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IN VITRO STUDIES OF BEATING HEART CELLS IN CULTURE

XVI MYOSIN TURNOVER AND THE EFFECT OF SERUM¹

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ABSTRACT

Myosin turnover in rat heart cells in culture was determined by measuring the rates of degradation and synthesis. The half life of myosin in complete growth medium was three and one half days.

Cells in medium without serum do not divide and show an increased degradation and decreased rate of synthesis. Non-dividing cells in complete growth medium turnover myosin at a normal rate. Serum contains factors which protect myosin against degradation and also stimulate synthesis.

Myosin synthesis seems to be dependent on RNA synthesis, since in the presence of actinomycin D a decrease in myosin occurs between 2 to 4 hours.

Cells in culture for one month maintain their ability to synthesize myosin.

The goal of studies carried out in this laboratory on primary cultures of beating rat heart cells (1, 2) has been to define the factors involved in the attainment and maintenance of the differentiated state of these cultures. The cell morphology (3), beating phenomenon (4), and the specific metabolism (5, 6), have been used as markers for this state. Recently our work has focused on the specific enzyme, myosin-ATPase, as a more fundamental indication of differentiation.

A correlation between growth rate of the cultures and their myosin-ATPase activity has been shown (7): the normal increase of ATPase with time is actually dependent on cell division within the cultures. Further, serum has been shown to be necessary for maintenance and increase of myosin-ATPase activity. The question of how these enzyme levels are changed by such factors and by culture conditions is of fundamental importance. This paper presents evidence that myosin turnover does occur rather rapidly and that the changes in myosin-ATPase activity observed are the result of changes in the turnover processes, the rates of both synthesis and degradation of myosin being affected by serum factors.

MATERIAL AND METHODS

Hearts obtained from neonatal rats were minced and trypsinized, and single cells were prepared and cultured essentially as described by Harary and Farley (8). The usual plating concentration was 5×10^6 cells per plate, in Falcon 20 x 100 mm plastic tissue culture dishes. The standard

growth medium was Ham's F10 (9) (Hyland), supplemented with 100,000 u/liter penicillin and 100 mg/liter streptomycin, and with 10% fetal calf serum and 10% horse serum. This medium is referred to as "complete growth medium" or "CGM"; and the same medium without the serum supplement is referred to as "minimal medium" or "MM". Cells were always plated and held for at least the first 24 hours in the complete growth medium. This was then replaced by the experimental medium which was subsequently changed every two or three days.

Cycloheximide (Sigma), puromycin HCl (NBC), colchicine (Calbiochem), and actinomycin D (Calbiochem) were all made up as sterile 100X concentrated stock solutions and kept frozen. Irradiation of cells was carried out on the monolayers in culture dishes at room temperature with a ^{60}Co gamma source, at a rate of approximately 125 rad/min. The usual dose of 2000 rads has been shown (10) to stop cell division and (7) to inhibit growth of heart cells to less than a two fold increase over two weeks, while the cells remained normal in appearance and in their beating activity.

Protein was determined by the method of Lowry (11), or by Eagle's modification of this for cultured cells (12), using crystalline bovine serum as a standard, and measuring optical density at 660 m μ .

Single and double label counting was carried out in a Packard Tri-Carb liquid scintillation counter, using Triton-toluene scintillant (13). Quenching was corrected for by the channels-ratio method.

ATPase activity units are expressed as nanomoles of inorganic phosphate produced from ATP per minute, and all values given are averages of duplicate assays. Myosin-ATPase was assayed by the method of Perry (14).

Cells were washed on the plates, scraped, and homogenized with a Virtis homogenizer in 0.05 M tris-HCl, pH 7.5. "Total protein" refers to this homogenate. The tris homogenate was centrifuged at 30,000 x G for 15 minutes, and the resulting pellet rehomogenized in 0.5 M KCl and 5 mM ATP. This second homogenate was centrifuged at 30,000X G for 15 minutes. The 0.5 M KCl supernatant is referred to as the "myofibrillar extract" and was the material used for the ATPase assay or for gel electrophoresis.

