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Methyl-Esterified Proteins in a Mammalian Cell Line†

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ABSTRACT: Methyl esterification of carboxylic acid residues in intact mouse S49 lymphoma cells was examined, and at least 24 proteins were found to be modified. Cell fractionation revealed that a distinct set of these proteins could be found in each of the four fractions. Nuclei contained 11 methyl-esterified proteins at 12, 15.5, 18, 19, 39, 41, 45, 70, 90, 105, and 130 kilodaltons (kDa). Five proteins copurified with the plasma membrane/mitochondrial fraction at 13, 24, 25, 27, and 28 kDa. Two proteins at 32 and 56 kDa were in the microsomal fraction, and six were soluble at 16.5, 21, 24, 26, 34, and 36 kDa. Eleven of these proteins were [^{3}H]methyl esterified when cell homogenates were incubated with *S*-adenosyl-L-[*methyl*-^{3}H]methionine. The steady-state level of methyl group incorporation into protein in intact cells was approximately 118 pmol/mg of protein. Assuming the average protein is 40 kDa, there appears to be 1 methyl group per 210 proteins. This was compared to phosphorylation which gave approximately one phosphoryl group for every four proteins. Exogenously added L-[*methyl*-^{3}H]methionine equilibrated with the cellular *S*-adenosylmethionine pool within 30 min which was sufficiently rapid to allow the rate of methyl group turnover to be determined. Most methyl-esterified proteins demethylated in a pulse–chase experiment with half-lives ranging from 2.6 to 9.3 h. When protein synthesis was blocked with puromycin, amino acid backbone incorporation of methionine was reduced to 2% of control. Methyl group incorporation, however, was 39% of the control. This level of methyl group incorporation could be attained whether label was added 15 or 105 min after the addition of puromycin, indicating that these proteins were being continuously methylated and demethylated. The reversible nature of this modification suggests that it may serve a regulatory function in these cells.

The reversible posttranslational methylation of protein carboxyl groups may be a mechanism for regulation of protein function in mammalian cells (Paik & Kim, 1980a,b; Dilberto, 1982). Methyl esterification activity has been isolated from a wide variety of mammalian tissues. Several proteins have been suggested as possible substrates for this enzyme, but an in vivo role for the modification has not yet been determined (Kim & Li, 1979; Kloog et al., 1980; Freitag & Clarke, 1981; Gagnon et al., 1981; Kloog & Saavedra, 1983; Billingsley et al., 1984). In bacteria, on the other hand, the methyl ester-

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ification of a set of membrane chemotaxis receptors has been well characterized. Motile cells adapt to stimuli by reversibly methylating specific L-glutamyl residues on the receptor proteins (Springer et al., 1979; Koshland, 1980). A similar function in mammalian cells has been searched for (Venkatasubramanian et al., 1980; Aksamit et al., 1983) but not found.

As a first step in the systematic characterization of methyl esterification in mammalian cells, we have investigated the extent and distribution of modified proteins in a mouse lymphoma cell line. This study was facilitated by the use of an assay which readily distinguishes methyl esters from other methylation as well as from methionine incorporation into protein (Chelsky et al., 1984). The reversible nature of the methylation was also studied. The ability to demethylate and remethylate substrate proteins is a prerequisite for a regulatory role for this modification. In addition, methylation in cell extracts was compared with results in intact cells as a first step in the characterization of the components of the modification/demodification pathway.

Experimental Procedures

Cells and Reagents. Mouse S49 lymphoma suspension cultures (clone 24.3.2 from H. R. Bourne, University of California, San Francisco) were grown in Dulbecco's modified Eagle's medium (DME)[1] plus 10% heat-inactivated horse serum in a 7% CO_2 atmosphere (Coffino et al., 1975). Cells were harvested by centrifugation and washed once in phosphate-buffered saline (PBS: 10 mM sodium phosphate, pH 7.3, and 150 mM NaCl).

L-[*methyl*-^{3}H]Methionine ([^{3}H]Met) was repurified before use by high-pressure liquid chromatography (HPLC) on a 4.6 mm × 25 cm LC18 column (Supelco). Retention time for methionine was 7 min in 90% methanol and 0.005% trifluoroacetic acid at a flow rate of 1 mL/min. [^{3}H]Met, used directly from any supplier was cytotoxic to the S49 lymphoma line (Figure 1). Gel reagents were from Bio-Rad. Tissue culture medium and supplies were from GIBCO laboratories.

Gel Electrophoresis. Labeled cells were pelleted in a Beckman microfuge, resuspended in sodium dodecyl sulfate (SDS) sample buffer (Laemmli, 1970), and boiled for 1 min. SDS gels were 1.5 mm thick and contained 12.5% acrylamide [0.33% bis(acrylamide)] (Laemmli, 1970). After being stirred with Coomassie brilliant blue R-250 in 10% acetic acid and 25% 2-propanol and being destained in 10% acetic acid, gel lanes were sliced into 2.2-mm pieces with a Hoeffer razor blade gel slicer. After being dried under heat and vacuum on a filter paper backing, the gel pieces were separated with scissors and analyzed for the presence of methyl esters by the diffusion assay (Chelsky et al., 1984). To compare results with work from other laboratories, a sample was also analyzed by an SDS gel system at pH 2.4 (Table I) (Fairbanks & Avruch, 1972).

