Journal of Cellular Biochemistry 20:393–407 (1982)

Effect of Phalloidin on Liver Actin Distribution, Content, and Turnover

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Phalloidin increases F-actin microfilament content and actin-directed immunofluorescence in hepatocytes in vivo and also increases actin polymerization and the stability of F-actin in vitro. We studied the sensitivity of immunofluorescent staining of actin to an actin depolymerizing factor (ADF) as well as actin content, degree of polymerization, and turnover in livers of in vivo phalloidin-treated rats. Pretreatment with ADF abolished anti-actin antibody (AAA) staining of normal liver but did not modify staining of livers from phalloidin-treated animals. Planimetric analyses of SDS-polyacrylamide gels showed the percent actin of total protein was increased by approximately 40% and the absolute amount of actin by approximately 43%, ten days after daily phalloidin treatment (50 μg/100 gm body weight). Similar but smaller changes could be seen after one day of treatment. Ultracentrifugational analyses of liver extracts indicated no change in the amount or proportion of G-actin but a 194% increase in the proportion of F-actin in ten-day treated animals, changes also apparent in one day animals. Neither the relative fractional rate of actin synthesis nor its synthesis as a percent of total protein synthesis was altered either at one-day or ten-day post-phalloidin treatment. Dual-isotope experiments indicated that the rate of actin degradation was decreased selectively in the one- to three-day period following drug treatment. Thus, phalloidin appears to stabilize actin against the depolymerizing actions of ADF, increases the proportion of F-actin without altering the size of the G-actin pool, and causes accumulation of actin by decreasing its relative rate of degradation.

Key words: liver, phalloidin, actin turnover, immunofluorescence, actin polymerization

Actin is present in relatively high amounts when compared with myosin in nonmuscle cells. This has suggested that, in addition to the well-established contractile activity, through interaction with myosin, actin exerts a cytoskeletal role that regulates cell shape as has been proposed for limulus sperm achrosomal process [1] and isolated rat hepatocytes [2]. This cytoskeletal role is probably based on changes of the equilibrium between G and F cytoplasmic actin [1–3]. The mechanisms of regulation of this equilibrium are presently not clearly known [for review, see 3].

Phalloidin, the cyclic peptide toxin from the poisonous mushroom Amanita phalloides [4] is well known to interact directly with actin, stabilizing the filamentous form and preventing actin depolymerization [5–7]. This toxin is becoming an impor-

Received April 28, 1981; accepted October 1, 1982.

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tant tool for investigation of the contractile and cytoskeletal roles of actin, including its role in liver cell function [2,4,8].

Administration of phalloidin to rats results in a marked increase in actin microfilaments at the periphery of hepatocytes, particularly around bile canaliculi [2] and is accompanied by cholestosis [2,9–11] suggesting that microfilament dysfunction may alter bile secretion [10]. These in vivo effects correlate with increased immunofluorescent staining of hepatocytes with actin antibodies [12].

Elias et al [11] reported that phalloidin administration increases actin content, particularly the F-actin pool, also indicating no change in G-actin content, though data were not provided regarding the latter. It was suggested that the increased actin content was due to increased synthesis.

A model is emerging which suggests cytoskeletal assembly at the level of the polyribosome [13,14]. This would require the coordinate synthesis of the involved cytoskeletal elements, including actin. It becomes important in this context to determine whether actin accumulation caused by phalloidin is due to increased actin synthesis, which might be expected, in turn, to alter the synthesis of other cytoskeletal elements or to decreased actin degradation.

The purpose of the present studies was to reevaluate changes in actin content and polymerization and to measure actin synthesis and degradation in the liver after phalloidin treatment. These experiments have been presented in preliminary form [15].

MATERIALS AND METHODS

Animals

Male Wistar or Sprague Dawley rats, 130–170 gm, were maintained on Purina laboratory chow ad libitum for at least two weeks before and during experiments. No interstrain effects were observed on any of our results. Phalloidin was injected intraperitoneally once or daily at a dose of 500 μg/kg body weight for up to ten days, as indicated in Results. Control animals received saline, yielding the same results after one as after ten days of injections. Animals were studied 22–24 hr after a single injection or 20–24 hr after the last of daily injections, except in degradation experiments in which case animal sacrifice was 18 hr after the last phalloidin treatment. Animals were anesthetized with ether, their livers were perfused with saline and removed, and the animals sacrificed by exsanguination.

Immunofluorescence and Sensitivity to Actin Depolymerizing Factor (ADF)

Anti-actin antibodies (AAA) were obtained from the serum of a patient with chronic aggressive hepatitis [14,15]. The serum was passed through a column of sepharose covalently linked with rabbit skeletal muscle actin and the adsorbed antibody was eluted at pH 2.7. For immunofluorescent staining, 4-μm thick cryostat sections were incubated with AAA, followed by fluorescein-conjugated IgG fraction of goat anti-human IgG antiserum. After rewashing in phosphate-buffered saline (PBS), the level of fluorescence was compared with that found in control preparations treated by a solution of normal human Ig with a concentration of protein similar to that present in AAA. ADF was purified from human serum as previously described [16–18]. To determine the sensitivity of cytoplasmic actin to ADF, 4-μm thick cryostat sections were incubated with normal human serum (diluted 1:10) or with purified

ADF (approximately 25 μg/ml) and processed for immunofluorescence as described above. As control for ADF, we used: one, human IgG (3 mg/ml); and two, human albumin (2 mg/ml). Photographs were taken with a Zeiss ultraviolet photomicroscope equipped with epiillumination and a specific filter for fluorescein, using Plan Apo-Chromate 40×/1.0 objective on HP5 Ilford Black-and-White Film (Ilford Company, Basel, Switzerland) or Ektachrome 400 Color Slide Film (Kodak Company, Lausanne, Switzerland).