Myosin was isolated by electrophoresis on 3.6% polyacrylamide gels (15, 16) using the flat-sheet apparatus of Reid and Bielecki (17). The modifications of the general protein separation techniques required for work with myosin have been described (18), the chief ones being the tris glycine electrode buffers and tris-HCl gel buffers, and the use of 0.1% sodium lauryl sulfate. Protein bands were made visible by staining with 0.2% Coomassie Blue (Sigma) (19) in acetic acid-methanol. Stained bands were measured on a Photovolt recording densitometer equipped with an integrator. The correlation between amount of myosin as measured by densitometry of gels and myosin-ATPase activity held under all culture conditions (18). Because of the greater facility and accuracy the enzyme assay was usually employed for determining the amount of myosin in the cultures. Radioactive protein was measured by cutting the entire gel into 2 or 5 mm bands, or more often by cutting out only the stained myosin bands, and counting these gel strips. The gel was dissolved by heating in 30% H_2O_2 , and the solution added to Triton-toluene scintillator.

Protein degradation was measured by following the loss of radioactivity in protein after a single initial pulse of 1 μ Mole radioactive leucine (^{14}C UL, specific radioactivity 1 $\mu\text{C}/\mu$ Mole). This initial incubation usually began at the time of plating and continued for 48 hours. At this point, "Day 2", duplicate sets of four plates were sacrificed and the initial radioactivity in the total and myofibrillar protein and in the myosin band were determined. The remainder of the plates were changed to experimental or control medium 10 ml per plate, containing excess cold leucine (40 mg/liter). Duplicate sets of four plates were sacrificed for each subsequent point in the experiment. Medium was changed every two days to fresh chase medium. Radioactivity at each point was expressed as the percent of the initial radioactivity; and a straight line was fitted to these values plotted versus time in a semi-logarithmic fashion. From the slope of this line the decay rate constant and the half life could be determined.

Protein synthesis rates were usually determined from measured values of degradation rates and of absolute amounts of protein present at various times. The relation of these processes may be expressed (20) as:

$$\frac{dp}{dt} = A - BP$$

where P is the amount of protein present, A is the zero order rate constant for the synthesis and B is the first order rate constant for degradation. An approximate solution to this yields the following expression which we used for evaluating protein synthesis rate:

$$P_s = P_1 - P_o + B(P_o)$$

where P_s is μg protein synthesized, P_o = initial amount of protein and P_1 = protein level at time t_1 ; therefore the protein synthesized

equals the absolute change in amount plus the protein degraded.

Protein synthesis rates were also measured independently of degradation rates by measuring uptake of radioactive amino acids from short pulses at various times of culture. Comparison of these values normalized for differences in radioactive pool sizes indicates relative synthesis rates. Absolute rates of synthesis of myosin could be obtained when the amino acid composition of the protein was taken into account.

RESULTS

The first indications that a substantial turnover of myosin occurs in the heart cell cultures came in preliminary studies on the effect of protein synthesis inhibitors on the myosin-ATPase levels. Myosin appeared to be remarkably short-lived (Fig. 1). Under conditions in which 95% of protein synthesis was inhibited with either cycloheximide or puromycin, the level of the enzyme decreased quite rapidly, implying a marked degradation rate. This observation, coupled with the finding of a steady increase of myosin-ATPase under normal culture conditions (7), suggested there was a rapid rate of myosin synthesis in the cells: synthesis sufficient to account for the actual increase in level, despite the large amounts of myosin constantly being degraded. To investigate this further, myosin synthesis and degradation rates were measured directly under normal culture condition.

The heart cell cultures usually grow in a linear fashion with time (7) over the first week or two. The total protein (a direct measure of

cell number (Fig. 2) typically doubles in the first two days and increases by the same increment for each two day period thereafter, up to a three-to five-fold increase, at which point (corresponding to the time at which cells are confluent on the plates) the increase continues at a lesser rate, as previously observed. The protein in the myofibrillar extract increased at a slightly faster rate than the total protein, starting out as roughly 20% of the total protein, rising to 30 or 40% of the total after one week of culture. During this period myosin-ATPase increased at a rate of 50 to 70% as compared to that of the total protein.