Labeling of Intact S49 Lymphoma Cells and Cellular Fractionation. Cells (3×10^7) were labeled in 30 mL of minus-Met DME plus 40 μM [^{3}H]Met (3.6 Ci/mM, Amersham) for 12 h and harvested by washing twice in cold PBS. All subsequent steps were carried out at 4 °C. The cells were resuspended in 1 mL of HME buffer (Ross et al., 1977) (20 mM HEPES, pH 7.0, 2 mM $MgCl_2$, and 1 mM EDTA) plus 75 mM NaCl and lysed with 40 strokes in a tight-fitting Potter Elvehjem glass homogenizer. Nuclei were isolated by centrifugation of the lysate for 5 min at 1500*g*. The pellet was washed twice in HME plus 150 mM NaCl followed by two washes in detergent (10 mM Tris, pH 7.0, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, and 0.3 M sucrose) and a final wash in the same buffer with 0.1 mM $CaCl_2$ replacing the Nonidet P-40 (Weintraub & Groudine, 1976). The supernatant from the 1500*g* spin was then centrifuged for 10 min at 30000*g* and the pellet washed twice with HME plus 150 mM NaCl. This pellet contained the plasma membranes and mitochondria as determined by assays for the ouabain-sensitive ATPase (Jorgensen, 1974; Goldmark & Linn, 1972) and cytochrome oxidase (Wharton & Tzagoloff, 1967), respectively. The supernatant was then centrifuged at 230000*g* for 1 h to pellet microsomes. The supernatant of the final spin, the cytosol, was precipitated with 10% trichloroacetic acid and washed twice with acetone/ether (1:1). All samples were dissolved in SDS gel sample buffer and the proteins separated by gel electrophoresis. Volatile/base-labile radioactivity was determined by the diffusion assay (Chelsky et al., 1984).

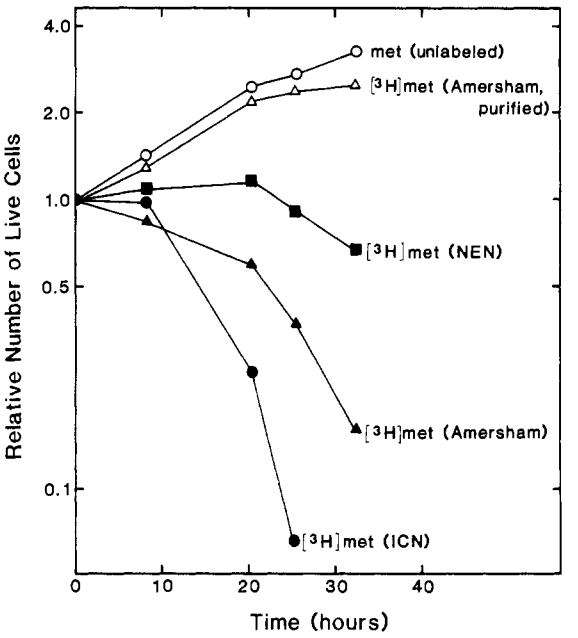


FIGURE 1: Effect of [^{3}H]Met on cell growth before and after purification by high-performance liquid chromatography. A culture of S49 lymphoma cells was grown to 6×10^5 cells/mL in minus-Met DME (UCSF tissue culture facility) plus 100 μM Met and 10% heat-inactivated horse serum. Cells were aliquoted into six sterile centrifuge tubes (3 mL each) and pelleted in a table-top centrifuge. The medium was removed, 3 mL of minus-Met DME was added back with the additions indicated in the figure, and the cells were transferred to 6-cm tissue culture plates. The cell density was determined at the indicated times with a hemocytometer and normalized to the initial density. Radioactive Met was present at 75 μCi per plate, diluted with unlabeled Met (Sigma) to 25 μM final concentration; NEN (12 Ci/mM), Amersham (15 Ci/mM), ICN (1 Ci/mM).

Labeling and Fractionation of Cell Homogenates. S49 lymphoma cells (2×10^7) were washed in 25 mM HEPES, pH 7.0, plus 150 mM NaCl and resuspended in 0.6 mL of 12.5 mM HEPES, pH 7.0, plus 75 mM NaCl (5-fold dilution). The cells were lysed by 50 manual strokes in a 2-mL siliconized Dounce homogenizer on ice. The efficiency of lysis was determined by using a hemocytometer. The suspension was incubated at 37 °C for 1 h after addition of 0.14 mL (0.14 mCi) of *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet) (140 Ci/mM, Amersham) and centrifuged at 1500*g* for 5 min. Nuclei were extracted from this pellet by washing in Nonidet P-40 as described above.

[1] Abbreviations: DME, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; Met, methionine; SDS, sodium dodecyl sulfate; AdoMet, *S*-adenosylmethionine; kDa, kilodalton(s); HPLC, high-pressure liquid chromatography; PEI, poly(ethylenimine); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

The supernatant of the 1500*g* spin was centrifuged at 35000*g* for 90 min to pellet membranes and mitochondria. The supernatant from this spin (cytosol) was precipitated with 5% trichloroacetic acid. The trichloroacetic acid pellet was washed twice with 1 mL of acetone/ether (1:1) and dried.

