Biochemistry

Volume 8, Number 9 September 1969

3-Methylhistidine Metabolism in Proteins from Cultured Mammalian Muscle Cells*

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ABSTRACT: Primary cultures of rat leg muscle cells were found capable of converting [^{14}C]histidine as well as [^{14}C-*methyl*]-methionine into protein bound [^{14}C]3-methylhistidine. The cultured cells were used to study the half-life of the labeled amino acids in acid-precipitable proteins undergoing turnover. The initial rates of turnover for histidine were estimated to be dependent not only on age of the muscle cell cultures, but also on types of proteins sampled from the cultures. Constant rates of decrease in radioactive 3-methylhistidine were noted in proteins from cells after 8 days in culture. These constant rates were obtained in preparations of total cell proteins, soluble proteins of cell sap, or preparations of actin and myosin sampled from these cultures. Turnover rates for preparations of actin and myosin from older cells of the same cultures were analogous.

On the other hand, initial turnover rates for histidine and 3-methylhistidine isolated from actin and myosin preparations of young cultures were different.

3-Methylhistidine has been reported in actin and myosin isolated from vertebrate muscle (Asatoor and Armstrong, 1967; Johnson *et al.*, 1967). Asatoor and Armstrong (1967) found that the ratio of 3-MeHis/His in purified rabbit actin was 0.126. The data of Johnson *et al.* (1967) agreed with this value. These latter workers also reported that actin from 28-day rabbit foetus contained smaller amounts of 3-MeHis. The presence of this amino acid in adult myosin was also reported by Perry's group (Johnson *et al.*, 1967) and the 3-MeHis/His of their preparations was 0.026. Later, it was reported by Trayer *et al.* (1968) that 3-MeHis could not be detected in myosin isolated from 28-day-old rabbit foetus. More information is needed for correct interpretation of the present data (Gerdaz *et al.*, 1968) and for explaining the methylation of unique residues of histidine in actin and myosin.

Answers to the following questions were sought in our investigation. (1) Do muscle cells grown in culture convert [^{14}C]His into [^{14}C]3-MeHis? (2) How does the amount of [^{14}C]3-MeHis vary with respect to [^{14}C]His at various times in differentiating cells in culture and can it be used as an indicator for synthesis of contractile proteins? (3) Can the system be used for a further study of turnover and interaction of actin and myosin during differentiation of muscle cells?

Methods and Materials

Cell Culture. Methods were adapted from techniques originally used by Konigsberg (1963) for culturing monolayers of chick leg muscle cells. Leg muscle from 24- to 48-hr rat pups (Holtzman strain) was dissected out and cleaned free of crude gristle. The tissue was minced with scissors and incubated for 15 min at 37° in 0.1% trypsin made up in Hank's balanced salt solution (Hanks and Wallace, 1949) devoid of calcium. An equal volume of complete medium was added after this incubation to curtail further trypsinization. Single cells were collected by centrifugation after filtration through gauze and nylon (204 pore size). The centrifuged cells were resuspended in complete medium and plated in gelatin-covered 60-mm Falcon plastics petri plates (Hauschka and Konigsberg, 1966), at a concentration of 10^5 cells/ml. Each plate was pretreated with 5 μg of gelatin; 4 ml of medium was used per plate with medium changed every 2 days until day 6 and daily thereafter. The pretested medium used for growth of these cells consisted of F10 (Ham, 1963)–horse serum–fetal calf serum (85:10:5). The calcium content of the complete medium was adjusted to 3 mM after analysis in a Perkin-Elmer atomic absorption spectrophotometer. The cells were incubated in 5% CO_2–95% air

* Contribution No. 351 from C. F. Kettering Research Laboratory, Yellow Springs, Ohio 45387. *Received March 17, 1969.* Parts of these data were presented at the 8th Annual Meeting of the American Society for Cell Biology, Boston, Mass. 1968.

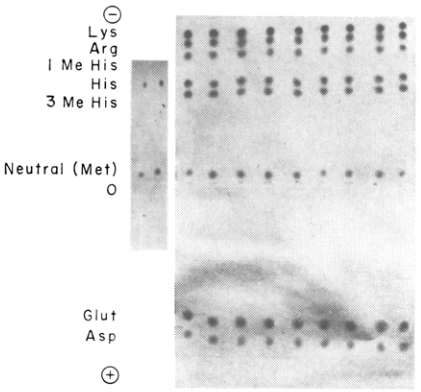

FIGURE 1: Paper electrophoresis of protein hydrolysates from cultured muscle cells; 0.05 μmole of each of the following amino acids were added to the hydrolysates; starting from the top: lysine, arginine, 1-methylhistidine, histidine, 3-methylhistidine, alanine (neutral), glutamic, and aspartic acid. The electrophoresis run was for 35 min at 5000 V. The pyridine–acetic acid buffer used was at pH 6.5. Inset shows autoradiograph of an electrophoresis paper of a total protein hydrolysate of cells from another experiment with [^{14}C]His and [^{14}C-*methyl*]Met (neutral) added after hydrolysis of proteins from these *unlabeled* cells. Trailing of radioactivity is absent indicating absence of interfering substances in the hydrolysates.

in a humidified incubator at 37°. Cultures were sampled regularly for normalcy of growth and per cent myogenic nuclei. These examinations were carried out after hematoxylin–eosin staining with ten fields being scored per plate. The cultures showed 55–60% myogenic nuclei at 4 days in culture but this figure was reduced to 47–50% by day 9 of culture. Occasional examination of the cells was also carried out with the aid of an electron microscope in cooperation with Dr. H. Mollenhauer.