Actin Analyses

Two-dimensional polyacrylamide gel electrophoresis of whole liver extracts was according to O'Farrell [23] using liver tissue homogenized directly in isoelectric focusing buffer.

Total actin content and distribution in G- and F-forms was determined as described previously [20] and involved ultracentrifugational analyses of liver extracts. Briefly, perfused livers rinsed in ice-cold saline were homogenized in modified H-buffer of Bray and Thomas [21] and total extracts, 120,000g supernates and 120,000g pellets analyzed by single-dimension sodium dodecyl sulfate (SDS) polyacrylamide slab gel electrophoresis [22] containing 10% acrylamide. The actin peak was identified by use of an actin standard run with each slab, by coelectrophoresis of standard actin with liver extracts, and by diminished appearance of the band after chromatography of extracts through a DNase I actin affinity column [24]. Densitometer traces of such gels were analyzed for percent actin content on the basis of percent of total Coomassie Brilliant Blue protein stain on the gel in the actin peak (see Fig. 3).

Actin was also recovered from acetone powders [25] by chromatography on the DNase I affinity column (see Fig. 2) exactly as described by Lazarides and Lindberg [24].

Synthesis Experiments

Control and experimental rats each were injected with L-[3,4,5-^{3}H(N)]-leucine, specific activity 111.2 Ci/mmole, intraperitoneally in 0.5 ml sterile saline in isotopic amounts given in figure and table legends. Animals were sacrificed 3 hr later and excised, perfused livers minced with scissors and razor blades on ice in a 4°C cold room. A small portion was extracted with ten volumes SDS-polyacrylamide gel sample buffer by polytron homogenization and boiling for 5 min before radioactivity in hot trichloroacetic acid (TCA)-precipitable protein [26] and protein content [27] were determined. A second portion was submitted to SDS-polyacrylamide gel electrophoresis in 8-mm inner diameter glass tubes using gel recipes as for slab gels (see above) and loading 500–700 μg protein on each gel. Radioactivity in 1-mm serial slices [28] subsequently was measured.

The majority of the liver mince was extracted for actin by DNase I affinity chromatography of acetone powders, again for determination of isotopic and protein contents of the purified preparations.

Degradation Experiments

Two untreated rats were injected, one with 500 μCi L-[3,4,5-^{3}H(N)]-leucine, 50 Ci/mmole, and the other with 400 μCi of L-[U-^{14}C]-leucine, 342 μCi/mmole. Eight, 32, and 56 hr later, phalloidin (500 μg/kg body weight) was injected intraperitoneally in 0.5 ml saline into the ^{14}C-labeled rat, while an equal volume of saline

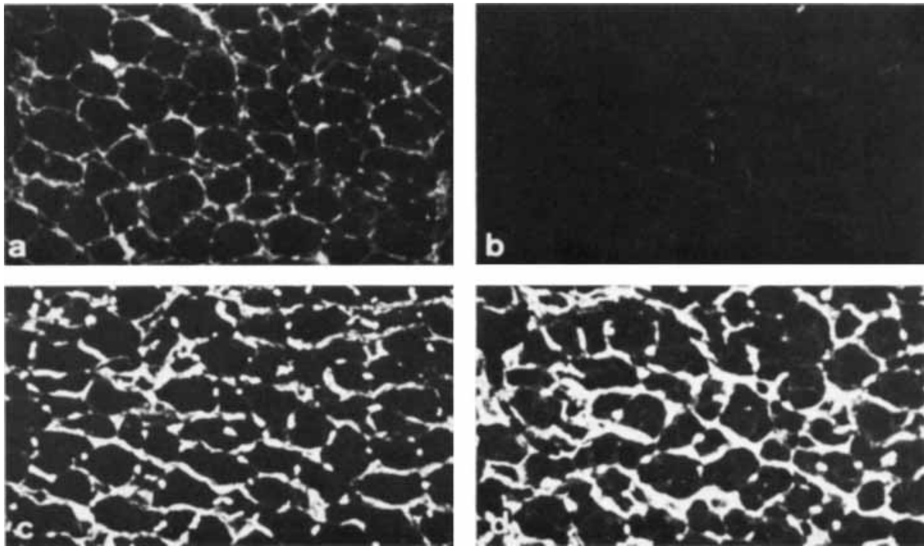

Fig. 1. Crytostat sections of liver from normal rats (a and b) and from rats injected with phalloidin for ten days (c and d). The sections were treated for indirect immunofluorescent staining with AAA alone (a and c) or after pretreatment with ADF (b and d). a) Normal liver shows a "polygonal pattern" of cytoplasmic staining with several spots corresponding to bile canaliculi. b) ADF pretreatment in normal rats abolishes AAA staining. c) In phalloidin-treated rats, the cytoplasmic peripheral staining and the spots corresponding to bile canaliculi are more intensely stained when compared with control rats and d) pretreatment with ADF does not abolish the intensity and pattern of immunofluorescent staining.

was administered to the ^{3}H-labeled rat. Animals were sacrificed 18 hr later, and perfused livers mixed and thoroughly minced together before the $^3H/^{14}C$ ratio of total mix and DNase column purified actin determined [26].