Degradation and Synthesis of Myosin. Degradation of the total and myofibrillar protein fractions is first order when measured directly by following the loss of radioactive protein from pre-labelled cultures (Fig. 3). The half life of the two fractions in this experiment was three and one half days, representative of that found in all experiments involving CGM.

The degradation of myosin (Fig. 3), measured as radioactivity lost from prelabelled myosin, also occurs quite rapidly, at a rate similar to that of total and myofibrillar protein, or a half life again of about three and one half days.

The increase in myosin level can be measured not only by following the myosin-ATPase activity but also by measuring the actual amount of myosin present at various times in culture, by isolating the protein electrophoretically. The increase of myosin with time in culture is qualitatively similar to that already described for myosin-ATPase activity (7). The

increase in amount of myosin present, together with the rapid degradation indicates that myosin synthesis is occurring throughout the experimental period.

This observation was verified by measuring the synthesis of myosin directly. Cells were incubated with radioactive amino acids for short (3 hour) periods on the days indicated (Fig. 4) and immediately harvested and assayed for radioactive myosin. The amount of label incorporated into myosin increased with time, suggesting that the myosin synthesis rate was increasing. The results of this type of experiment in a growing, changing system are subject to a considerable number of qualifications but approximations of absolute synthesis rates can be made.

Using the ^{14}C leucine incorporation data of Fig. 4, the experimentally determined specific radioactivities of the leucine in the medium and in the cells, and the number of leucine residues in myosin (amino acid analysis, unpublished data), we have calculated that, 34, 36, and 40 micrograms of myosin per plate were synthesized on days 4, 6, and 8 respectively, of this experiment.

Effect of Protein synthesis inhibitors on degradation and synthesis.

Our initial studies of myosin turnover involved measuring disappearance of enzyme activity after treatment with protein synthesis inhibitors (Fig. 1). At the concentrations used, the incorporation of radioactive amino acids into TCA precipitable material was decreased 95%. There was little or no effect on the appearance of the cells over 2 days of treatment, and their effect was reversible during this period. In the presence of the inhibitors the normal increase in total protein is stopped, (Fig. 5a) and the levels actually almost always decreased after two days

of exposure to the inhibitors. Puromycin was less effective than cycloheximide, even at near toxic doses, as has been reported by others (21) and was thus not always used in experiments of this type. The effect on total protein degradation is apparently slight, sometimes increasing, sometimes decreasing and sometimes not affecting the rate (Fig. 5b). Some reincorporation of label may be occurring under conditions where synthesis is allowed to continue. Evidence for this was occasionally seen in experiments in which the degradation curve of the protein was biphasic, with a very slow rate on the first two days, perhaps because of reincorporation of label during the day two to four period. Often when this situation was seen, the straight line through the radioactivity values for the last three days was extrapolated back to a hypothetical 100% value for the first day.

The effects of inhibitors on levels and degradation of myofibrillar protein were quite similar to those on total protein. Myosin degradation often appeared to be increased upon addition of inhibitors during the first period after the radioactive incubation (Fig. 6), perhaps due to reincorporation in the controls, but was normally unaffected or actually slightly decreased at other times during the course of the experiment. The decrease in rate of myosin degradation was seen quite consistently although the difference between inhibitor-treated cells and controls was often so slight that the significance of this observation is somewhat doubtful.

As will be discussed later, rates of synthesis were calculated from these degradation rates. As expected, the synthesis was quite low,

usually zero, in the presence of protein synthesis inhibitors (Table I).

Non-dividing cultures. Previous work from this laboratory demonstrated that the normally occurring increase in total myosin-ATPase in culture is dependent upon cell division. To determine whether synthesis and degradation or activation and inactivation processes were being affected under certain non-growing situations, we studied turnover rates in three such systems: 1) cultures in minimal medium (MM): Ham's F10 with added antibiotics, but no serum, and not containing the albumin used in earlier work in this laboratory; 2) cultures in CGM treated with colchicine, $5 \times 10^{-7} M$ (0.2 ug/ml). Twice the previously used concentration was found to be necessary to inhibit cell division with the present (F10 base) medium; and 3) cultures irradiated as described in "Methods". Table I shows the effect on levels and degradation of total and myofibrillar protein.