Uniformly labeled [^{14}C]*histidine or* [^{14}C-*methyl*]*methionine* was added to the complete medium. Small quantities of these ^{14}C-containing media were added to the 4 ml of nonradioactive medium already present in culture plates as detailed in the figure and table legends below. Upon analysis of the free amino acid composition of the completed medium, the His content of the fresh medium was found to be 1.08×10^{-4} M while 3-MeHis content was 3×10^{-6} M and the methionine content was 6.6×10^{-5} M.

Protein Preparations. Preparations were made by pooling contents of several plates. The radioactive medium was aspirated off the exposed cultures and the monolayers of cells at the bottom of the plates washed with a total of 10 ml of ice-cold sucrose (0.3 M)–Tris (0.01 M) adjusted to a pH of 7.4. Cells were sampled after removal of the sucrose–Tris buffer.

Total proteins were sampled by adding cold 10% trichloroacetic acid to the cultures and repeatedly scraping the cells. The trichloroacetic acid precipitates were collected by centrifugation and stored at −70° since all the samples were hydrolyzed after each experimental series was completed.

All protein preparations were treated in a uniform manner for acid hydrolysis. Proteins were precipited in cold trichloroacetic acid and centrifuged. The precipitates were then washed with ethanol–ether (1:1) and with hot 70% ethanol and finally treated with 5% perchloric acid at 70° for 20 min. The residue was dried and hydrolyzed with 6 N HCl in evacuated glass tubes for 22 hr at 104°. After hydrolysis, the acid was removed by evaporation in a stream of dry nitrogen followed by vacuum desiccation over pellets of NaOH. This hydrolysate was resuspended in 0.1–0.4 ml of a water–ethanol mixture (3:1). The suspended matter occasionally found associated with hydrolysates of total cell protein was removed by centrifugation and the clear supernatant used for separation of amino acids. A rehydrolysis of the initial hydrolysates did not change radioactive counts associated with the isolated amino acids.

Actin was isolated by the method of Carstens and Mommaerts (1963) and subjected to three conversion cycles between globular G-actin and F-actin.

The initial extracting solution for myosin was 0.3 M KCl, 75 mM KH_2PO_4–75 mM K_2HPO_4 (pH 6.8), and 0.5 mM ATP. For reextractions of myosin the methods of Perry (1955) were used.

Reference actin and myosin preparations were isolated from adult female rats. The rats employed for this purpose were mothers of pups used in various cell culture experiments. Such adult preparations of actin and myosin were used in preliminary experiments for coextraction of similar proteins from cultured muscle cells, to indicate similar proteins extracted solely from cultured cells and for confirming the reported ratios of 3-MeHis/His in the hydrolyzed proteins (Asatoor and Armstrong, 1967; Johnson *et al.*, 1967). The acrylamide gel electrophoresis method of Small *et al.* (1961) was used according to the modifications of Heywood *et al.* (1967) for protein identification while amino acid analyses of such hydrolyzed proteins were made on physiological fluids column (Asatoor and Armstrong, 1967).

A vertical gel electrophoresis apparatus from Buchler Instruments was used.

Proteins of cell sap were prepared by homogenizing the cultured cells with a Dounce homogenizer in a medium consisting of 0.25 M KCl, 0.01 M MgCl, and 0.01 M Tris buffered at pH 7.2. The supernatant remaining after centrifugation at 106,000*g* for 30 min was used. Soluble proteins of the cell sap were precipitated by adding trichloroacetic acid to a final concentration of 5%.

Amino acid analysis were conducted with a Spinco Model 120 amino acid snalyzer or a Phoenix Precision Co. amino acid analyzer Model 8000B.

Protein was estimated after dissolving the washed perchloric acid residue in 1 N NaOH. The method of Lowry *et al.* (1951) was used.

Separation of Hydrolysates and Radioactive Measurements. Paper electrophoresis was performed with a Gilson Medical Electronics Model D high-voltage tank electrophoresis apparatus; 1–5-μl samples were applied to Whatmann No. 3MM paper 60 cm in length and electrophoresis was carried out for 40 min at 5000 V. The buffer mixture of Offord (1966) was used under a layer of "Varsol." The following nonradioactive amino acids (0.1 μmole each) were always spotted together with the hydrolysates: Lys, Arg, 1-MeHis, His, 3-MeHis, Gly, Asp, and Glu. The dried chromatograms were sprayed with 0.4% ninhydrin in acetone and separated samples were collected within 3 hr (see Figure 1). With samples from cultures labeled with [^{14}C]His for longer than 24 hr, the entire strip was counted by punching out adjacent circles 15 mm in diameter. It was found that radioactivity was not present in significant amounts in spots other than those rep-

resented by His, 3-MeHis, and neutral glycine as a standard amino acids when cells were labeled for periods less than 24 hr. The paper circles were cut in four smaller segments, placed in glass or nylon scintillation vials (Nuclear-Chicago), and extracted in 0.1 N NaOH; 10 ml of Bray's solution was used for scintillation counting (Bray, 1960) in a Unilux II Model Nuclear-Chicago scintillation spectrometer. Blank circles cut from each sample strip after electrophoresis showed background of 19–21 cpm. Counting efficiency was graphically determined (Takahashi *et al.*, 1961) and was estimated at 80% without and 72% with the electrophoresis paper present in the vials. The reported counts were not corrected for this counting efficiency. Each sample was separated by electrophoresis at least three times and each spot collected was counted three times. The standard error of the mean for each sample varied between 5 and 7%.