Phalloidin was purchased from Boehringer (Mannheim, West Germany) or from Sigma Chemical Company (St. Louis, Missouri). Isotopically labeled amino acids were obtained from New England Nuclear Corp., 549 Albany Street, Boston, Massachusetts or Amersham Corp., Arlington Heights, Illinois. Fluorescein-conjugated goat anti-human IgG was obtained from Behring Werke AG, Marbourg Lahn, West Germany. Wistar rats were bred and raised in the Department of Pathology, University of Geneva, Geneva, Switzerland; Sprague Dawley rats were supplied by Canadian Breeding Laboratories, St. Constance, Quebec, Canada.

RESULTS

Immunofluorescence and Sensitivity to ADF

Immunofluorescent staining of cryostat liver sections with AAA resulted in a bright fluorescence of the muscular layer of hepatic vessels and bile ducts (Fig. 1a). In addition, hepatocytes had a definite "polygonal pattern" of staining at the periphery of their cytoplasm. After ten-day phalloidin treatment (and to a lesser extent also after 24 and 48 hr) the amount of peripheral fluorescence after incubation with AAA was significantly increased (Fig. 1c) when compared with normal animals. Bright fluorescent spots apposed to the plasma membrane of many hepatocytes were facing similar spots in adjacent cells suggesting that the distribution of such spots was corresponding

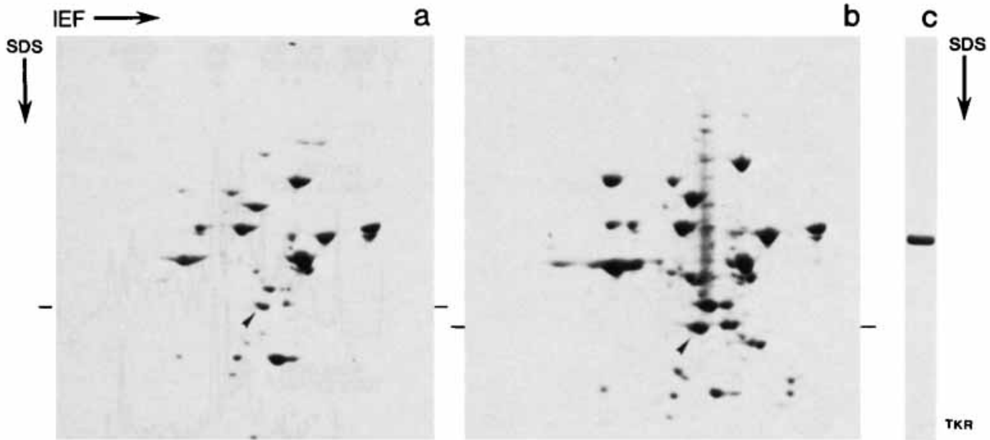

Fig. 2. Polyacrylamide gel electrophoresis of liver extracts from normal and ten-day phalloidin-treated rats. a) Two-dimensional electrophoresis of proteins from total liver of control rats extracted directly into first-dimension isoelectric focusing (IEF) buffer. Arrow indicates actin. Hash marks on each side of the gel indicate the 42,000 molecular weight region of the gel. b) Two-dimensional electrophoresis of proteins from total liver of phalloidin-treated rats. c) One-dimensional SDS-polyacrylamide gel electrophoresis of DNase I column purified actin. This preparation was one of those analyzed in the actin synthesis experiments described in Table III. The migration of actin is not the same as in the two-dimensional gels because of differences in the acrylamide concentration.

to that of bile canaliculi. In addition, fluorescent spots were randomly distributed throughout the cytoplasm of hepatocytes (only in ten-day-treated rats). In sections treated with ADF, AAA immunofluorescent staining was abolished in normal hepatocytes (Fig. 1b) but remained intense in livers sampled after 48 hr and ten-day phalloidin treatment (Fig. 1d). In all cases, pretreatment of tissue sections with control fractions (containing IgG or albumin) did not change the intensity and distribution of AAA staining (data not shown).

Actin Analyses

Two-dimensional polyacrylamide gel electrophoretograms of extracts of total liver from control and phalloidin-treated rats (eight day) are shown in Figures 2A and 2B. The actin spot was identified by approximate isoelectric point in the first dimension, by approximate mobility in the second dimension and by adding DNase I column purified actin (Fig. 2c) to the origin extracts to enrich the actin spot. Important to subsequent analyses using one-dimensional SDS gel is that there was but one other major spot in the actin molecular weight range but of slightly higher molecular weight than actin. In particular, the enrichment of actin in extracts from phalloidin-treated animals (Fig. 2b) would be the only major contributor to increases of band intensity that would be visualized in single-dimension SDS gels.