The effect in minimal medium was as previously shown. The total and myofibrillar protein levels remained essentially constant. The degradation of these protein fractions under starvation conditions proceeded quite rapidly. In these cultures the myosin-ATPase also dropped quite rapidly over the period of a week with a concurrent rapid degradation of myosin (Figure 7 and Table I) much more rapidly than that in cultures which contain serum.

Under the other two non-dividing conditions, irradiation and colchicine, total and myofibrillar protein levels were held constant or at least to less than a two-fold increase as had been previously described. Degradation under these conditions was at an intermediate rate between the CGM

and the MM situations (Table I). Myosin-ATPase was maintained at a constant level, while degradation of myosin was only slightly, if at all, increased by maintenance under these condition (Fig. 7). We have shown that under these three experimental conditions the myosin-ATPase activity is a measure of absolute amounts of myosin present (18). Thus synthetic rates can be calculated validly from these enzyme levels and the degradation data.

The degradation rates of myosin in minimal medium cultures was markedly greater than that in controls. It is known that these cells are not dividing (7), but mere absence of cell division does not seem to account for this increase in degradation, since cells in other non-dividing situations do not differ so markedly in degradation rates from controls (Fig. 7).

To determine whether the MM effects were due to deprivation of serum factors, cells were incubated in minimal medium, and changed back to complete medium with or without colchicine (5×10^{-7} M). Colchicine-treated cells were also treated with cycloheximide or actinomycin D. The reversibility of some of the effects of starvation upon return of the cells to complete medium has been documented (7). In the present experiments (Table II) the total protein remained constant or decreased slightly in minimal medium, increased at the control rate when cells were returned to CGM, and remained constant when returned to CGM plus colchicine. The degradation rate of total protein was decreased drastically by return to complete medium and decreased to some extent in colchicine-treated cells. As was often seen, the degradation rate was decreased in the presence of cycloheximide.

The myosin-ATPase decreased in MM, increased slightly (often more than seen here (7)) in cells returned to CGM and remained constant in colchicine-treated cells. Cycloheximide and actinomycin D prevented the increase in activity in cells returned to CGM. The degradation rate of myosin was seen to decrease upon the return to complete medium, with the colchicine effect intermediate, and again cycloheximide apparently having its unusual effect of decreasing the degradation rate compared with non treated cells.

Protein synthesis rates. Protein synthesis rates were calculated from degradation rates and absolute levels of protein fractions and can be summarized thus for culture in normal medium: Synthesis of total and extracted protein remains constant at 300 and 100 ug/plate/day respectively and may increase with time over the culture period to 800 and 300 ug/plate/day. The myosin rate (Table III) varies up to two-fold from culture to culture with a similar variation in degradation rates and in absolute amounts of protein. Because of this considerable variation in the control cultures, the effects of various treatments on synthesis rates were compared by expressing the rates as percent of the control for each day in each individual experiment. Culture in minimal medium (Table IV) resulted in a great decrease in the ability to synthesize total protein, and an even greater decrease in myosin synthesis. Colchicine-treated and irradiated cells while losing some synthetic ability, still were capable of considerable synthesis, of total, myofibrillar and myosin fractions. The data from the experiment with protein synthesis inhibitors furnishes a check on this method of calculation since the synthesis rates are low or zero as would be expected. The

relative synthesis rates for the cells grown on MM and restored to CGM, indicated that return to CGM reversed the MM effect on synthesis as well as degradation, even, to some extent, in the presence of colchicine (Table V).

Actinomycin D. The effect of actinomycin D treatment on the heart cells (at a level, 0.2 ug/ml, which inhibits at least 95% of RNA synthesis within four hours) was a reduction in both total protein and myosin levels (Table VI). There was usually a slight increase in degradation rate of total protein, but no change or perhaps a decrease in myosin degradation after addition of the inhibitor. The loss of myosin-ATPase with no change in degradation indicated that continued synthesis of myosin required RNA synthesis. In an effort to measure this dependence, that is to obtain a better idea of the decay of myosin messenger activity, the ability to synthesize myosin at shorter times after administration of the drug was determined. Total and myofibrillar protein are maintained or decreased slightly in level after 24 hours, while the ability to incorporate amino acids into these fractions decreased rapidly, to 50% of the control in seven hours and to 10% in 24 hours (Table VII).