Incorporation of Exogenous Methionine into the Cellular S-Adenosyl-L-methionine Pool. Cells (10^7) were incubated in 10 mL of 4 μM [2,8-^{3}H]adenine (1.25 Ci/mM) in DME for 9 h. They were then centrifuged and resuspended in 10 mL of minus-Met DME containing [^{3}H]adenine at the same specific activity along with 15 μM [^{35}S]Met (0.69 Ci/mM, Amersham). Aliquots of 0.5 mL were removed periodically and centrifuged in a Beckman microfuge followed by addition of 0.05 mL of 1 M formic acid to the pellet. Samples were then vortexed, placed on ice for 30 min, and centrifuged in a Beckman microfuge for 2 min to pellet acid-insoluble material. The acid-soluble fraction of each sample was lyophilized, resuspended in 3 μL of distilled water, and applied to a PEI–cellulose F thin-layer chromatography plate (EM Reagents) along with 10 μg of unlabeled AdoMet. Plates were developed 9 cm with 0.7 M ammonium borate, pH 9, washed with methanol, and developed 5 cm in the second dimension with 3 M ammonium sulfate (Bochner & Ames, 1982). Plates were sprayed with EN^{3}HANCE (New England Nuclear), and subsequent autofluorography indicated that the AdoMet spot was well resolved from other radioactive material on the plate. AdoMet was detected with a UV lamp, and the spot was cut out and placed in a scintillation vial with 0.6 mL of 0.5 M sodium phosphate, pH 6, and incubated at 22 °C for 30 min with shaking. Scintillation fluid was then added, and the samples were counted for ^{3}H and ^{35}S.

Methyl Ester and Phosphate Incorporation into Protein. Cells (5×10^6) were labeled in 2.3 mL of minus-Met DME containing 15 μM [^{3}H]Met (12 Ci/mM) and 10% heat-inactivated horse serum for 10 h. Alternatively, 5×10^6 cells were labeled for 10 h in 2.3 mL of DME containing 15 μM unlabeled Met, 10% serum, and 2 mM [^{32}P]orthophosphate (43 mCi/mM, Amersham). In both cases, each cell aliquot was analyzed by gel electrophoresis as described above. The lane containing ^{3}H-labeled cells was stained and destained, cut into 2.2-mm sections, and analyzed by the diffusion assay. The lane containing cells labeled with ^{32}P was treated with hot 5% trichloroacetic acid followed by 10% acetic acid as described by Bhorjee & Pederson (1976). This procedure removes all but serine and threonine phosphates (Bhorjee & Pederson, 1976). The ^{32}P lane was then cut into 2.2-mm slices which were each dissolved in 1 mL of hydrogen peroxide at 70 °C for 5 h in capped glass scintillation vials. Radioactivity was determined after addition of 10 mL of scintillation fluid. The protein content of lymphoma cells was determined by the method of Peterson (1977).

Results

Methyl Ester Formation in Intact Cells. The occurrence of methyl-esterified proteins was studied by incubating cells in [^{3}H]Met. Radioactive methionine is converted by the cells into [^{3}H]AdoMet, the methyl donor in most methylation reactions including the methyl esterification of carboxyl groups (Paik & Kim, 1980a,b). Proteins from labeled lymphoma cells were separated on Laemmli-type SDS gels which were then sliced into 130 uniform pieces per lane. Since the methyl esters can be base hydrolyzed to form methanol, the volatile/base-labile radioactivity from each slice was determined and plotted to produce a profile of methyl-esterified proteins (Figure 2). The number of peaks of base-labile radioactivity was too large for complete resolution on this gel. The predominant methyl-esterified proteins migrate with apparent molecular weights of 21K, 24K, 27K, 28K, 36K, and 70K. The limit of detection in these experiments is approximately 1000 copies per cell of a given [^{3}H]methyl-esterified protein.

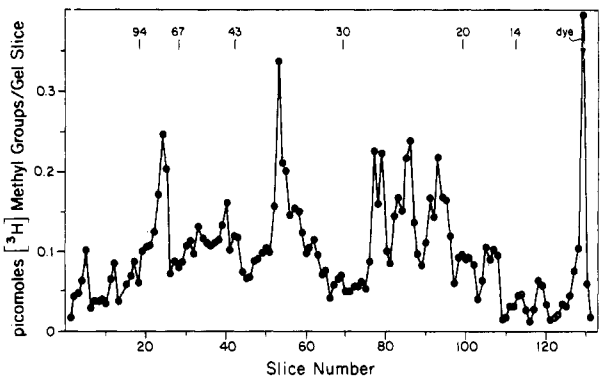


FIGURE 2: Profile of methyl-esterified proteins in lymphoma cells. Cells (10^7) were labeled in 10 mL of minus-Met DME plus 11 μM [^{3}H]Met (90 Ci/mM) for 4 h and analyzed by gel electrophoresis and the diffusion assay as described under Experimental Procedures.

Table I: Detected Incorporation of Methyl Ester Groups following SDS Gel Electrophoresis[a]

gel system	sample	methyl groups recovered (pmol/mg)	% max recovery
pH 2.4	control	64.2	100
pH 8.3	control	61.7	96
	+RNase A (0.5 μg)	60.0	93
	+trypsin (1.0 μg)	4.6	7
	+NaOH (pH 11)	1.6	2

[a] Samples were prepared from labeled intact cells as described under Experimental Procedures, heated to 50 °C for 4 min following addition of the appropriate sample buffer, and subsequently treated with 0.75 μg of DNase I (Worthington) for 1 h at 20 °C. Identical samples in gel buffer were also pretreated with the indicated addition for another 3 h at 20 °C followed by boiling for 1 min. The base-treated sample was neutralized with Tris-HCl.

In addition to analysis by pH 8.3 Laemmli-type SDS gels (Laemmli, 1970), another SDS gel system was used which separates protein at pH 2.4 (Fairbanks & Avruch, 1972). A low-pH gel system has been found necessary by some researchers for full recovery of base-labile methyl groups (Kloog et al., 1980; Freitag & Clarke, 1981; Billingsley et al., 1984). However, methyl esterifications of purified rat non-histone chromosomal proteins (Quick et al., 1981) as well as bovine photoreceptor rod outer segments (Swanson & Applebury, 1983) are stable during Laemmli gel electrophoresis. We find little difference between the two systems (Table I) and suspect that the SDS acts to protect the methyl esters from hydrolysis as suggested previously by others (Kleene et al., 1977; Galletti et al., 1978). The stability on SDS gels of the methyl esters found here is similar to that found for regulatory methyl esterification of bacterial membrane receptors.