Single-dimension SDS-polyacrylamide gel analyses were next used to determine the effect of in vivo phalloidin administration on total actin content as well as the G-actin/F-actin ratio in order to establish more firmly the nature of the biochemical change caused by the cyclic peptide and to establish a time course of change over which turnover experiments would be made. Figure 3 shows densitometric traces of representative polyacrylamide gels of total extracts, 120,000g supernates (S120) and

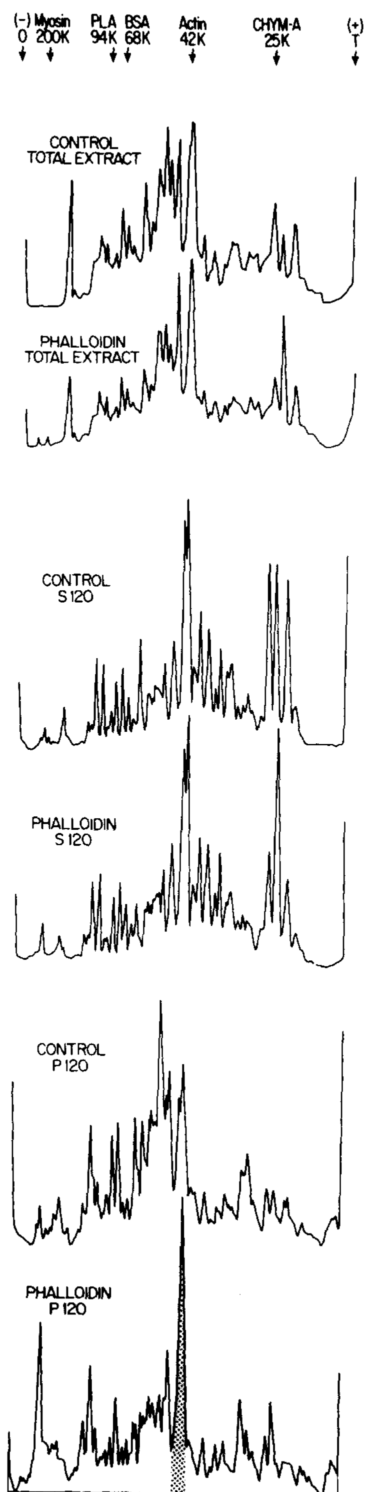

Fig. 3. Densitometric traces of SDS-polyacrylamide gel electrophoretograms of liver extracts from normal and ten-day phalloidin-treated rats. The method for quantitating the relative size of the actin band (stippled) is shown in the P120 extract of liver from phalloidin-treated rats. Shown by arrows are the locations of actin and other molecular weight markers. S120, 120,000g supernate; P120, 120,000g pellet; PLA, phosphorylase A; BSA, bovine serum albumin; CHYM-A, chymotrypsinogen A.

120,000g pellets (P120) of liver extracts of control animals and animals treated daily with phalloidin for 10 days. A number of changes in relative band intensity were observed in extracts of liver from control and phalloidin-treated animals. There was a shoulder on the high molecular weight side of the actin band, doubtless the band of slightly high molecular weight seen in the two-dimensional gels. Visual inspection suggested the relative size of the actin band was increased slightly, but definitely, in total extracts, not altered in S120 extracts but increased substantially in P120 extracts. Planimetric analyses of such densitometric scans provided results in Table I. The mean actin content as a percent of protein content in total extracts was increased by approximately 40% ($P < .001$) in three experiments, while there was no difference in percent actin content of S120 extracts. The biggest change in pooled data from three experiments, an increase of approximately 183% ($P < .001$) was in the P120 extracts from phalloidin-treated rats when compared with control rats. Analyses of a single experiment involving one-day treated rats indicated changes already were occurring, as was suggested by our immunofluorescence data.

The change in actin content after ten days of phalloidin treatment was nearly the same when expressed per equivalent amount of liver wet weight (Table I). Furthermore, there was an increase of 64 $\pm$ 8.0% (mean $\pm$ SEM) in total liver weight. Thus, total liver actin concentration and content both were increased by in vivo phalloidin treatment.

The fractionation protocol was designed to analyze G-actin and F-actin content in normal and phalloidin-treated liver, by partitioning G-actin into the S120 and F-actin into the P120 fractions, respectively. The data on that basis indicate the proportion of G-actin as a percent of liver protein was not changed appreciably, while the proportion of F-actin was increased substantially. We calculated the percent F-actin of total actin based on the total protein and percent actin content of S120 and P120 fractions from equivalent amounts of liver, with the result that 7.3 $\pm$ 0.7% (mean $\pm$ SEM) of total actin in normal liver was F-actin, but 15.8 $\pm$ 2.1% was F-actin in livers from phalloidin-treated animals.

It was necessary to provide evidence that significant exchange of actin between the unpolymerized G-actin (S120) and the polymerized F-actin (P120) pool had not occurred during isolation procedures [20,29]. Radiolabeled G-actin (S120) and F-actin (P120) fractions were prepared from rats injected 3 hr prior to sacrifice with 500 μCi ^{3}H-leucine. These ^{3}H-labeled fractions were then each added to a separate, fresh homogenate of unlabeled liver, which subsequently was fractionated to yield a second S120 and P120 fraction. Only 15.8 $\pm$ 4.2% ($n=3$) percent of labeled actin in the original, labeled S120 fraction redistributed to the new P120 fraction for control liver and 17.8 $\pm$ 4.3% ($n=3$) for liver from phalloidin-treated animals. At the same time, 13.4 $\pm$ 3.8% ($n=3$) of P120-labeled actin redistributed to the new S120 fraction for control livers and 8.2 $\pm$ 2.9% ($n=3$) for liver extracts from phalloidin-treated rats. The differences shown in Table I remained significant even when data were reanalyzed assuming maximal interconversion of actin during the isolation procedures.

The P120 fractions of livers from ten-day phalloidin-treated rats also were enriched for a protein that comigrates with known skeletal muscle myosin heavy chains; as also was the case to a smaller extent for total extracts (Fig. 3).