In some experiments in which labeled histidine was introduced for short periods, thin-layer chromatography on silica-coated glass-fiber paper (Mallinckrodt) was used to separate 3-MeHis from His. These results were used to confirm those obtained with paper electrophoresis with good success. The developing system used was arrived at by trial and error with combinations of simpler systems listed in the literature (Pataki, 1968). Our system was methyl ethyl ketone–pyridine–water–methanol–formic acid (16:7:7:5:0.8, v/v). In this system, Asp, Lys, and Arg are not well separated from His. However, the system is useful because these amino acids show negligible radioactivity after short-term exposures of cultures to [^{14}C]His, and because 3-MeHis was well separated. After the solvent front had moved 30 cm, the following R_F values were obtained: Ser, 0.63; Ala, 0.60; Glu, 0.56; *His*, 0.47; Asp, 0.45; Lys, 0.44; Arg, 0.43; 1-MeHis, 0.32; and *3-MeHis, 0.30.*

Reagents and Chemicals. Two sources of [^{14}C]His were used: lot no. 284–256 from New England Nuclear Corp. with specific activity of 239 mCi/mmole and Stanstar lot no. 6901 from Schwartz BioResearch with a specific activity of 50 mCi/mmole. The amount of radioactivity in the 3-MeHis spot after paper electrophoresis of the original samples was determined before use. Repeated runs at various dilutions of the original [^{14}C]-histidines were made so as to give 3–9 $\times$ 10^5 cpm after electrophoresis and collection of the spots corresponding to His (see Figure 1). The amount of radioactivity at the 3-MeHis spot was less than 0.1 per cent of the recovered counts. The standard error of such counts was less than 3%.

[^{14}C-*methyl*]Methionine was obtained from Amersham–Searle with a specific activity of 53.6 mCi/mole (CFA 152 Batch 51).

Trypsin (three-times crystallized) from Worthington Biochemical Corp. was used for cell dissociation from muscle tissue. HCl-extracted high-strength porkskin gelatin (lot no. 01030) from Whitten Co. of Winchester, Mass., was used for treatment of plastic culture plates.

Hank's balance salt solution (Hanks and Wallace, 1949) and F10 medium of Ham (1963) were mixed in our laboratory in all but one experiment cited (one of the cultures used for isolation of actin and myosin preparations was grown in stock medium bought from Schwarz BioResearch). Animal sera were pretested in preliminary experiments before use and were bought from Baltimore Biological Laboratories or Grand Island Biological Co.

Amino acids and other biochemicals used for making culture media were supplied by Mann Biochemical or Calbiochem.

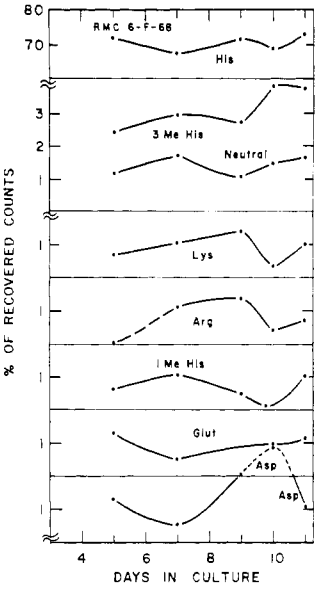

FIGURE 2: Per cent redistribution of [^{14}C]histidine radioactivity in hydrolysates of total cell proteins collected at different periods of culture. [^{14}C]Histidine (0.05 μCi/4 ml of medium) with original specific activity of 239 mCi/mmole was introduced into the culture medium starting from day 3 in culture and the radioactive medium renewed after each sampling. On days 5 and 7, duplicate sets of ten and five plates were pooled, respectively. Duplicate sets of four plates were pooled for other sampling periods. The total protein associated (cpm/plate) was 3877, 14,790, 14,350, 18,750, and 21,800 for days 5, 7, 9, 10, and 11, respectively. (Culture code is in top left corner.)

Scintillation fluors and naphthalene were bought from Nuclear-Chicago.

Urea used for gel electrophoresis was supplied by Schwarz while ingredients for the polyacrylamide were bought from Kodak.

Common chemicals of highest purity were bought from Mallinckrodt or Fisher Scientific Co. Deionized and doubly distilled water was used for every phase of this work including rinsing of glassware.

Results

The Distribution of [^{14}C]Histidine in Total Cell Proteins. The uptake of [^{14}C]His into total cell proteins was followed in a number of experiments. Cells were cultured in isotope-containing medium starting at 2–4 days after seeding. The radioactive medium was renewed on the same schedule as the controls (refer to Methods and figure legends).