Other tests of the nature of the methyl group included increasing the pH of the sample buffer to 11, which resulted in loss of the methyl ester groups (Table I). Ladders of DNA have been seen on SDS gels of bacterial samples (Wang & Koshland, 1978), suggesting the possibility of non-protein-associated molecules being fractionated by these gels. Therefore, the effect of both DNase I and RNase A was tested, but neither altered the recovery of base-labile radioactivity on SDS gels. Treatment with trypsin, however, caused all of the methyl esters to migrate at the dye front (Table I).

Cellular Fractionation. The locations of the methylated proteins were determined by fractionating the cells into four

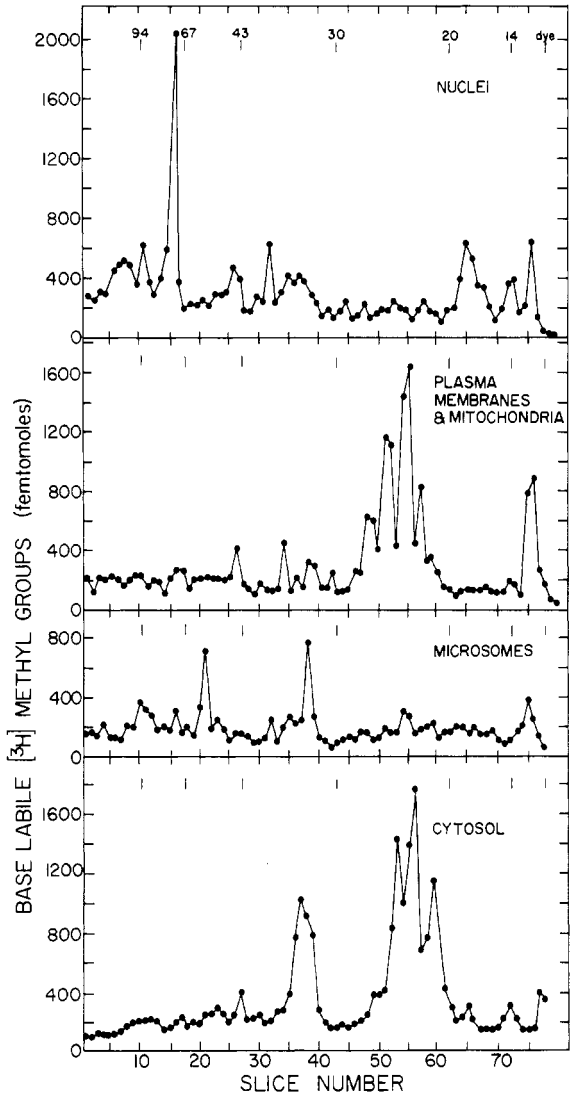


FIGURE 3: Cell fractionation following labeling of intact cells.

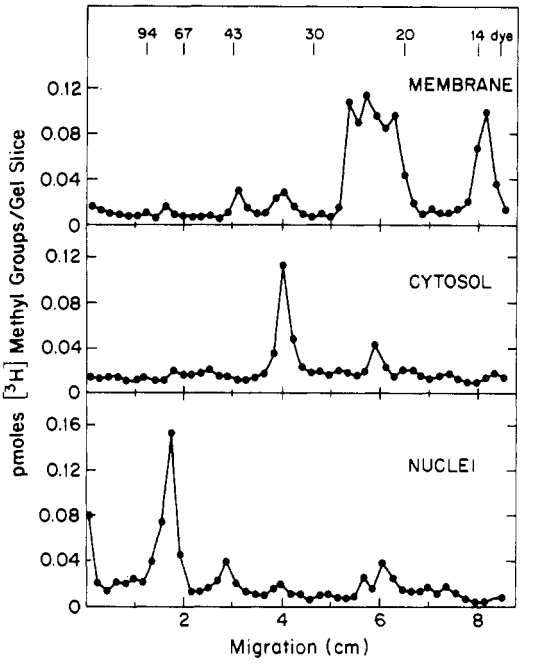


FIGURE 4: Cell fractionation following labeling of a cell homogenate.

Table II[a]

sample	incorporation (pmol/mg)	rel incorporation (%)
intact cells		
control	20.5	100
+puromycin	8.0	39
+cyclohexamide	7.8	38
cell homogenates		
control	3.5	100
+75 μM *S*-adenosylhomocysteine	0.7	20

[a] Intact S49 lymphoma cells were labeled and analyzed as described under Figure 7. Puromycin or cyclohexamide (0.2 mM each) was added 15 min before addition of label. Cell homogenates were labeled and analyzed as described under Experimental Procedures. AdoMet was present in the cell homogenates at 4 μM.

components whose major constituents were nuclei, plasma membranes and mitochondria, microsomes, and cytosol (Figure 3). Twenty-four reproducibly distinguishable peaks of base-labile radioactivity were obtained. Five proteins were in the plasma membrane/mitochondrial fraction at 13, 24, 25, 27, and 28 kDa. The two predominant peaks in the microsomal fraction migrated at 32 and 56 kDa. The cytosol contained six proteins at 16.5, 21, 24, 26, 34, and 36 kDa of which the 34- and 36-kDa proteins did not resolve here. The nucleus contained 11 methylated proteins at 12, 15.5, 18, 19, 34, 41, 45, 70, 90, and 105 kDa.