TABLE I. Liver Actin Content of Normal and Phalloidin-Treated Rats*

	Percent actin of total protein[a]				
		Phalloidin			
Fraction	Control	1-day	10-day	Percent change for 10-day treatment	Percent increase in actin content per equivalent amount of liver for 10-day treatment[b]
Total extract	6.9 ± 0.4	7.3	9.6 ± 1.1^c	40 ± 11^c	44 ± 9^c
	(n = 6)	(n = 2)	(n = 6)	(n = 6)	(n = 6)
120,000g supernate	8.5 ± 1.4	8.1	8.2 ± 1.2	-2 ± 12	-3 ± 13
	(n = 6)	(n = 2)	(n = 6)	(n = 6)	(n = 6)
120,000g pellet	4.6 ± 0.3	7.6	13.0 ± 0.9^c	183 ± 14^c	194 ± 16^c
	(n = 6)	(n = 2)	(n = 6)	(n = 6)	(n = 6)

*Each value for control and ten-day phalloidin extracts is the mean ± SD of data from three separate experiments, each analyzed in duplicate. Data from ten-day phalloidin extracts are mean values of duplicate samples from a single experiment: variability $\leqslant$ 15%.

[a]Weight of paper under actin peak divided by the weight of paper under the total trace.

[b]Weight of paper under actin peak from livers of ten-day phalloidin-treated animals divided by that from corresponding liver fractions, for traces of gels through which extracts for equivalent amounts of liver (wet weight) were electrophoresed.

[c]$P < .01$ vs control by Students t-test for paired samples run in adjacent tracks of the same gels.

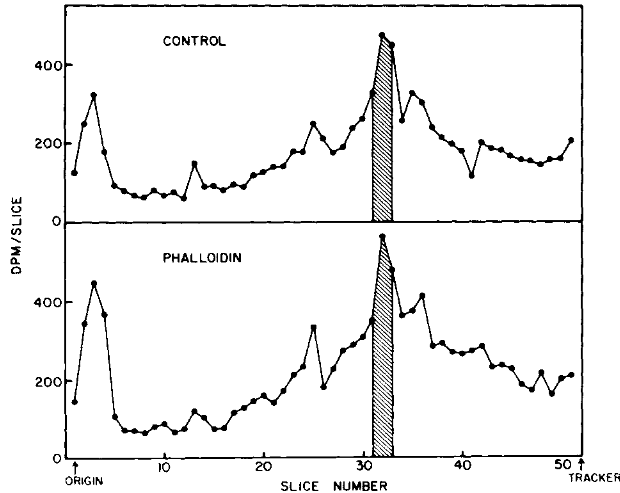

Fig. 4. Isotopic labeling of SDS-polyacrylamide gel electrophoretograms. Gels containing radiolabeled protein were cut into 1-mm serial slices and the isotopic content of each slice determined as described in Materials and Methods. The hatched area indicates that region of each gel, the radioactivity of which was attributed to actin.

Synthesis

We performed a series of experiments to test the possibility that the observed increases in liver actin content caused by phalloidin were due to increased actin synthesis. Control rats and rats given phalloidin either once or daily for ten days were injected intraperitoneally with ^{3}H-leucine and sacrificed 3 hr later. SDS extracts of total liver protein were displayed on SDS-polyacrylamide gels, which were then sliced to measure radioactivity. There appeared to be no obvious increase in the isotopic content of the actin peak of livers from ten-day treated animals as a percent of total incorporation (Fig. 4). Analyses of gels from three separate experiments confirmed this impression (Table II). Results were the same when the experiment was performed with rats treated only one day with phalloidin (data not shown).

The actin band region is complex, and indeed, a definite shoulder exists in most preparations (Fig. 3). Therefore, we wished to confirm our results by studying ^{3}H-leucine incorporation into purified actin. The isotopic experiments were repeated, but this time we compared the specific activities of DNase affinity column purified actin and total liver protein (Table III). Figure 2c shows that the column-purified actin was highly purified (>98%). The only radioactivity in the gel was located with the actin band. For obviation of problems with differences in precursor amino acid pools in control and experimental animals, incorporation of ^{3}H-leucine was expressed as the quotients of the specific activities of actin and total cell proteins [30]. This approach assumes that major changes have not occurred in the pattern of proteins synthesized in control and experimental tissues, as appears to be the case in our studies (Fig. 4). In livers from control animals, the specific activity of actin was the same as (experiments 2 and 3) or slightly less (experiment 1) than that of total cellular protein indicating a similar or slightly lower fractional rate of actin synthesis as compared with total protein synthesis. In livers from phalloidin-treated animals the fractional

TABLE II. Actin Synthesis in Normal and Phalloidin-Treated Rats*

	Percent of total radioactivity in actin peak	
	Control	10-day Phalloidin
Experiment #1	8.5	8.3
Experiment #2	8.1	8.7
Experiment #3	8.3	8.1

*Control rats and rats treated with phalloidin daily for 10 days, each were injected intraperitoneally with 500 μCi of ^{3}H-leucine. Animals were sacrificed 3 hr later, and SDS extracts of total liver protein were displayed on SDS-polyacrylamide gels prior to isotopic analysis (See Materials and Methods). Data are single determinations: replicate analyses of a single sample indicated a variability of ± 15% (not shown).

