electrophoretogram of radioactive peptides from muscle actin and the presumptive fibroblast actin

The proteins were recovered by elution from stained gels and made radioactive by reaction with ^{125}I (Bray & Brownlee, 1973). They were digested with chymotrypsin and electrophoresed on Whatman 3MM paper for 90 min at 3 kV in the pH 3.5 buffer (5ml of pyridine, 50ml of acetic acid, 945ml of water). The radioactive peptides were detected on X-ray film after exposure for 6h. Other comparisons (not shown), were made by digestion with trypsin and electrophoresis at pH 6.5 buffer, but revealed no large differences in the two proteins. A, Chicken muscle actin; B, presumptive fibroblast actin.

EXPLANATION OF PLATE 2

Fibroblast proteins at different stages of culture, fractionated on 8% polyacrylamide gel

Cultures of skin fibroblasts were prepared in parallel and harvested at various stages of growth. They were dissolved in sodium dodecyl sulphate solution and fractionated on 8% sodium dodecyl sulphate-polyacrylamide gels. (a) Cells in a rounded-up form before inoculation into the culture dish; (b) in a rapidly growing stage, 6h after inoculation; (c) just after reaching confluence, 3 days after inoculation; (d) confluent culture, 7 days after inoculation.

Fig. 2. Chromatography of the N^{ϵ} -methylhistidine from fibroblast actin

The spot containing N^{ϵ} -methylhistidine in Fig. 1 was eluted with water, concentrated by evaporation and chromatographed in butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.) for 2 days. The chromatogram was stained with fluorescamine and the radioactivity of 2cm strips determined in a scintillation counter. These values, in c.p.m., are given on the ordinate. His, histidine; 1MeHis, N^{ϵ} -methylhistidine; 3MeHis, N^{ϵ} -methylhistidine; O, origin.

for the gel, whereas for the bovine serum albumin used as an internal standard, the baseline was obtained directly from gels lacking this additional protein. The areas of the actin or myosin peaks were then compared with that of bovine serum albumin which had been electrophoresed on the same gel, and which made up a known fraction (2–14%) of the total protein present. This avoided many of the inaccuracies of gel densitometry, some of which are discussed below, but was still subject to non-linearities in staining and to variations in the amount of stain taken up by different protein species (Fishbein, 1972). To correct for this, mixtures of actin and bovine serum albumin containing the same amounts of protein as determined by the Lowry *et al.* (1951) procedure were run on gels and stained. Their relative intensities were then determined by scanning over the same range of concentration, and electrophoretic conditions identical with those used for the extracts. It was found with Coomassie Brilliant Blue that bovine serum albumin bound rather more stain than actin, and the average ratio for 16 determinations was 1.5:1 (Fig. 4). The comparable ratio for Fast Green, a less sensitive protein stain (Gorovsky *et al.*, 1970), was 1.2:1.

The results of 25 such determinations, made on gels which were selected for their good resolution and freedom from minor imperfections, are collected in Table 1. They have been corrected for the different staining abilities of actin and bovine serum albumin. The final values range from 6 to 11% of the total fibroblast protein, with an average of approx. 8.5%.

In two experiments the amount of actin was estimated by its incorporation of radioactive

Fig. 3. Densitometer tracings of gels containing fibroblast extract

The gels were prepared as described in the text and contained 50 μ g of fibroblast protein. The lower gel also contained 1 μ g of bovine serum albumin. The additional peak due to bovine serum albumin, which represents 2% of the total protein, was compared with that of the presumptive actin peak. When allowance was made for the difference in staining ability of the two proteins this gave an estimate of the amount of actin present.

Fig. 4. Comparison of the staining ability of actin and bovine serum albumin

A mixture of muscle actin and bovine serum albumin containing 1 mg of each protein/ml as determined by the Lowry *et al.* (1951) assay was prepared. Various amounts, ranging from 2 to 10 μ g of total protein, were applied to 8% sodium dodecyl sulphate-polyacrylamide gels and fractionated. The two stained peaks were measured by densitometry and their areas plotted in arbitrary units.