The appearance of methyl-esterified proteins of similar molecular weights in the different fractions does not appear to be due to cross-contamination because of the clean separation acheived for other proteins. For example, the 19.5- and 70-kDa proteins are found almost exclusively in the nucleus while the 56-kDa protein is present only in the microsomal fraction. Similarly, the 27-kDa protein is found only in the plasma membrane/mitochondrial fraction.

Incorporation of Methyl Groups in Cell Homogenates. To determine the extent of protein methyl esterification in a cell homogenate, mouse lymphoma cells were lysed into a buffered salt solution and incubated with [^{3}H]AdoMet. No other additions were made, and after 1 h, the cell homogenate was fractionated into nuclei, cytosol, and a fraction containing plasma membranes and mitochondria. Proteins from each fraction were separated by SDS gel electrophoresis and analyzed for volatile/base-labile methyl esters by the diffusion assay. A number of protein peaks with significant base-labile radioactivity were obtained (Figure 4). The approximate sizes of the putative protein methyl esters were 23 and 36 kDa in the cytosol; 22, 45, and 70 kDa in the nuclei, and 13, 21, 24, 26, 38, and 41 kDa in the membrane fraction. These proteins appear to be a subset of those methylated in intact cells because of the correlation of gel mobility and cellular localization. The same proteins are methylated if the cells are first fractionated into nuclei, membranes, and cytosol and subsequently methylated by the addition of [^{3}H]AdoMet to the fractions (not shown). Incorporation of radioactivity into these proteins was inhibited by 70% when cells were labeled in the presence of the methylation inhibitor *S*-adenosylhomocysteine (Table II).

Methionine Exchange into the S-Adenosylmethionine Pool. Our ability to determine the incorporation of methyl esters per cell as well as their turnover rates is dependent on the assumption that labeled methionine, added exogenously, exchanges rapidly with the cellular AdoMet pool. To test this, cells were first labeled with [^{3}H]adenine for 9 h to ensure complete equilibrium. [^{35}S]Met was then added, and at intervals, aliquots of the cells were extracted in formic acid, and AdoMet was purified by two-dimensional thin-layer chromatography (Bochner & Ames, 1982). After 30 min, the

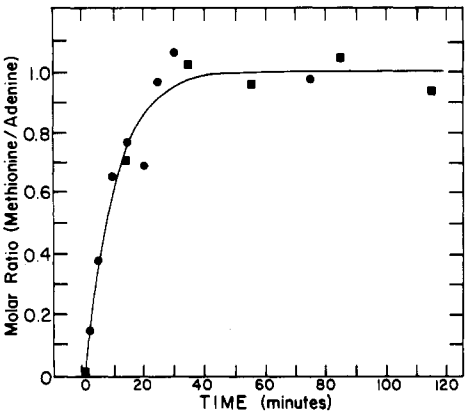


FIGURE 5: Incorporation of radioactive methionine into the cellular AdoMet pool. [^{35}S]Met was added to cells, which had been preequilibrated with [^{3}H]adenine, as described under Experimental Procedures. AdoMet was extracted from aliquots of these cells at intervals, and the molar ratio of the two labels was determined. Circles and squares represent two independent experiments.

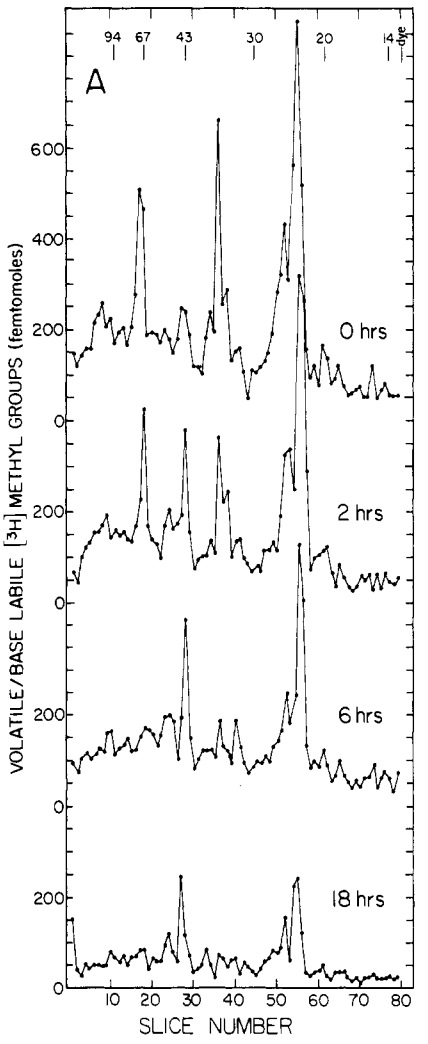
[^{35}S]Met had completely equilibrated with the [^{3}H]adenine at a molar ratio of unity (Figure 5). This ratio indicates that the entire AdoMet pool is undergoing exchange.

Turnover of Methyl Esters. To test whether methyl-esterified proteins are subsequently demethylated, lymphoma cells were labeled with a long pulse (17 h) of [^{3}H]Met followed by a chase period in which the label was removed and aliquots taken for up to 18 h. At each time point, the methylated proteins were analyzed by SDS gel electrophoresis. Figure 6A shows the pattern of methyl-esterified proteins as a function of time. From the profiles in this figure, the rates of decay were plotted for several of the major base-labile peaks (Figure 6B). The total radioactivity (including methionine incorporation) in the samples was determined as well. At least two of the base-labile peaks, at 22 and 25 kDa, represent multiple proteins, and their decay rates should be considered the sum of multiple rates.

