Atti Accademia Nazionale dei Lincei

Classe Scienze Fisiche Matematiche Naturali

Rendiconti

Tamilla Eremenko, Patrizia Cimarra, Antonio Giuditta, Pietro Volpe

Synthesis and methylation of proteins during the HeLa cell cycle

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Serie 8, Vol. **66** (1979), n.2, p. 153–162.

Accademia Nazionale dei Lincei

<http://www.bdim.eu/item?id=RLINA_1979_8_66_2_153_0>

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Accademia Nazionale dei Lincei, 1979.

Biochimica. — *Synthesis and methylation of proteins during the* HeLa *cell cycle* (*). Nota di TAMILLA EREMENKO, PATRIZIA CIMARRA, ANTONIO GIUDITTA e PIETRO VOLPE, presentata (**) dal Corrisp. A. RUFFO.

RIASSUNTO. — La velocità iniziale di sintesi e metilazione delle proteine è stata esaminata durante il ciclo mitotico di cellule *HeLa* mantenute in culture sincronizzate. Le proteine sono state separate in una frazione solubile in mezzo salino e in una frazione solubile in SDS. Ognuna di esse è stata ulteriormente frazionata per elettroforesi su gel contenente SDS.

Durante il ciclo cellulare il tasso sintetico varia in maniera sensibile in tutte le frazioni proteiche raggiungendo un massimo in G_1 e in minor misura in G_2. Le oscillazioni di maggiore ampiezza si ritrovano in un gruppo di proteine solubili in mezzo salino e di media grandezza. Le variazioni minime hanno luogo in un gruppo di proteine di basso peso molecolare solubili in SDS. La radioattività delle proteine solubili in SDS è considerevolmente maggiore (2–4 volte) di quella delle proteine solubili in mezzo salino.

Anche la velocità di metilazione delle proteine solubili in SDS va incontro a notevoli variazioni raggiungendo un massimo nella fase S. Il tasso di metilazione delle proteine solubili in mezzo salino rimane invece costante e a un livello inferiore per tutto il ciclo cellulare.

Questi dati confermano i nostri precedenti risultati e convalidano l'ipotesi che l'aumentata sintesi proteica non coinvolga indiscriminatamente tutte le proteine. La presenza di un massimo di metilazione nella fase S per le proteine solubili in SDS ma non per quelle solubili in mezzo salino sottolinea la selettività temporale e di tipo molecolare delle modificazioni postsintetiche delle proteine durante il ciclo mitotico.

1. INTRODUCTION

Protein synthesis during the mitotic cycle has been investigated in several cell systems using different methods of synchronization and adopting periods of incorporation of various lengths. These variations may explain some of the contradictory results present in the literature [1, 2]. Other controversial data may be attributed to the peculiar features of the mitotic cycle in some cell types. The G_1-phase, for instance, is completely absent in some systems but is rather long in others [3].

Previous results from this laboratory have indicated that the initial rate of peptide synthesis on *HeLa* cell polysomes, as measured with a 3 min pulse, shows two peaks which occur in G_1 and G_2 respectively [4]. The peaks are independent of the amino acid pool and the rate of amino acid transport [4,5]. They take place approximately one hour after the appearance of similar peaks

(*) International Institute of Genetics and Biophysics, Naples, Italy, and Zoological Station, Naples, Italy.

(**) Nella seduta del 13 gennaio 1979.

of newly-synthesized pre-mRNA [6] and correspond to the increase in polysome content which is considerably higher in G_2 than in G_1 [5]. On the other hand, the rate of peptide synthesis on polysomes is higher in G_1 than in G_2 [4] and polysomes are generally smaller in G_1 than in G_2 [4]. These findings have led to the hypothesis that large polysomes might exist in an inactive state in G_2 [4] and have provided some support for the suggestion that the elongation rate might be inversely related to the length of mRNA [7].

As a first test of these possibilities we examined the size of the proteins synthesized during the mitotic cycle, using SDS-gel electrophoresis to fractionate the protein mixture. In addition, we measured the rate of protein