Table 1. Estimated actin content of fibroblasts

The values were obtained by densitometry of gels by the procedures described in the text. A correction for the differential staining of actin and bovine serum albumin has been applied. Measured values were multiplied by 1.5 for Coomassie Brilliant Blue, and 1.2 for Fast Green. The corrected values have an average of 8.5% of the total protein.

Method of detection	Protein on gel (μ g)		Actin (% total protein)
	Fibroblast	Bovine serum albumin	
Coomassie Brilliant Blue	25	1	10.1
	50	1	8.1
	50	2	6.9, 7.0, 7.3, 8.1, 8.1, 8.2, 8.5, 8.6, 9.0, 10.2, 10.2
	50	4	8.0
	50	6	8.7
	50	8	10.8
	50	2	6.0, 7.1, 7.2, 7.4, 7.6, 9.0, 9.0, 9.7
	50	4	10.6
Fast Green			

methionine. Cultures were grown for five generations in the presence of [35 S]methionine and fractionated on 8% gels as described above. The gels were frozen and sliced transversely. The slices were dissolved in H_2O_2 and their radioactivity was determined (see the Experimental section). In the two experiments actin was found to contain 14.5 and 15.5% of the total radioactivity. Although no correction has been made for the high methionine content of actin (Elzinga *et al.*, 1973), this is probably 2-3 times that of the average protein (Dayhoff, 1972).

The fibroblast extracts contained a protein which co-migrated with the heavy chain of muscle myosin (Plate 1a). The intensity of this component after staining was also measured and compared with that of the added albumin. A correction for the differential staining of muscle myosin and bovine serum albumin was determined from standard mixtures of these two proteins. It was found that myosin took up much less stain than albumin, and for the myosin heavy chain, which comprises 84% of the myosin protein, this correction is 1:2.0. The estimated amount of myosin after making this correction was 2.5% (average of four determinations), and the myosin/actin ratio was therefore close to 1:3.

Although all of our quantitative estimates were carried out on cultures just at confluence, we also examined cultures at other stages of growth. Extracts of cells before plating, that is in the rounded-up state, of cells in rapid growth, and after several days growth at confluence, were examined. The results (Plate 2) were not quantitative but appeared to show that the amount of actin and myosin per cell increased with the age of culture.

Discussion

The yield of purified actin obtained by Yang & Perdue (1972) was 0.2% of the total fibroblast protein, very much lower than that expected from our estimate of 8.5%. An even greater discrepancy exists in *Acanthamoeba*, in which estimates of 10-15% by gel electrophoresis are to be compared with 0.2% recovered as actin (Pollard & Weihing, 1974). These results indicate, we believe, the considerable losses in the present preparative procedures. Methods that have been developed for striated muscle probably depend on its unique ultrastructure, and are grossly inefficient for other tissues.

Analysis by gel electrophoresis has the advantage that essentially all of the protein is analysed without prior fractionation, but is subject to many other uncertainties. There is, first of all, the question of the identity of the gel band. It must be shown that actin is the major component present and, if possible,

some estimate of its purity must be obtained. Then the many difficulties inherent in quantitative gel densitometry must be considered (Fishbein, 1972). Losses may occur during application to the gel or, especially in low-molecular-weight compounds, during fixation. The amount of stain taken up will vary with the porosity of the gel, the identity of the protein and the distance it has migrated. It is proportional to the quantity of protein only within a limited range.

In the present work we have analysed the gel component for the pattern of radioactive peptides it gives after reaction with Na^{125}I and digestion with proteolytic enzymes, and for its content of N^{ϵ} -methylhistidine. Both of these show that the band is predominantly composed of a protein very like actin, but neither provides an accurate estimate of purity. Assessment of the amount of protein in this band was made by a novel method of densitometry which avoids many of the usual inaccuracies. A small amount of bovine serum albumin was added to the fibroblast protein so that it made up a known fraction of the total protein and served as an internal standard. The amount of stain taken up by bovine serum albumin was then compared with that taken up by the presumptive actin on the same gel, so that losses in the course of sample application were immaterial. The staining ability of the two proteins, actin and bovine serum albumin, were compared in standard mixtures which had been electrophoresed and stained under conditions identical with those used for the test samples.