It was necessary to know how much of the [^{14}C]His radioactivity was converted to amino acids other than 3-MeHis and eventually reintroduced into total cell proteins when cultures were grown in the radioactive medium for periods as long as 9 days. The results from one experiment are shown in Figure 2. This pattern of count distribution was repeated in three experiments. Between 68 and 73% of the counts recovered from total cell proteins were in His. 3-MeHis accounted for 2–4% of the radioactivity, while smaller fraction of counts were distributed in other amino acids as shown in Figure 2. Radioactivity in very low amounts was also detected in an

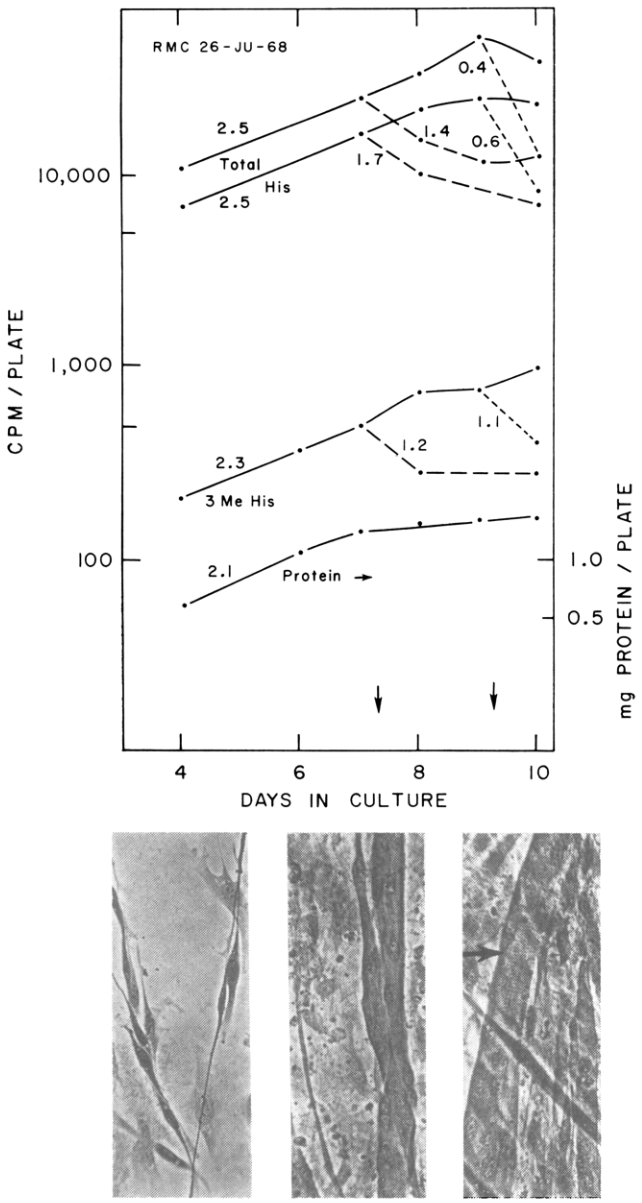

FIGURE 3: Radioactivities of histidine and 3-MeHis from total proteins of cultured rat muscle cells at different periods and conditions of culture. Starting from day 2 in culture, each plate was incubated with 4 ml of complete medium to which 0.1 μCi of uniformly labeled [^{14}C]histidine (original specific activity 239 mCi/mmole) was added. The radioactive medium was renewed in some plates as indicated in Methods. In other plates, the radioactive medium was replaced by nonradioactive medium on days 7 and 9 (↓), respectively. The plates were sampled for radioactivity in duplicate batches as in experiment shown in Figure 2. (Culture code number is in top left corner. The numbers beside each curve indicate $t_{1/2}$ (or 2t) values.) All three photomicrographs shown at the bottom of the figure were made with a phase microscope at 400× magnification and indicate appearance of cultured cells at days 4, 7, and 10. Arrow indicates cross-striations in a mature myofibril. The replicate plates used in cultures were stained with hematoxylin.

unidentified region above the acidic amino acids on the paper after electrophoresis.

Analysis of the fresh, complete medium showed that free His and 3-MeHis were present at 1.1 × 10^{-4} and 3 × 10^{-6} mole per l., respectively. The free His was added as an ingredient of the F10 nutrient mixture while 3-MeHis was presumably present in the animal sera. In one experiment, this medium was exposed to muscle cells on day 8 of culture, and the free amino acids were reanalyzed in the amino acid analyzer after collection from the culture plates. The His and 3-MeHis content of the exposed medium were 1.95 × 10^{-4} and 7 × 10^{-6} mole per l., respectively. Carnosine, anserine, and 1-MeHis could not be detected. The difference in concentration of His and 3-MeHis suggested that there was turnover of these amino acids in some cellular proteins. Other amino acids also showed increase or decrease in concentration between the unexposed and exposed media. Methionine at a concentration of 6.6 × 10^{-5} M in fresh media could not be detected even as methionine sulfone in media exposed to cultured cells. Since these concentration shifts were peculiar to each amino acid, the changes could not be attributed to artifacts.

When cpm incorporated were expressed on the basis of total cell protein in experiments like that of Figure 2, it became evident that there was a decrease in [^{14}C]His radioactivity per milligram of protein in cultures older than 8 days. Under our conditions of culture, rat leg muscle cells remained viable and firmly attached to the plates for 12–15 days. Our experiments were terminated by 10 days to avoid sampling unwanted cells from overgrowth of fibroblasts.