TABLE III. Effect of Phalloidin on Liver Actin Synthesis*

	Leucine incorporation (DPM/mg)		Actin synthesis	
	Actin	Total liver protein	(DPM per mg actin) / DPM per mg total liver protein	Control / Phalloidin
Expt #1				
Control	31,000 ± 200	36,400 ±1,100	0.85 ±0.03	—
Phalloidin (ten days)	29,500 ±1,600	38,400 ±1,400	0.77 ±0.03	1.11[a] ±0.02
Expt #2				
Control	46,700 ± 300	37,800 ±1,300	0.94 ±0.03	—
Phalloidin (ten days)	37,500 ±1,000	36,200 ±1,200	1.04 ±0.01	0.91 ±0.04
Expt #3				
Control	45,600 ± 500	44,000 ±3,600	0.95 ±0.04	—
Phalloidin (ten days)	40,900 ± 900	49,600 ± 900	0.83 ±0.02	1.15[a] ±0.04
Phalloidin (1 day)	42,100 ±1,900	50,000 ±1,400	0.84 ±0.04	1.14[a] ±0.01

*Control rats and rats treated with phalloidin for the times indicated each were injected 24 hr after the last phalloidin treatment with 500 μCi (experiments 1 and 2) of 600 μCi (experiment 3) of ^{3}H-leucine. Animals were sacrificed 3 hr later, and the specific activity of total protein and DNase column purified actin was determined as described in Materials and Methods. Data are given as mean ± SD of triplicate determination.

[a]Ratios significantly different from 1.0 ($P < 0.1$) by Wilcoxon signed ranks test [40].

rate of actin synthesis appeared the same for total protein, but it was lower in the other two experiments. These differences can be seen expressed as specific activities (Table III, columns 1,2) and normalized specific activities (Table III, column 3: a ratio of less than 1.0 indicates a slower relative fractional rate of synthesis for actin than for total cellular proteins). There was a reduction in the normalized fractional rate of actin synthesis in livers from phalloidin-treated animals compared with control in experiments 1 and 3 (Table III, column 4, ratio > 1.0) and no change in experiment 2. The data indicate that, at the time of our isotopic studies after phalloidin treatment,

TABLE IV. Actin Degradation in Normal and Phalloidin-Treated Rats*

	$^3H/^{14}C$ ratio	
	Experiment #1	Experiment #2
Total extract	4.7 ± 0.1	4.1 ± 0.2
DNase purified actin	2.9 ± 0.2[a]	2.1 ± 0.3[a]

*See Materials and Methods and Results for experimental details. Data are from two experiments and represent the mean ± 1 SD of triplicate measurements on each of two replicate samples in each experiment.
[a]$P < .01$ vs control.

there was no relative increase in actin synthesis which could explain the observed increase in relative actin content (Fig. 3, Table I: see Discussion).

Degradation

We next wished to know if phalloidin had a selective effect upon the degradation of actin. Rats were injected with ^3H- or ^{14}C-leucine prior to receiving either phalloidin or saline at 7 hr, 32 hr, and 56 hr (see Materials and Methods). Animals were sacrificed 18 hr after the last phalloidin or saline injection, and the $^3H/^{14}C$ ratio of actin and total proteins measured. Results from two such experiments are shown in Table IV. The $^3H/^{14}C$ ratio of column-purified actin was significantly less than that for total liver proteins. This indicates that the rate of actin degradation relative to total protein degradation was depressed in livers from phalloidin-treated animals, an effect that would explain the changes in liver actin content we have observed.

DISCUSSION

The purpose of our experiments was to determine the effects of phalloidin on liver actin distribution, content, and turnover. We began by investigating phalloidin-induced changes in the pattern of actin-directed immunofluorescence in hepatocytes using cryostat sections untreated or treated with ADF for correlation with biochemical measurements of liver actin content, G/F ratio, and turnover.

Immunofluorescent results confirm the previous observations [12] of AAA-positive actin microfilament accumulation in hepatocytes following the administration of phalloidin. In addition, we observed that pretreatment with ADF of cryostat sections of normal liver abolished AAA immunofluorescent staining, while the same pretreatment did not modify AAA staining in sections of phalloidin-treated livers. Further control experiments showed that this lack of activity was seen using larger amounts of ADF and longer times of incubation than those reported here, thus suggesting that phalloidin stabilizes F-actin against the action of ADF (data not shown). ADF depolymerizes actin very rapidly (about 20 sec when incubated with F-actin in vitro [19]) in a molar ratio of 1 molecule of ADF to 20 molecules of actin. There is no change in actin mobility on SDS-polyacrylamide gel electrophoresis after incubation of F-actin with ADF. The combined use of ADF and phalloidin appears to represent a useful tool for the study of the mechanisms of actin polymerization and depolymerization.

The actin content of liver from phalloidin-treated animals was increased substantially by ten days, although the effect was apparent after only one day of treatment

with the alkaloid. This change was accompanied by an even bigger increase in the proportion of F-actin in livers of drug-treated animals, the F-actin pool more than doubling within ten days. At the same time, the G-actin pool was not altered substantially. These results were anticipated from our studies of phalloidin-induced changes in actin-directed immunofluorescence, based upon our recent studies of regenerating and neoplastic epithelial tissues [20].

Elias et al [11] reported a much smaller percent increase in the F-actin pool (approximately 15% increase after seven days of treatment) than we observed even after one day of drug treatment (Table I) for reasons that are not clear. The improved resolution of our gel electrophoretograms might offer an explanation. The changes in actin content we observed were similar whether data were expressed as percent actin per total extractable protein in each fraction we analyzed or as per equivalent amount of liver, the latter important to determine that the percent increase in actin content was not due to relative decreases in the amounts of other proteins. The total weight of liver also is increased substantially by in vivo treatment of phalloidin.