Myosin-ATPase and myosin level (in ug/plate) decreased somewhat in actinomycin treated cells. In some experiments the decrease was more marked than that shown there. Again, myosin synthesizing ability decreased rapidly with time, to 50% of control rate in nine hours and to 15% in 24 hours.

Long Term Cultures. Almost all studies reported here were carried out on the cells within the first 10 days of culture, during which time the proportion of myocardial cells within the growing cultures can be

estimated. When cultures were observed over the period of one month, the following were noted: (Table VIII) Total protein synthesis increased steadily then usually leveled off after 10-15 days (1, 2). Degradation of both myosin and total protein (measured by loss of initial label over two intervals, days 2 to 6 and days 10 to 28) became markedly slower in older cultures. Synthesis as measured directly by incorporation (3 hour pulses) increased in total protein and remained rather constant for myosin. The incorporation data give qualitative indication of myosin synthesis throughout the period, and calculation of synthesis from degradation rates supports this observation.

DISCUSSION

Heart cells in culture have a significant turnover of myosin. The maintenance and increase in myosin and the reversal of the loss of myosin in MM by serum, is the result of an effect of the serum on both synthesis and degradation. A factor or factors in serum increase myosin synthesis and decrease its degradation. This is independent of the increase in total myosin which occurs in CCM dependent cell division. It would seem therefore that multiple factors and conditions affect the level of myosin.

The half life for myosin ranged from 4 to 6.5 days under normal culture conditions, and the synthesis of the protein occurred throughout the culture periods studied, at a rate of from 10 to 40 micrograms per plate per day. The variation in growth and synthesis rates, as well as differences in initial protein and ATPase levels, in control cultures

reflect a problem inherent in this type of system. Uncontrolled parameters can vary so that initial cell populations, general growth characteristics, function, differentiation and dedifferentiation processes may vary from culture to culture. Comparison of absolute values in different experiments thus may be difficult, but values relative to controls are readily comparable and reproducible.

Our estimate of the rate of myosin synthesis is comparable with the only other value determined for an absolute rate of synthesis of myosin in chick embryo (22). The values of 34, 36, and 40 ug of myosin synthesized per plate, measured directly by incorporation of radioactive amino acid (Fig. 4) compares quite favorably with the values of 21, 26 and 25 calculated from degradation rates (Table III) in the same experiment. This similarity indicates the validity of our measurements in our system. Our determination of the myosin one half life differs markedly from that found in vivo by Velick (23), Dreyfus et al. (24) and Schapira et al. (25, 26). Four independent types of experiments presented here support the half life measurement: 1) The rapid loss of myosin-ATPase following administration of protein synthesis inhibitors (Fig. 1); 2) The actual measurement of loss of myosin label following an initial pulse (Fig. 3); 3) The calculation of degradation rates from experimentally determined synthesis rates and myosin accumulation rates and 4) the rapid loss of ATPase in cultures maintained on minimal medium presumably carrying on a minimal amount of protein synthesis.

The previous work, mentioned above, on the turnover of myosin in vivo pointed to a very slow degradation (23), and long life time, rather than the short half life characteristic of a number of enzymes in cell cultures (20) and that found by us for myosin. The turnover rate in cell cultures

may differ from that seen in vivo. However, Goldberg in elegant and detailed work on protein turnover in fractions of rabbit muscle (26, 27) found a more rapid turnover than previous workers. Further data presented as evidence of a long myosin lifetime in rabbit muscle (25), may indicate that extensive reincorporation and reutilization of once-degraded protein may occur. The problem of reutilization of label is a universal one in this type of experiment. The extent of this reincorporation clearly affects the magnitude of degradation observed. Evidence that it does occur in our experiments; that is, that radioactive amino acids continued to be incorporated after the initial pulse is stopped, is two fold. First, we observed that protein synthesis inhibitors increases the apparent degradation rate of myosin and total protein especially in the first two days after the pulse. This was ascribed in part to cell death from these drugs, but is undoubtedly also a sign that under ordinary conditions, with protein synthesis permitted, label continues to be incorporated. Second, we often see an apparent biphasic degradation curve, with degradation quite slow for two days after the pulse, then increasing for subsequent measurements. The fact that all except the first points on the degradation curves in these cases, fell on a straight line indicates either that degradation rates change with time in culture, or that the first measurement is not really the 100% of maximum point of labelling. Since we have observed that, if anything, the degradation rate decreases with time, the indication is that the very slow rate, in the control, seen during the first period does not represent the actual degradation rate.