The experiment outlined in Figure 3 was performed to further study changes in His content of culture media as well as the decrease in [^{14}C]His relative to cell protein. Radioactive His was included in the culture medium of some plates from day 3 to 10 and the plates were sampled at regular intervals for total counts in the protein residue as well as counts in His and 3-MeHis isolated from the total cell proteins after hydrolysis. These are shown in the top three curves. The bottom curve with scale at right indicates protein content per plate. The dashed curves represent radioactivity in total cell proteins of cultures sampled after withdrawal of the [^{14}C]histidine on days 7 and 9, respectively. The numbers on increasing slopes of the curves represent the time in days for a doubling of the radioactivity or protein (2t). The numbers beside each broken curve represent time in days for radioactivity in total proteins to be halved. It has been assumed that the initial decrease in radioactivity is linear on the semilog scale and is designated as $t_{1/2}$. The $t_{1/2}$ for His changed rapidly from 1.7 to 0.6 when radioactive medium was withdrawn at day 7 as compared with day 9 of culture. The $t_{1/2}$ for 3-MeHis remained unchanged at these time intervals with values of 1.2 and 1.1.

Between 5 and 6 days our cultures consisted of few myoblasts undergoing proliferation, muscle cells in process of fusion, and relatively few multinucleated myotubes. Between 8 and 9 days, the cultures showed multinucleated myotubes only and the muscle cells had stopped dividing (fibroblast-type cells are capable of some division even in confluent cultures). These changes are indicated by photomicrographs at the bottom of Figure 3 showing appearance of the cells at days 4, 7, and 10 in culture, all at a magnification of 400×. The change in $t_{1/2}$ for His occurred in cultures undergoing transition from a proliferative to a differentiative phase. Since $t_{1/2}$ for 3-MeHis did not change, it was of interest to determine decrease in radioactivity of 3-MeHis in different protein preparations from cells at different time intervals.

Changes in 3-[^{14}C-methyl]Histidine in Soluble Protein sof Cell Sap. The initial half-life for turnover of 3-MeHis was

also studied in soluble proteins of the cell sap, *i.e.*, the supernatant remaining after the cell homogenate was centrifuged at 106,000*g* for 30 min. [^{14}C-*methyl*]Methionine was used to: (i) establish the role of precursor for this amino acid in formation of 3-MeHis in cultured rat leg muscle cells and (ii) obtain sufficiently radioactive protein associated 3-MeHis in short incubation periods devoid of interference from radioactive His. Figure 4A,B shows experiments from two different cultures. In the experiment of Figure 4A, the increase in specific activity (counts per minute per milligram of isolated protein) of methionine and 3-MeHis associated with cell sap proteins is shown and indicates linear incorporation of methionine and its conversion into 3-MeHis for 4.5 hr after addition of the label to 7-day-old cells. In Figure 4B, the cells were loaded with [^{14}C]methionine for 24 hr, from day 7 to 8, and the label was withdrawn on day 8. The $t_{1/2}$ values for methionine and 3-MeHis were estimated from a line passing thru values for 0, 3, 6, and 16 hr, to be 24.8 and 21 hr, respectively. The delay of 1–2 hr in decrease of radioactive label from 3-MeHis has been confirmed by supplementary experiments. A careful examination also reveals a slight change of $t_{1/2}$ for 3-MeHis after 20–24 hr. Decrease in protein-associated methionine radioactivity does not reveal small latent changes in $t_{1/2}$. The [^{14}C-*methyl*]methionine also labeled 1-MeHis associated with cell sap protein. The values are erratic and less than 10% of label found with 3-MeHis is found with 1-MeHis in cell sap proteins. At least two major spots of radioactivity have been noted in association with soluble proteins of the cytoplasm. Each of these spots show two distinct slopes for decrease in radioactive label derived from [^{14}C-*methyl*]-methionine. Identification and further study of these spots found in regions close to Lys and Arg on the electrophoresis paper are needed. No radioactivity associated with acidic amino acids is found in protein hydrolysates of [^{14}C-*methyl*]methionine-labeled cells after a 24-hr labeling period.

Distribution of [^{14}C]*Histidine Incorporated in Actin and Myosin Preparations from Cultured Cells.* Single cells were dissociated from rat leg muscles, seeded, and grown in the complete medium. On day 5, medium containing [^{14}C]His was introduced into some culture plates. After 24 hr, half of the culture plates were collected and frozen at −70°. The medium from the remaining culture plates was replaced with fresh medium devoid of [^{14}C]His. The cells from the latter plates were collected after another 24 hr. This treatment was repeated with cells from replicate plates of the same culture at a later period, *viz.*, days 7, 8, and 9. The cultures marked A_1 and A_2 were collected on days 6 and 7, after being exposed to the [^{14}C]His medium between days 5 and 6 of cultures; these were used for preparations of actin marked A_1 and A_2. Actin preparations A_3 and A_4 were collected from similarly treated older cells. Additional plates from the same culture were used for myosin preparations. These preparations were labeled M_1–M_2 and M_3–M_4 corresponding to parallel collections on days 6 and 7 and again on days 8 and 9 of culture. These protein preparations were treated as described in Methods (the 3-MeHis/His ratio of a similar adult rat actin preparation was 0.12, while similar adult rat myosin preparations exhibited 3-MeHis/His ratios between 0.016 and 0.018).

Representative data from one of two experiments is given in Tables IA,B and II.