At the same time, the protein pattern of the SDS gels is complex, and the actin band contains at least one shoulder (Fig. 3). Our two-dimensional gel analyses indicate the preponderant peak is actin. Nonetheless, some caution should be taken regarding the absolute magnitude of the calculated alterations in actin content. Importantly, the change in protein content in the actin molecular weight range due to phalloidin is probably all due to changes in actin content itself (Fig. 2).

A different result occurs with treatment of freshly isolated hepatocytes with phalloidin in vitro in which case the amount of F-actin increases while the amount of G-actin decreases [2]. Presumably, there was not enough time in the in vitro experiments for phalloidin-induced changes in actin turnover to become apparent.

We did not find increased relative rates of actin synthesis in phalloidin-treated liver, although this has been suggested to explain increased liver actin content [see 11]. Our observations were made at two different times post-phalloidin treatment, at least one of which (one-day treatment) occurred when actin content was increasing to reach levels obtained at later times (e.g., at ten days). For altered synthesis to explain a relative increase in content of a protein would require that the relative fractional rate of synthesis of that protein compared with other proteins be increased at some point over the time course during which content is increasing. Experiments with freshly isolated hepatocytes from control and phalloidin-treated rats provided the same negative results with regard to altered relative rates of actin synthesis (data not shown). At the same time, it remains possible that the relative fractional rates of actin synthesis in phalloidin-treated animals were elevated at times we did not examine, e.g., immediately after phalloidin treatment. Detailed time-course studies would be necessary to evaluate this possibility.

Our data allow no conclusions regarding absolute rates of total protein or actin synthesis since analyses of proper precursor amino acid pools were not made [see 31]. It is for this same reason that caution must be exerted in interpreting the conclusion of Gravela and Poli [32] that phalloidin inhibits protein synthesis by isolated hepatocytes. It also has been reported that phalloidin dissociates ribosomes of intact cells but does not appear to alter protein synthesis when added to cell-free systems [33].

We utilized a dual-isotope method to ascertain whether or not phalloidin altered relative rates of actin degradation. Administration of isotope to identical animals

insured similar patterns of incorporation of label into protein prior to phalloidin or saline treatment. Animals were studied approximately 2.5 days later, a time approximately equal to the half-life of liver proteins [see 34] and when true differences in rate of loss of isotope from prelabeled proteins would be visualized most easily. Our data indicate that actin degradation is depressed relative to that for total liver proteins based upon the fact that the $^{3}H/^{14}C$ ratio was significantly less for actin than for total liver protein. The difference was far greater than the error of the method [35,36, data not shown].

Pollender and Gruda [37] have shown that phalloidin protects actin against trypsin- or pronase-induced proteolysis in vitro. It is well known that proteins most susceptible to in vitro proteolysis have short in vivo half-lives [34]. Thus, the experiments of Pollender and Gruda [37] offer strong supportive evidence to ours that the major mechanism by which phalloidin induces increases in liver actin content in vivo is by inhibiting actin degradation.

As with our synthesis experiments, our turnover studies were not designed for determining what effect phalloidin has on absolute rates of protein degradation. Such measurements are even more difficult than are measurements of synthesis using isotopically labeled amino acid [See 38]. At the same time, studies of the effects of phalloidin on protein degradation might provide insight into translocation mechanisms postulated for protein degradative processes [34] given the effect of phalloidin on actin distribution and function.

Our studies of DNase column purified actin require that the actin extracted from acetone powders by this method is representative of the actin present in livers of normal and phalloidin-treated animals. Our synthesis experiments suggest this is true, since the same results were obtained whether total actin or DNase column purified actin were analyzed for isotopic content. We do not know if the same is true for the degradation experiments. Bray and Thomas [21], however, have provided evidence that there is no precursor-product relationship in the synthesis of F- and G-actin in fibroblasts. Furthermore, results were the same when actin extraction was in the presence of 0.74 M guanidine hydrochloride, a treatment thought to depolymerize all actin [39; data not shown]. Thus, we believe that phalloidin inhibits the degradation of F-actin.

Fulton et al [13,14] postulate the coordinate assembly of cytoskeletal elements during or immediately after their synthesis on polyribosomes. If true, this would have predicted that increases in actin content due to increased synthesis would have been accompanied by similar increases in the content and synthesis of other cytoskeletal proteins such as the keratins of liver hepatocytes (molecular weight range, 50,000–60,000). Inspection of Figures 3 and 4, respectively, indicate that neither has occurred. This provides further evidence, admittedly circumstantial, against phalloidin-induced increases in actin content being due to increased synthesis, as it also precludes our results as a test of the Fulton hypothesis.

Our results leave open the question as to what regulates the actin content of cells. Demands for appropriate cytoskeletal function may play a role, especially if the turnover of individual cytoskeletal proteins must be coordinate. It becomes important to understand in the case of phalloidin treatment whether the toxin siphons off actin with which it is complexed away from the degradative machinery of the cell, leaving uncomplexed actin functional and turning over together with other cytoskeletal elements at a normal rate. Again, it would appear that phalloidin will be an important

tool for the cell biologist to understand the role of actin in cell function as well as to understand the control of actin turnover.