One result of the work reported here is that the differentiated state of the cultures may now be defined by myosin synthesizing ability.

Synthesis continues through the first seven to nine days of culture at approximately the original level indicating that the cultures as a whole maintain this differentiated state. In fact, in longer experiments myosin is synthesized even after beating ceases, although the synthesis rate is considerably less. That the continued synthesis occurs, in part at least, in already differentiated cells is indicated by its occurring in non-dividing cultures. This may be both a renewal or replacement of already-formed myofibrillar myosin; and as well, a continued development of partially differentiated cells. The unexpectedly rapid turnover rate of myosin found in cultured cells suggests however, some further work in this system, and perhaps in the area of general muscle protein catabolism is necessary.

It is clearly seen that serum factors play a role in maintaining myosin, by protecting against degradation, stimulating synthesis, and promoting cell division. Work is continuing on the nature and mechanism of action of these factors.

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TABLE I

PROTEIN LEVEL AND DEGRADATION IN NON DIVIDING CULTURES^a

Experimental conditions	Day 2	4	7	9	1/2 life (days)
Total protein (mg/plate)					
CGM	.5	1.3	1.5	1.8	
CGM + Colchicine	-	.85	1.0	1.1	
CGM + Irradiation	-	1.1	1.0	1.25	
MM	-	0.5	0.45	0.3	
Total protein degradation (% of initial radioactivity)					
CGM	100 ^b	90	67	51	7
CGM + Colchicine	-	72	40	30	4.2
CGM + Irradiation	-	88	40	24	4.2
MM	-	60	28	15	3
Myofibrillar protein level (mg/plate)					
CGM	0.18	0.42	0.62	0.8	
CGM + Colchicine	-	0.25	0.42	0.38	
CGM + Irradiation	-	0.35	0.37	0.38	
MM	-	0.15	0.15	0.10	
Myofibrillar protein degradation (% of initial radioactivity)					
CGM	100	85	73	65	10
CGM + Colchicine	-	80	60	50	7
CGM + Irradiation	-	78	52	40	5.7
MM	-	60	28	18	3
Myosin-ATPase levels (units/plate)					
CGM	75	105	105	130	
CGM + Colchicine	-	78	75	65	
CGM + Irradiation	-	105	103	90	
MM	-	60	30	20	

^a All cells were plated and maintained for 48 hours in CGM. After this period some were transferred to medium without serum MM medium containing 10^{-7} M colchicine, or were irradiated. At each day duplicate sets of plates with each treatment were harvested and analyzed.

^b Corrected for reincorporation.

TABLE II
RECOVERY OF CELLS FROM MINIMAL MEDIUM^a

Experimental conditions	Day 2	4	6	2	4	6
	Levels (mg/plate)			Total protein	Degradation	
					(% of original radioactivity)	
CGM	0.4	0.97	1.25	100	67	48
MM	"	0.42	0.30		40	14
MM → CGM	"	0.42	0.80		40	32
MM → CGM + colchicine	"	0.42	0.43		40	23
MM → CGM + colchicine + cycloheximide	"	0.42	0.36		40	30
MM → CGM + colchicine + actinomycin D	"	0.42	0.20		40	22

Experimental conditions	Myosin ATPase level			Myosin		1/2 Life
				Degradation		
				(% of original radioactivity)		
CGM	60	80	115	100	68	4
MM		32	10		40	1.8
MM → CGM		32	38		40	3.5
MM → CGM + colchicine		32	30		40	2.5
MM → CGM + colchicine + cycloheximide		32	15		40	4.5
MM → CGM + colchicine + actinomycin D		32	10		40	2

^a Cells were cultured from day 0 to 2 in CGM, changed to MM for two days then maintained on MM, returned to CGM plus drugs on day 4. Degradation was measured as percent of radioactivity present of the initial pulse of ¹⁴C leucine.