In myosin preparations (Table IA), the ratio [^{14}C]3-MeHis/His did not change appreciably with age of the culture or after

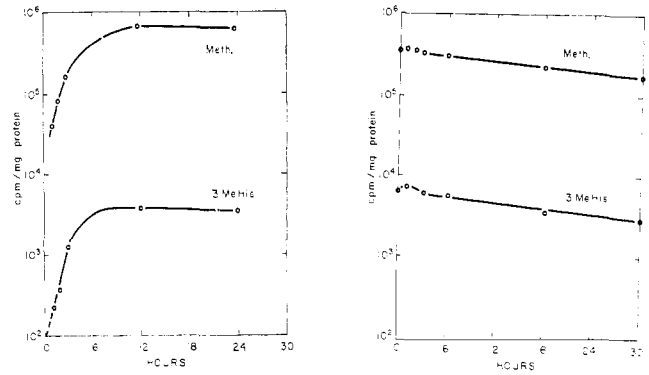

FIGURE 4: Increase (A, left) and decrease (B, right) of radioactivity in methionine and 3-MeHis isolated from soluble proteins associated with cell sap; 1 μCi of [^{14}C-*methyl*]methionine/4 ml of medium was added and eight plates were pooled for obtaining each sample point at the time (in hours) indicated. A 7-day culture was used (see text for details). Eight plates were pooled for obtaining each sample point at the time (in hours) indicated after withdrawal of the radioactive medium and replacing this with fresh medium; 1 μCi of [^{14}C-*methyl*]methionine was added per 4 ml of medium to another culture on day 7. On day 8, the radioactive medium was removed, the cells were washed with fresh medium, and fresh medium was reintroduced for further sampling of cells on day 9. A $t_{1/2}$ value of 21 hr is estimated for 3-MeHis (see text for further details).

replacement of the radioactive medium with regular medium. In actin preparations from the same cultures (Table IB) two types of increases in the [^{14}C]-MeHis/His ratios were noted. One increase was associated with the age of culture. The second increase of the ratio was noted in actin prepared from cultures in which radioactive medium had been replaced by control medium.

The data from Table I were also plotted on semilog paper. The counts per minute isolated in either His or 3-MeHis per milligram of the respective protein preparations were plotted *vs.* time. Each pair of points for actin and myosin were used to estimate $t_{1/2}$. It was assumed that the decrease in radioactivity of the preparations was exponential within the 24-hr period. As indicated by data in Figure 4, this assumption may be valid even in the case of 3-MeHis. The $t_{1/2}$ values are given in Table II.

The His-associated radioactivity was lost at a faster rate in myosin preparations from older as compared with younger cells. The His-associated radioactivity was lost at a slower rate in actin preparations from older cells. The $t_{1/2}$ for actin and myosin preparations from older cells was 18 hr.

The value of $t_{1/2}$ for 3-MeHis was close to 21 hr in both actin and myosin preparations from older cells. In the case of 3-MeHis, even the preparations from younger cells showed $t_{1/2}$ values of 17 and 21 hr for actin and myosin, respectively. This value of $t_{1/2}$ for 3-MeHis from isolated protein preparations compares reasonably with analogous loss of radioactivity in 3-MeHis associated with total cell proteins or cell sap proteins (Figures 3 and 4) when it is considered that the various experiments were conducted many months apart with primary cell cultures made from offspring provided by different rats. The doubling rate for the culture used in Figure 3 is slower than the usual doubling rate of 1.4–1.7 as in the experiment shown in Tables I and II.

The remaining protein from the cells sampled for actin and

TABLE I: Uptake of [^{14}C]Histidine and Conversion into 3-Methylhistidine in Preparations of Myosin (M) and Actin (A) from Cultured Muscle Cells.

	Sample Treatment (days)				cpm/mg of Protein			
	[^{14}C]His Medium	Control Medium	Day Sampled	mg of Protein Hydrolyzed	His	3-MeHis	3-MeHis/His	% Recov[a]
				Part A				
M_1	5–6		6	0.720	58,600	1,850	0.031	96
M_2	5–6	6–7	7	0.544	39,010	1,102	0.028	93
M_3	7–8		8	0.505	95,010	2,110	0.022	100
M_4	7–8	8–9	9	0.490	39,100	885	0.022	94
				Part B				
A_1	5–6		6	0.058	63,900	3,100	0.048	97
A_2	5–6	6–7	7	0.092	10,500	648	0.064	90
A_3	7–8		8	0.046	61,500	3,840	0.062	88
A_4	7–8	8–9	9	0.065	24,100	1,850	0.077	95

[a] [^{14}C]Histidine was added to each of nine plates per sample at a level of 0.45 μCi/plate (specific activity of histidine was 50 mCi/mmole). Eight plates per sample were used from older cultures. Each plate contained 4 ml of medium. After 24 hr, this medium was replaced with control medium. Cells were harvested in 0.25 M sucrose–Tris-HCl (0.05 M, pH 7.4) and frozen at −70°. Actin and myosin preparations were separately made for each sample as outlined in Methods. The resultant protein pellets were treated with 10% trichloroacetic acid and the ether–ethanol-washed trichloroacetic acid pellet was hydrolyzed in 6 N HCl *in vacuo*. Per cent recovery refers to per cent of total applied counts recovered after electrophoresis on the average, from histidine and 3-methylhistidine spots only.

myosin was also hydrolyzed and the amino acids were separated by electrophoresis. Between 6000 and 9000 cpm per sample were used to determine 3-MeHis/His ratios. The residues from cells sampled for actin showed the following ratios: A_1, 0.004; A_2, 0.008; A_3, 0.004; and A_4, 0.007. The residues from cells sampled for myosin showed the 3-MeHis/His ratios to be 0.006 for M_1, 0.01 for M_2, 0.001 for M_3, and 0.011 for M_4.