As seen in Figure 6B, the total radioactive incorporation into protein decreased during the chase period in this experiment ($t_{1/2}$ = 10 h). The average rate of decrease, however, was slower than the decrease in base-labile radioactivity except in the case of the 45-kDa methyl-esterified protein ($t_{1/2}$ = 34 h). Half-lives for the loss of base-labile radioactivity from the 22, 25, 36, 70, and 130-kDa methyl-esterified proteins were 9.3, 6.0, 3.0, 2.6, and 7.3 h, respectively.

Methyl Esterification in the Absence of Protein Synthesis. When lymphoma cells were pretreated for 15 min with puromycin before addition of label, incorporation of methionine into protein was only 2% of that of the untreated sample after 90 min. The level of methyl esterification, however, was 39% that of the untreated sample. Analysis by SDS gel electrophoresis (Figure 7) indicated that blocking protein synthesis had differential effects on the various methylated proteins. Methylation of the 36-kDa protein was nearly independent of protein synthesis (72% of control) while the 70-kDa protein appeared to be tightly linked (<8% of control). Results with cycloheximide in place of puromycin were essentially identical (Table II).

To ensure that the incorporation of methyl groups in the absence of protein synthesis was not due to the slow processing of recently synthesized protein, the ability to methylate these proteins over time was tested. Aliquots of cells were labeled for 90-min intervals beginning at 15, 45, 75, and 105 min after the addition of puromycin. The total base-labile incorporation of radioactivity for the four samples showed little change at 1.11, 1.17, 1.09, and 1.09 pmol, respectively (Figure 8). Each



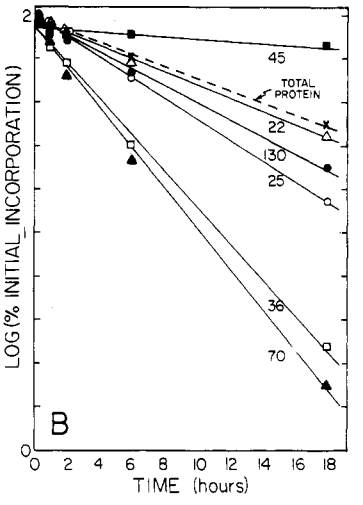


FIGURE 6: Pulse–chase analysis of methyl-esterified proteins. (A) Loss of radioactivity following replacement of [^{3}H]Met with excess unlabeled methionine was monitored as a function of time. Cells (1.25 × 10^{7}) were labeled for 17 h in minus-Met DME with 15 μM [^{3}H]Met (15 Ci/mM), washed twice to remove label, and incubated in normal DME (100 μM Met). At the indicated times following removal of label, an aliquot of 2.5 × 10^{6} cells was removed and analyzed by SDS gel electrophoresis and the diffusion assay as described under Experimental Procedures. (B) Base-labile radioactivity remaining in each major peak in (A) was summed for each time point and normalized to the zero time point. Total radioactivity in the entire sample was also determined for each time point and is shown in comparison.

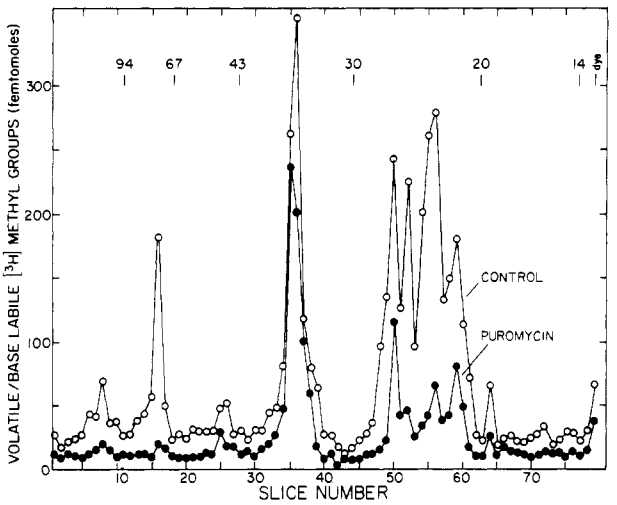


FIGURE 7: Methyl esterification of proteins in the presence of puromycin. Two aliquots of 5×10^6 lymphoma cells were each labeled in 5 mL of minus-Met DME containing 10% heat-inactivated horse serum and 13 μM [^{3}H]Met (15 Ci/mM) for 1.5 h. One aliquot of cells was treated with 0.2 mM puromycin beginning 15 min before addition of label. Samples were analyzed by gel electrophoresis and the diffusion assay as described under Experimental Procedures.

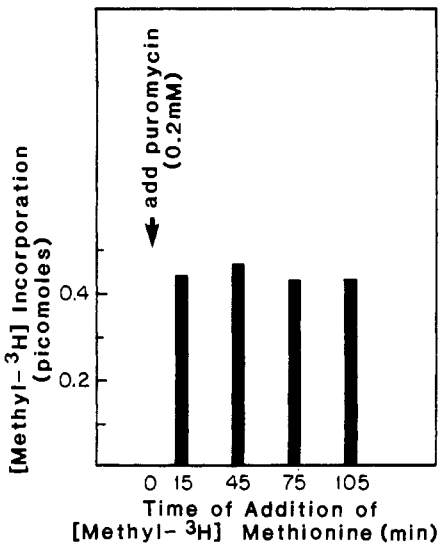


FIGURE 8: Incorporation of methyl esters as a function of time following inhibition of protein synthesis. Four aliquots of 5×10^6 lymphoma cells were each incubated in 3.5 mL of minus-Met DME plus 10% heat-inactivated horse serum. Puromycin (1 mM) was added to each aliquot at time zero, followed by addition of 5 μM [^{3}H]Met (15 Ci/mM) at the times indicated. After incubation for 90 min, in the presence of label, cells were analyzed as in Figure 5, and total methyl group incorporation is presented here.

sample contained 0.25 mg of protein. In addition, the gel profiles of base-labile radioactivity were all essentially identical and resembled that shown in Figure 8 (data not shown). Incorporation into the 36-kDa protein for the same time points was 0.40, 0.39, 0.38, and 0.39 pmol.