TABLE III

VARIATION OF MYOSIN SYNTHESIS RATES WITH TIME IN CULTURE^a

Experiment #	Day 2-4	4-6	7-9
135	23	19	25
149	21	26	25
163	12	15	
164	14	24	

^aAll values were calculated from previously determined degradation rates, and are expressed in μg myosin per plate per day. Experiments represent those in which cells were grown on Complete Growth Medium; each point is the average of determinations made on duplicate sets of four plates.

TABLE IV
EFFECT OF VARIOUS TREATMENTS ON PROTEIN SYNTHESIS RATES^a

Experimental conditions	Day 2	3	4	5	7	8
Total protein						
CGM	100	100	100	100	100	100
MM		48	36			2
CGM + Colchicine		82	79			70
CGM + Cycloheximide		24	16			
CGM + Irradiation	135	125				150
Myofibrillar protein						
CGM	100	100		100	100	100
MM		27		35		0
CGM + Colchicine		43		105		29
CGM + Cycloheximide		6		1		
CGM + Irradiation		78		52		70
Myosin						
CGM	100	100		100	100	100
MM		53		15	0	0
CGM + Colchicine		67		80		15
CGM + Cycloheximide		7		0	0	
CGM + Puromycin		50		0		
CGM + Irradiation		130		160		15

^aAll values are expressed as percent of control. Conditions as indicated in Table 1 and text for irradiation.

TABLE V
RECOVERY OF SYNTHESIS RATE^a

	Total protein	Myosin
CGM	100	100
MM	25	3
MM, returned to CGM	65	40
MM, returned to CGM + colchicine	25	20

^aConditions as in Table II. Figures represent synthesis rates as compared to controls on day 6.

TABLE VI
EFFECT OF ACTINOMYCIN D^a

Experimental Conditions	Day 2	2.5	3	4	6
Total protein (mg/plate)					
CGM	0.6	0.7	0.82	0.83	1.10
CGM + Actinomycin D			0.58	0.45	
Myosin-ATPase (units/plate)					
CGM	48	52	65	53	82
CGM + Actinomycin D			48	35	
Total protein degradation (% of original radioactivity)					
CGM	100	(92)	85	40	50
CGM + Actinomycin D			67	32	
Myosin degradation (% of original radioactivity)					
CGM	100	73	61	50	
		82	71		

^aCells were plated and maintained for 2.5 days on complete growth medium, then treated with 0.2 ug/ml Actinomycin D and assayed after 12 and 36 hours of treatment, for protein levels and amounts of radioactivity in protein fractions.

TABLE VII

SHORT TERM EFFECT OF ACTINOMYCIN D^a

Hours on actinomycin D	0	2	4	8	12	24
PROTEIN LEVELS						
Total protein (mg/plate)						
CGM	1.4					1.7
CGM + Actinomycin D		1.5	1.3	1.5	1.4	1.3
Myofibrillar protein (mg/plate)						
CGM	0.45					0.55
CGM + Actinomycin D		0.53	0.43	0.43	0.38	0.38
Myosin (ug/plate)						
CGM	60					80
CGM + Actinomycin D		65	45	35	35	35
PROTEIN SYNTHESIS (DPM INCORPORATED/PLATE)						
Total protein ($\times 10^5$)						
CGM	2.6					2.65
CGM + Actinomycin D		2.6	2.0	1.3	0.9	0.2
Myofibrillar protein ($\times 10^5$)						
CGM	1.0					1.05
CGM + Actinomycin D		1.2	0.9	0.65	0.5	0.08
Myosin ($\times 10^3$)						
CGM	5.8					6
CGM + Actinomycin D	7.6	5.7	3.8	2.5		1.3