Discussion

The methylation of histidine residues reported in these experiments utilized only [^{14}C]His and [^{14}C]methionine labels.

TABLE II: Calculated $t_{1/2}$ of Protein Preparations from Cultured Muscle Cells Grown in [^{14}C]Histidine Medium.

Sample Pairs	$t_{1/2}$[a] Value (hr)	
	[^{14}C]Histidine	[^{14}C]3-MeHis
A_1–A_2	10	17
M_1–M_2	41	21
A_3–A_4	18	23
M_3–M_4	19	20

[a] $t_{1/2}$ indicates time calculated for halving of [^{14}C]radioactivity (cpm/mg of protein) in various fractions as taken from slopes of a semilog plot of data as given in Table IA,B.

In other experiments not reported here, analysis of protein hydrolysates with an amino acid analyzer confirmed the increasing presence of MeHis with increase in mature myofibrils in culture (the use of [^{14}C-*methyl*]methionine in long-term incubation with the culture medium was avoided because of the instability of the label and because the amino acid is readily metabolized). The presence of 3-MeHis in cultured muscle cells as well as its increase in the incubated medium makes it improbable that it is formed in another organ and then transported to the muscle. This confirms the observations of Asatoor and Armstrong (1967) who reported that the specific activity of [^{14}C]histidine (uniformally labeled) was unchanged after isolation of the injected label from muscle hydrolysates as 3-MeHis.

The redistribution of the His label into other amino acids, especially 1-MeHis, was minimal as shown in Figure 2. The per cent of counts recovered in 3-MeHis spots increased as the cells completed proliferation and entered the major phase of differentiation. Although it is not obvious from the data given for Figures 2 and 3, the [^{14}C]His counts accumulated per milligram of total protein decreased in cultures after day 8, *i.e.*, when the cells entered the major period of differentiation. There was no decrease in the number of cells at this time as shown by counting the number of nuclei in replicate culture plates (see Methods).

The decrease in labeled His per milligram of total protein was due to a faster loss of amino acid at the later period of culture (broken curves in Figure 3). This rapid His loss could be ascribed to an increased initial turnover in some major protein(s) of the older cells. The unknown proteins cannot be identified until further experimental points are obtained for

estimating initial values of $t_{1/2}$. These would include $t_{1/2}$ values for total proteins of the culture together with the major structural and contractile protein components. Actin may not be the protein influencing the initial loss of [^{14}C]His in older cells since its turnover estimate with this isotope is restricted with time.

The turnover characteristics of 3-MeHis isolated from cultured cell proteins were consistent at a variety of time periods. The 3-MeHis may be restricted to contractile proteins because the remaining protein from cells exhibited 3-MeHis/His ratios of 0.01 or less after removal of actin and myosin.

The ratio of 3-MeHis/His is useful for estimating contractile proteins in hydrolysates of adult tissue preparations where turnover of proteins is more constant. The use of labeled antibody has proved successful in estimation of total myosin in differentiating muscle (Coleman and Coleman, 1968). Antibody to highly purified mammalian actin cannot be conventionally made in rabbit sera and exotic antisera for actin have not been reported as aids to estimation of foetal actin. The measurement of 3-MeHis for determination of foetal actin requires a knowledge of the extent of actin methylation at different periods of normal development.

The gradual age-dependent histidine methylation of actin indicated in cultured muscle cells agrees with data published on foetal rabbit actin by Johnson *et al.* (1967). The methylation of myosin is limited but it is present in our preparations both from early and late cultures. This does not agree with data of Trayer *et al.* (1968) on foetal rabbit myosin. Detailed studies are necessary for both types of systems before offering an explanation for the discrepancy.

If myosin preparations from cultured cells were contaminated with actin, the 3-MeHis/His ratio would be more variable. If the preparative method used for myosin tends to yield only a constant fraction of actin contaminant, 3-MeHis/His ratio would increase in preparations from mature cells. If the preparative method for myosin tends to yield only a certain type of actin-contaminating myosin, the fact argues in favor of changes in interaction between these proteins with age of the cultured cells. The 3-MeHis eventually ends up in a stable compartment where the turnover rate is quite slow. The limited data from cultured cells argue in favor of concerted control for actin and myosin methylation because the $t_{1/2}$ values of 3-MeHis and His in both the isolated proteins from 8 to 9 day cultures was close to 21 hr.

The methylation mechanism probably involves *S*-adenosylmethionine (Asatoor and Armstrong, 1967). This mechanism is likely to differ from the imidazole *N*-methyltransferase from brain (Brown *et al.*, 1959) or the 1-methylhistidine formation *via* carnosine *N*-methyltransferase (McManus and Benson, 1967). Indeed carnosine and anserine could barely be detected by the amino acid analyzer in the medium exposed to cells between 8 and 10 days of culture. The nature of the labeled products from [^{14}C-*methyl*]methionine at locations other than 3-MeHis on the electrophoresis paper and their possible role in cell metabolism cannot be assessed at the present time. The question of whether histidine can be incorporated in methylated form remains to be established despite the suggestion that methylation is slow and gradual as indicated above, or the data of Cowgill and Freeburg (1957), indicating that up to 90% of injected methylhistidines are found in excreta of mammals and birds. Amino acid acceptor activity experiments are indicated for establishment of this question.