The results obtained with gels stained with Coomassie Brilliant Blue show that actin comprised about 8.6% of the total protein (Table 1). Estimates with Fast Green, a less sensitive protein stain, which is reported to show less variation with the type of protein (Gorovsky *et al.*, 1970), gave similar values (average 8.2%). In two experiments the amount of radioactive methionine which was taken up into actin was about 15% of the total. When corrected for the high methionine content of actin, 2–3 times that of an 'average' protein (Elzinga *et al.*, 1973; Dayhoff, 1972), this gives a value of 5–8%. Taken together, a value of 8.5% of the total protein appears as the best estimate.

Despite all the precautions it is unlikely that any of these procedures are of very great accuracy. The unknown amount of protein other than actin in the gel band, the difficulty in assigning accurate background values on the densitometer scans, and the vagaries of individual gels, especially in their staining and destaining, all contribute uncertainties. None of these is likely to result in major errors, however, and the essential agreement obtained by the three methods encourages us to believe them to be reliable approximations to the true value.

On a weight basis, actin is probably the most abundant protein of fibroblasts. It constitutes a smaller fraction than in fresh muscle, for which values between 17 and 34% have been reported (Hanson & Huxley, 1957), but is still a major component. The total protein content of the cells used in this study was 150 pg per cell, so that each cell contained about 12 pg of actin. The volume of each cell is probably close to $3000\mu\text{m}^3$ based on a flattened cell area of $800\mu\text{m}^2$ and a cell thickness of $4\mu\text{m}$ (Abercrombie *et al.*, 1971). This value falls within the range estimated for cultured baby hamster kidney cells (Follett & Goldman, 1970). The apparent concentration of actin, therefore, if present in a freely diffusing monomeric form is 4 mg/ml or 0.1 M (Elzinga *et al.*, 1973). Alternatively, if all the actin is present as double-helical filaments, then each cell would contain a total length of about 40 cm. A more realistic picture is that about half of the actin is present in an unpolymerized form at about 2 mg/ml (D. Bray & C. Thomas, unpublished work), and the remaining filamentous actin is distributed largely in bundles beneath the cell cortex and within filopodia (Goldman & Knipe, 1973; Perdue, 1973; Spooner *et al.*, 1973).

Many non-muscle cells contain a protein similar to myosin, and this is true of cultured fibroblasts (Adelstein *et al.*, 1972). Although it is not exactly the same as its counterpart in muscle (BurrIDGE, 1974), it co-migrates with muscle myosin on sodium dodecyl sulphate-polyacrylamide gels (Plate 1a). If this band consisted entirely of the heavy chain of muscle myosin then its amount, estimated in the above fashion, would be 2.5% of the total protein. This includes a considerable correction for the lower staining ability of myosin than albumin on these 8% polyacrylamide gels, and lies within the range of values obtained by Ostlund *et al.* (1974) by measurements of myosin adenosine triphosphatase activity. The ratio of presumptive myosin to actin is then 1:3.2, to be compared with the value of 1:0.6 reported for rabbit muscle (Tregear & Squire, 1973; Potter, 1974) and 1:3.5 for vertebrate smooth muscle (J. Kendrick-Jones, unpublished work). It should be emphasized, however, that these are maximum values which assume that the band in question consists entirely of myosin.

All of the detailed analyses described here were carried out on cultures of fibroblasts in which the cells had just formed a confluent layer over the dish. A comparison of gels prepared at other stages, however, with those of cells dissociated with trypsin, at early stages of growth and several days after reaching confluence showed essentially the same patterns (Plate 2). Differences in the relative amounts of various bands appeared, however, and this was true of actin and myosin. Although quantitative measurements were not made, it was

clear that these two increased considerably in amount with the age of the culture. Ostlund *et al.* (1974) have shown that myosin contents measured by adenosine triphosphatase activity after partial purification, vary in different kinds of cell cultures, and in particular, that they are higher in cells grown on dishes than in suspension. These may be indications of a control mechanism which regulates the amounts of actomyosin in the cell.

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