Identity of the Methylated Residue. Digestion of methyl-labeled *Escherichia coli* membranes with 1 mg/mL *Streptococcus griseus* protease in 0.05 M *N*-ethylmorpholine acetate, pH 5.2, for 18 h at 37 °C resulted in an 80% yield of the base-labile radioactivity in the original sample as glutamic acid γ-methyl ester. This was determined by derivatization of the digest with *o*-pthalaldehyde and subsequent analysis by reverse-phase high-performance liquid chromatography (not shown). This result is consistent with previous findings (Van der Werf & Koshland, 1977; Kleene et al.,

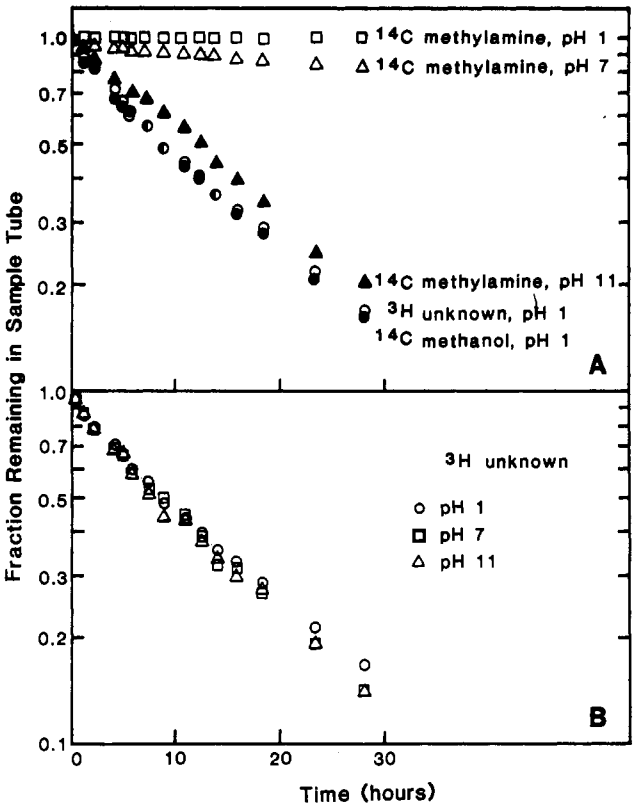


FIGURE 9: Rate of diffusion of the [^{3}H]methyl group released by mild alkalai treatment as compared to that of [^{14}C]methanol and [^{14}C]-methylamine at pH 1, 7, and 11. A sample of [^{3}H]Met-labeled S49 cells was fractionated by Laemmli SDS gel electrophoresis. The gel lane was stained, destained, and dried. The 36- and 70-kDa regions of the gel were placed in a 1.5-mL screw cap tube with 0.5 mL of 0.2 N NaOH. After 18 h at 30 °C, the tube was cooled and opened, and aliquots were added to either [^{14}C]methanol (NEN) or [^{14}C]-methylamine (ICN) at pH 1, 7, or 11. Samples were placed into a 1.5-mL tube which was set into a 15-mL scintillation vial containing 6 mL of Scint A (Beckman). Scintillation fluid did not enter the sample tube. The capped scintillation vial was set in a scintillation counter at 22 °C, and at intervals, the ^{14}C and ^{3}H radioactivity which had diffused into the scintillation fluid from the sample tube was determined. This value was subtracted from the total radioactivity in the sample tube, and the result was plotted on a semilog graph.

1977). A similar analysis of mammalian proteins produced neither the methyl ester of glutamate or aspartate. However, the proteolyzed methylated group reacts with *o*-pthalaldehyde and dansyl chloride, indicating that it contains a free amino group. It also is labile at pH 9 but not at pH 5, consistent with a methyl ester. In addition, the ^{3}H-labeled product of the hydrolysis at pH 9 is methanol as determined by comparison of the rate of diffusion with that of an authentic [^{14}C]methanol standard (Figure 9A). Methylamine which can be released at low yield by hydrolysis of N^{γ}-methyl-L-arginine (Paik & Kim, 1980a,b) was also compared to the ^{3}H-labeled unknown (Figure 9A). At pH 11, the rate of diffusion of the methylamine standard was slower than that of methanol, consistent with its higher molecular weight. When the solution was titrated to pH 1 with HCl, the resulting methylamine hydrochloride was nonvolatile. Rates of diffusion of the [^{14}C]methanol standard and the ^{3}H-labeled unknown, however, were idential whether the pH of the sample was adjusted to 1, 7, or 11 following hydrolysis at pH 9 (Figure 9B).

Comparison of the Levels of Methyl Esterification and Phosphorylation. When cells were labeled for 10 h, 28.7 pmol of [^{3}H]methyl esters was incorporated into 243 μg of protein (118 pmol of methyl groups/mg of protein). The incorporation of phosphate into protein was determined under the same

conditions as those used for methyl ester incorporation. Unlabeled methionine was substituted for [^{3}H]Met, and [^{32}P]-orthophosphate was added followed by incubation for 10 h. In this time, the cells incorporated 5.9 nmol of phosphoryl groups/mg of protein. This number is consistent with that obtained previously by Forsberg et al. (1969) of 6–10 nmol/mg of protein.