The use of cultured cell system makes it possible to examine methylation of histidine in contractile proteins also as a problem in protein turnover. Do cell-specific proteins exhibit distinct turnover rares at various stages of differentiation as alluded in this study or are the rates *in vivo* similar to turnover rates from adult mammalian muscle proteins as measured by McManus and Mueller (1966), Dreyfus *et al.* (1960), and Velick (1956)? Turnover rates of proteins *in vivo* can be obscured by inclusion of interstitial and matrix fluids as well as changes in specific activity of the amino acid pools. The cultured cell system avoids some of these difficulties and cloned culture systems (Yaffe, 1968; Coon, 1966; Cahn and Cahn, 1966) may be preferred in future studies.

Acknowledgment

Skilled technical assistance for cell cultures was provided by Miss Grace Norris. The assistance of Mr. Dan Crevensten and G. Loewer for other phases of this work is also acknowledged. I am especially grateful to Dr. M. Armstrong and Dr. Keelin Fry for help and encouragement in all phases of this work and Drs. M. Lamborg and R. Darrow for their suggestions and comments.

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Vitamin B_{12} Dependent Methionine Biosynthesis in Cultured Mammalian Cells*

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ABSTRACT: Several established mammalian cell lines were cultured in such a way that they appeared to be dependent upon vitamin B_{12}. Methionine was replaced by homocysteine in the growth medium and the cells survived and proliferated only when vitamin B_{12} was added. The level of 5-methyltetrahydrofolate–homocysteine transmethylase activity was elevated some 10–20-fold in these cells. Elevated enzyme levels were also observed in cells cultured in medium containing methionine. However, this only occurred when vitamin B_{12} was also included in the medium. The cofactor requirements for the transmethylase obtained from cultured mammalian cells were found to be similar to those observed for mammalian tissues.

The terminal reaction in methionine biosynthesis involves the transfer of a methyl group from N^5-methyltetrahydrofolate to homocysteine (Larrabee *et al.*, 1963; Sakami and Ukstins, 1961). Enzyme systems catalyzing this reaction have been

$$N^5\text{-methyltetrahydrofolate} + \text{homocysteine} \longrightarrow \text{methionine} + \text{tetrahydrofolate}$$

partially purified from *Escherichia coli* (Stravrianopoulos and Jaenicke, 1967; Taylor and Weissbach, 1967) and from mammalian (Buchanan *et al.*, 1964; Kerwar *et al.*, 1966) and avian liver (Dickerman *et al.*, 1964). The cofactors which have been implicated in methionine biosynthesis include $FADH_2$ (Hatch *et al.*, 1959) and *S*-adenosylmethionine (Mangum and Scrimgeour, 1962). In addition, 5-methyltetrahydrofolate–homocysteine transmethylase isolated from *E. coli* has been shown to contain a bound form of vitamin B_{12} (Takeyama *et al.*, 1961). There is also evidence that methionine biosyntheis in animal tissues requires a vitamin B_{12} prosthetic group. The dietary requirement of rats for methionine can be replaced by homocysteine and vitamin B_{12} (du Vigneaud *et al.*, 1950). A nutritional deficiency of vitamin B_{12} in young chicks resulted in a marked reduction of the transmethylase activity (Dickerman *et al.*, 1964). A partial restoration of the activity occurred when the animals were subsequently provided vitamin B_{12}.

It has been shown that cultured mammalian cells require methionine but not vitamin B_{12}. However, vitamin B_{12} increased the rate of proliferation of a particular strain of L cells cultured in a medium deficient in deoxycytidine and thymine (Sanford and Dupree, 1964).

It was recently reported (Mangum and North, 1968) that HEp-2 cells cultured in a growth medium where methionine was replaced by homocysteine appeared to have an absolute dependency upon vitamin B_{12}. This dependency resulted in a marked elevation of 5-methyltetrahydrofolate–homocysteine transmethylase activity. The present study provides a more detailed examination of vitamin B_{12} dependent methionine synthesis in cultured mammalian cells. Conditions are also described for obtaining elevated levels of the transmethylase in cells that are not dependent upon *de novo* methyl group formation.

Materials and Methods

Minimum essential medium (Eagle, 1955) both with and without methionine and choline was purchased from Grand Island Biological Co. Chemicals were obtained from the following sources: vitamin B_{12} and FAD from Sigma Chemical Co.; [^{14}C]formaldehyde from New England Nuclear Corp; *S*-adenosylmethionine, folic acid, and Dowex 1-Cl^- from California Corp. for Biochemical Research; and DL-homocysteine from Nutritional Biochemical Corp.

Tetrahydrofolate was prepared by the catalytic hydrogenation of folic acid (Hatefi *et al.*, 1960) and [*methyl*-^{14}C]N^5-methyltetrahydrofolate was chemically synthesized by reduc-

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† U. S. Public Health Service Research Career Development Awardee K3-GM-10,215 of the National Institute of General Medical Sciences.