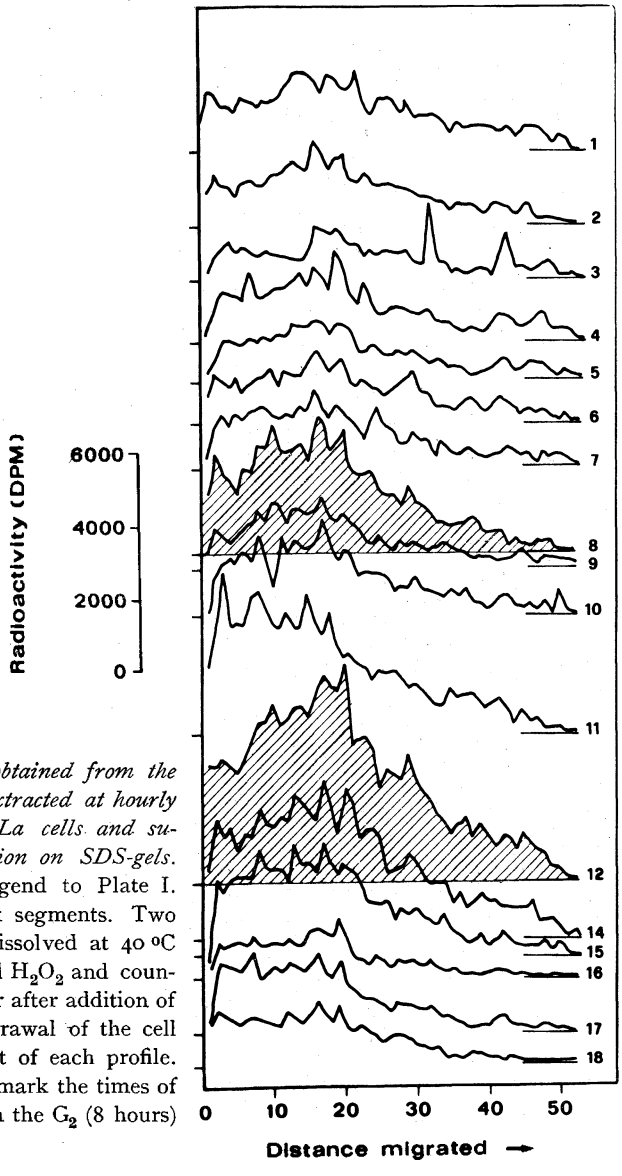


Fig. 1. – *Radioactive profiles obtained from the saline-soluble protein fraction extracted at hourly intervals from synchronized HeLa cells and subjected to electrophoretic separation on SDS-gels.* For experimental details see legend to Plate I. Gels were sliced into 1 mm thick segments. Two slices were placed in a vial, dissolved at 40 °C overnight with 1 ml concentrated H_2O_2 and counted in a scintillation spectrometer after addition of 10 ml Insta-gel. Times of withdrawal of the cell samples are shown on the right of each profile. Shaded profiles are intended to mark the times of maximal labelling which occur in the G_2 (8 hours) and in the G_1 (12 hours) phases.

methylation to compare it with the processes of DNA and RNA methylation which are known to take place during the S–phase [8, 9]. Knowledge of the relative rates of synthesis and post-synthetic modification of different groups of proteins during the *HeLa* cell cycle was also expected to provide some clues as to the problem of macromolecular programming during the mitotic cycle.

2. MATERIALS AND METHODS

HeLa cells were grown and synchronized in suspension [10] using calf serum depleted of free amino acids by chromatography on Sephadex G–50 [11]. The length of each phase of the cell cycle was monitored as reported earlier [12]. Rates of protein synthesis and methylation were examined as described in the figure legends starting from the second S-phase after removal of the synchronized cells from the thymidine-enriched medium. At hourly intervals throughout the cell cycle samples were withdrawn from the suspension culture, labelled with ^{3}H-leucine (New England Nuclear) as described earlier [4, 5], washed in Hank's salt solution and frozen at – 20 °C. Saline-soluble and SDS-soluble proteins were separated by homogenization in 14 mM Tris–33,3 mM glycine buffer pH 8.2 and centrifugation in a Sorvall SS-34

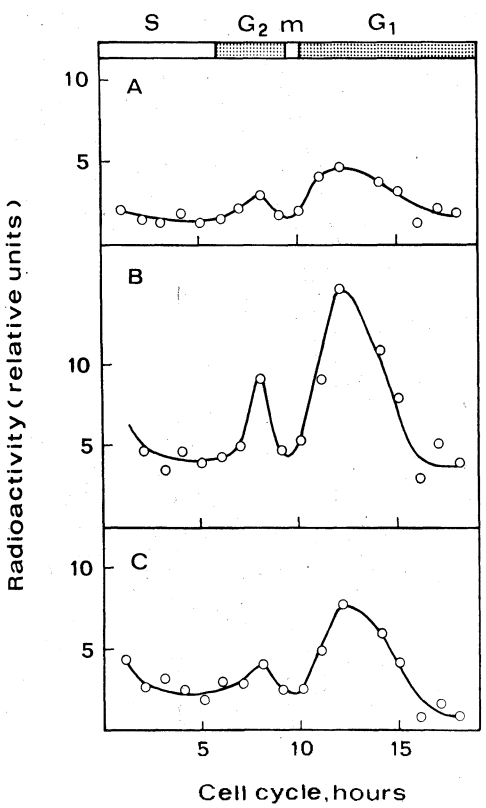


Fig. 2. – *Time course of the radioactivity associated with three groups of proteins separated from the saline-soluble fraction by SDS-gel electrophoresis.* The surface areas corresponding to fractions 0–6 (A), 7–25 (B) and 27–55 (C) of the radioactive profiles obtained at intervals of one hour throughout the *HeLa* cell cycle (Fig. 1) were expressed in terms of paper weight and reported as a function of time.