Discussion

There are at least 24 methyl-esterified proteins in intact mouse lymphoma cells. This result was determined by analysis of cells labeled with [^{3}H]Met using SDS gel electrophoresis. [^{3}H]Methyl groups, released as methanol at alkaline pH, are detected by diffusion assay of the gel slices. This assay does not detect other forms of methylation or methionine incorporation into protein. Therefore, it has been possible to analyze the extent of protein methyl esterification in growing cells rather than to rely on systems lacking any competing incorporation such as cell homogenates or red blood cells.

While 24 methyl-esterified proteins can be readily detected in these experiments, the actual number may be higher since the lower limit of detection here is approximately 1000 methyl groups in a given protein per cell. Another way to determine the extent of methyl esterification is to sum the incorporation across the gel profile which, at steady state, is 118 pmol/mg of protein. If an average protein is assumed to be 40 kDa in size (Sommer & Cohen, 1980), the number of methyl esters can be approximated at 1 for every 210 proteins in the cell. When protein phosphorylation was determined in an analogous manner, we found that there was one phosphate for every four proteins. This result is reflected in the glycolysis pathway, for example, where three out of the nine enzymes are regulated by phosphorylation. Methylation is present at only 2% the level of phosphorylation, but the number appears low only because of the prevalence of phosphorylation. It remains to be proven whether reversible methylation plays a regulatory role, but it could be a valuable alternate mechanism just as cGMP serves as an alternate to cAMP.

In order to analyze the components of the methylation/demethylation machinery, it would be necessary to establish conditions in which the proteins methylated in intact cells are also methylated in vitro. Therefore, packed cells were lysed, followed by addition of [^{3}H]AdoMet. The overall profile of incorporation was very similar to that found in intact cells. The cell homogenate was also fractionated following labeling to avoid relying completely on gel mobility for comparison with intact cells. The results indicated that the 11 proteins which were labeled in cell homogenates were a subset of those found in intact cells. Using this system, it should be possible, by means of further purification as well as mixing experiments, to identify the components of the methyl esterification process.

In previous studies, purified methyl transferase from a variety of sources has been added to a cell homogenate to determine the level of methyl-accepting proteins. The in vitro system used here has the advantage that the observed incorporation is dependent only on the endogenous cell components and the environment created by the lysis buffer. The methyl transferases purified to date have been isolated on the basis of their ability to methylate an arbitrary substrate such as ovalbumin (Kim & Paik, 1970) or a mixture of denatured proteins (Liss et al., 1969). It is possible that these enzymes may not be the same as those responsible for methylation of the proteins detected here.

Identity of the Methylated Compound. Amino acid analysis of methyl-esterified proteins in red blood cells has revealed the presence of D-aspartate β-methyl ester, suggesting the possibility of a repair pathway for proteins with racemized amino acids (McFadden & Clarke, 1982). A study using a model protein suggests that another possible substrate may be an isoaspartyl group (Aswad, 1984), generated by spontaneous exchange of the peptidyl α-carbonyl with the β-carboxyl side chain of aspartate to produce an α-carboxyl side chain. Other possible methyl acceptors are L-aspartyl and L-glutamyl residues (Paik & Kim, 1975) as well as the α-carboxyl group at a protein's C-terminus.

The actual group from which the base-labile radioactivity is derived has not been identified as it has been in bacteria. There is evidence, however, that it is a methyl ester of a protein carboxylic acid: (1) The radioactivity is derived from the methyl moiety of AdoMet as determined by the labeling of cell homogenates; (2) adenosylhomocysteine (demethylated AdoMet) inhibits the reaction in cell homogenates, indicating that the leaving group is the methyl moiety; (3) the product of the methylation is labile in mild alkaline solution which is characteristic of methyl esters but not of N-methylation; (4) the methyl group released in mild base has the same rate of diffusion as authentic methanol (see Figure 9) as expected for the hydrolysis of a methyl ester; (5) the rate of diffusion of the hydrolyzed methyl group is independent of pH, consistent with methanol but not methylamine; (6) if the labeled cells are treated with trypsin before SDS gel analysis, the base-labile/volatile radioactivity migrates at the dye front with the other protein-linked radioactivity. Treatment with DNase I and RNase A have no effect on gel migration.

Reversibility of Methylation. If methylation is being used to regulate the activity of these proteins, we would expect that both methylating and demethylating enzymes are present in the cells. At least one protein with methyl transferase activity has been identified in mammalian tissues (Paik & Kim, 1980a,b; Dilberto, 1982), and there are reports of methyl esterase activity as well (Venkatasubramanian et al., 1980; Quick et al., 1981). Demethylation was examined by using a pulse–chase experiment. Methyl groups were lost over time with an average half-life of approximately 5 h. At least two groups decayed with shorter half-lives of 2.6 and 3 h, while the methyl groups of one protein showed almost no loss.

If the proteins which show a loss of methyl groups in the pulse–chase experiment are being remethylated, we would expect to be able to detect a steady level of methyl group incorporation into these proteins long after they are synthesized. This would also argue against the possibility that the turnover in the pulse–chase experiment is due to protein degradation. When protein synthesis was blocked with puromycin, the pattern and level of methylation remained constant whether label was added 15 or 105 min after puromycin addition. For example, the ability to methylate the 36-kDa protein declined by less than 4% in 90 min while the loss of methyl groups in the pulse–chase experiment of the same protein was 30% in 90 min. Methylation and demethylation, therefore, appear to be occurring on a continuous basis which is essential for a regulatory modification.

Acknowledgments

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