In summary, we have found that in vivo treatment of rats with phalloidin causes a change in the distribution, stability against the actions of ADF and content of liver actin. Increases in actin content are due to selective increases in the F-actin compartment and appear to be accompanied by decreased relative rates of degradation but not increased relative rates of actin synthesis. Changes in the degree of stable polymerization of cellular actin may well be causally related to the changes that are known to occur in hepatocyte function after administration of the toxin. Altered sensitivity of actin to proteases induced by phalloidin appears a likely explanation for altered actin degradation and hence cellular build-up.

ACKNOWLEDGMENTS

We thank Mrs. Elizabeth Rungger-Brändle for helpful discussions; Mrs. F. Gabbiani, M. Bouland, and Miss V. Roethlisberger for technical help; Mr. J.-C. Rumbeli, and Mr. E. Denkinger for photograph work; and Mrs. L. Davis and Ms J. Bode for secretarial help with the manuscript.

This work was supported in part by the Swiss National Science Foundation grant No. 3.4450.79, NIH grant numbers HL14212 (SCOR) and AM21621, and by Fogarty International Fellowship (RBL) TW00316.

REFERENCES

1. Tilney LG, Clain JG, Tilney MS: J Cell Biol 81:229–253, 1979.
2. Prentki M, Chaponnier C, Jeanrenaud B, Gabbiani G: J Cell Biol 81:592–607, 1979.
3. Lindberg U, Carlsson L, Markey F, Nyström LE: Methods Achiev Exp Pathol 9:143–170, 1979.
4. Wieland T: Adv Enzyme Regul 15:285–300, 1977.
5. Lengsfeld AM, Low I, Wieland TH, Dancker P, Hasselbach W: Proc Natl Acad Sci USA 71:2803–2807, 1974.
6. Low I, Wieland T: FEBS Lett 44:340–343, 1974.
7. Dancker P, Low I, Hasselbach W, Wieland T: Biochim Biophys Acta 400:407–414, 1974.
8. Taylor DL, Wang Y-L, Heiple JM: J Cell Biol 86:590–598, 1980.
9. Gabbiani G, Montesano R, Tuchweber B, Salas M, Orci L: Lab Invest 33:562–569,1975.
10. Dubin M, Maurice M, Feldmann G, Erlinger S: Gastroenterology 75:450–455, 1978.
11. Elias E, Hruban Z, Wade JE, Boyer JL: Proc Natl Acad Sci USA 77:2229–2233, 1980.
12. Gabbiani G, Montesano R, Tuchweber B, Salas M, Ora L: Lab Invest 33:562–569, 1975.
13. Fulton AB, Wan KM, Penman S: Cell 20:849–857, 1980.
14. Fulton AB, Wan KM: J Cell Biol 91:418a, 1981.
15. Low RB, Gabbiani G: J Cell Biol 87:223a, 1980.
16. Gabbiani G, Ryan GB, Lamelin JP, Vassali P, Majno G, Bouvier C, Cruchaud A, Luscher EF: Am J Pathol 72:473–488, 1973.
17. Gabbiani G, Chaponnier C, Zumbe A, Vassalli P: Nature 297:697–698, 1977.
18. Chaponnier C: Thesis No. 1949, Faculty of Sciences, University of Geneva, Switzerland, 1980.
19. Chaponnier C, Borgia R, Rungger-Brandle E, Weil R, Gabbiani G: Experentia 35:1039–1040, 1979.
20. Low RB, Chaponnier C, Gabbiani G: Lab Invest 44:359–367, 1981.
21. Bray D, Thomas C: J Mol Biol 105:527–544, 1976.
22. Laemmli UK, Favre M: J Mol Biol 80:575–599, 1973.
23. O'Farrell PH: J Biol Chem 250:4007–4021, 1975.
24. Lazarides E, Lindberg U: Proc Natl Acad Sci USA 71:4742–4746, 1974.
25. Vandekerckhove J, Weber K: J Mol Biol 126:783–802, 1978.
26. Airhart J, Kelley J, Brayden JE, Low RB, Stirewalt WS: Anal Biochem 96:45–55, 1979.
27. Bradford MM: Anal Biochem 72:248–254, 1976.

28. Dice JF, Schimke RT: J Biol Chem 247:98–111, 1972.
29. Rubin R, Warren R, Lukeman DS, Clements E: J Cell Biol 78:28–35, 1978.
30. DePhillip R, Chadwick D, Ignotz R, Lynch W, Lieberman I: Biochemistry 18:4811–4819, 1979.
31. Rannels DE, McKee EE, Morgan HE: In: Litwack G (ed): "Biochemical Actions of Hormones IV." New York: Academic Press, 1977, pp 135–195.
32. Gravela E, Poli G: Experentia 33:603–604, 1977.
33. Gravela E, Zuretti ME, Poli G: Res Commun Chem Pathol Pharmacol 12:101–110,1975.
34. Goldberg AL, Dice JF: Annu Rev Biochem 43:835–869, 1974.
35. Arias I, Doyle D, Schimke RT: J Biol Chem 244:3303–3315, 1969.
36. Low RB, Cerauskis P: J Nutr 107:1244–1254, 1977.
37. Pollender JM, Gruda J: Can J Biol Chem 57:49–55, 1979.
38. Waterlow JC, Garlick PJ, Millward DJ: "Protein Turnover in Mammalian Tissues and in the Whole Body." Amsterdam: North-Holland Publishing Co, 1978.
39. Blikstand I, Markey F, Carlsson L, Persson T, Lindberg U: Cell 15:935–943,1978.
40. Dixon WJ, Massey FJ Jr: "Introduction to Statistical Analysis," Ed. 3. New York: McGraw-Hill, 1969.