^a See text

TABLE VIII
LONGTERM CULTURES^a

	Day 2	4	6	10	15	22	28
Total protein ($\mu\text{g}/\text{plate}$)	0.4	1.0	1.5	3.9	4.9	5.1	7.5
Protein degradation (% of initial radioactivity)	100	77	43	(100) ^b	40	28	18
Myosin-ATPase (Units/plate)	75	82	120	105	125	175	
Myosin degradation (% of initial radioactivity)	100	40	22	(100)	32	28	20
Myosin synthesis ^c (DPM/plate $\times 10^4$)	-	-	-	2	1.9	2.4	4.2
Total protein synthesis ^c (DPM/plate $\times 10^5$)	-	-	-	8	12	8	17
Calculated myosin synthesis ^d (rate $\mu\text{g}/\text{plate}/\text{day}$)	-	14	14	-	14	8	13
Calculated total protein synthesis ^d (rate $\mu\text{g}/\text{plate}/\text{day}$)	-	410	680	-	935	440	1067

^a See text. ^b To measure degradation rate two pulses of radioactive tyrosine

(1 μC per plate) were given one from day 0-2 and the second from day 8-10.

^c ^{14}C tyrosine was used for the initial single pulse (degradation) and ^3H valine (10 μC , 17.5 μg per plate) for the three hour pulses at each point (for synthesis). ^d The rate was determined on day shown and calculated as

rate per day for periods 0-2, 4-6, 10-15, 15-22, 22-27 days.

Fig. 1. Effect of protein synthesis inhibitors. Cells were prepared and plated in complete growth medium (CGM). After 2, 4 and 6 days of growth in this medium, cycloheximide (2 ug/ml) or puromycin (1 ug/ml) was added to some cells. After two days exposure to the drug, experimental and control cells were harvested and assayed for myosin-ATPase. - $\circ$ - CGM, - $\bigcirc$ - CGM + cycloheximide, - $\square$ - CGM + puromycin.

Fig. 2. Correlation of cell number and total protein. See text for details. CGM; - $\square$ - Total protein, - $\circ$ - Cell number. MM; - $\square$ - Total protein, - $\bigcirc$ - cell number.

Fig. 3. Protein degradation in complete medium. Cells were exposed to ^{14}C leucine for 48 hours (day 0 to 2) then harvested at two day intervals, during the chase period with excess cold leucine in the medium. Chase period begins on day 2. At each point duplicate plates were analyzed for total and myofibrillar protein and for radioactive myosin, isolated by gel electrophoresis. - $\circ$ - Total protein, - $\bigcirc$ - Myofibrillar protein, - Δ - myosin.

Fig. 4. Effect of growth in complete medium on myosin level and synthesis. At days 2, 4, 6, and 8 of culture, heart cells were given a 3 hour pulse of ^{14}C leucine. Cells were immediately harvested, and myosin extracted and isolated by gel electrophoresis. Total myosin was measured by dosimetry of the electrophoretic band, and radioactivity incorporated into myosin was calculated from counts found in this band. - $\circ$ - ^{14}C counts in myosin, - $\bigcirc$ - ug myosin.

Fig. 5. Effect of protein synthesis inhibitors on total protein. Cells were labeled (day 0-2) with ^{14}C leucine, then grown on CGM, plus excess leucine. At day 2, 4 and 6 of culture, cycloheximide (2 ug/ml) or puromycin (1 ug/ml) was added to cells in complete growth medium. Total protein levels and radioactivity in the fractions were determined for duplicate sets of plates on each day. A: Total protein level. B: Degradation of total protein. - $\circ$ - CGM, - $\bigcirc$ - CGM + cycloheximide, - $\square$ - CGM + puromycin.

Fig. 6. Effect of protein synthesis inhibitors on the degradation of myosin. Cells were incubated with ^{14}C leucine on days 0 to 2 then grown on CGM plus excess leucine. On days 2, 4 and 6 cycloheximide (2 ug/ml) or puromycin (1 ug/ml) was added. Two days later myosin was isolated and counted in these and control cultures. The radioactivity was expressed as percent of that present in control cultures at the beginning of the chase period (0 day 2). - $\circ$ - CGM, - $\circ$ - CGM + cycloheximide, - $\square$ - CGM + puromycin.

Fig. 7. Degradation of myosin under non-growing conditions. Culture conditions were those outlined in legend of Table 1. Degradation of myosin was determined by measuring loss of radioactivity in the electrophoretically isolated myosin. - $\circ$ - CGM, - $\square$ - colchicine, - Δ - irradiation, - $\circ$ - MM.

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