rotor at 18.000 rpm for 60 minutes. The soluble fraction was dialyzed overnight against 200 vol. of 10 mM sodium phosphate buffer pH 7.2 containing 0.1 % SDS and 0.1 % mercaptoethanol. The insoluble residue was delipidized with 2 ml ether-ethanol (1 : 1) followed by 2 ml ether. The residual solvent was evaporated at 40 °C and the protein fraction was resuspended in 2.0 ml of 10 mM sodium phosphate buffer pH 7.2 containing 1 % SDS and 1 % mercaptoethanol. The suspension was kept in a boiling water bath for 15 minutes.

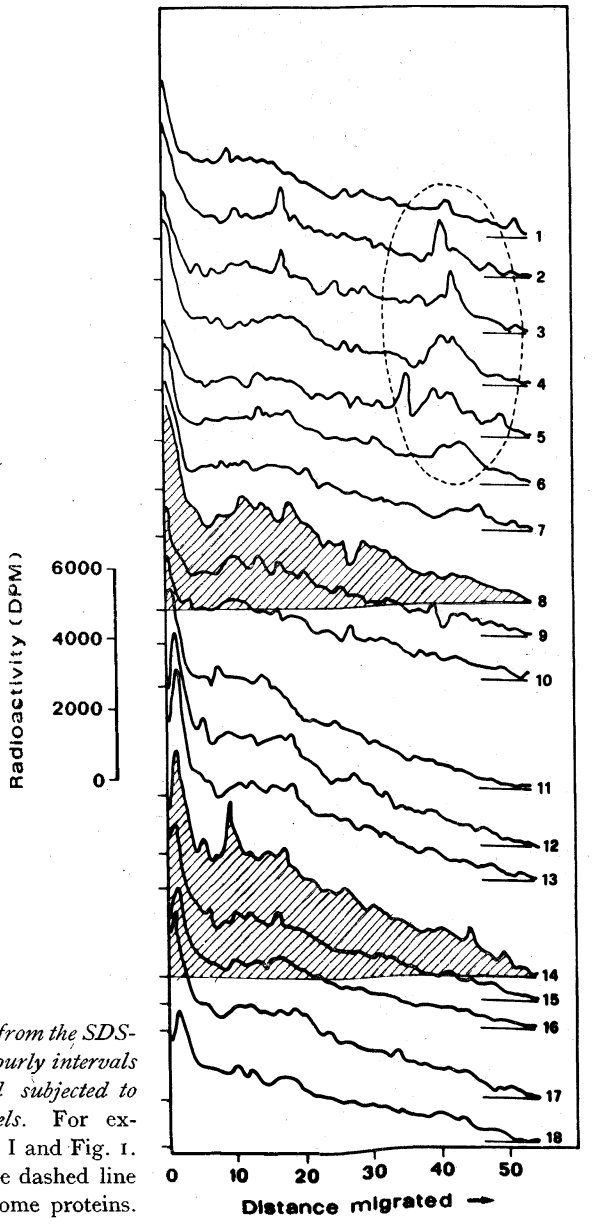


Fig. 3. – *Radioactive profiles obtained from the SDS-soluble protein fraction extracted at hourly intervals from synchronized HeLa cells and subjected to electrophoretic separation on SDS-gels.* For experimental details see legends to Pl. I and Fig. 1. The radioactive peaks circled by the dashed line are presumed to correspond to histome proteins.

The proteins which remained insoluble after this treatment (less than 5 %) were discarded by centrifugation as above. SDS-gel-electrophoresis was carried out according to the method of Weber and Osborne [13]. Protein concentration was detemined using the method of Lowry *et al.* [14]. Radioactivity of the gel fractions was measured in a Packard scintillation spectrometer after solubilization of the gel slices in concentrated H_2O_2 at 40 °C overnight.

3. RESULTS

3.1 – *Electrophoretic fractionation of the proteins.*

HeLa cell proteins obtained at hourly intervals during the mitotic cycle were separated into saline-soluble and SDS-soluble fractions as described in Methods. The protein mixture from each fraction was separated electrophoretically according to the method of Weber and Osborn [13].

As shown in Pl. I, each of the two fractions was separated into approximately 20 bands which were present throughout the cell cycle in essentially the same proportions. A comparison of the two sets of gels indicates that proteins of relatively large molecular weight prevail in the saline-soluble fraction,while proteins of smaller size prevail in the fraction solubilized with the SDS-buffer. The most heavily stained bands of the latter fraction (arrow in Pl. I, B) migrate in a region corresponding to some of the histone proteins.

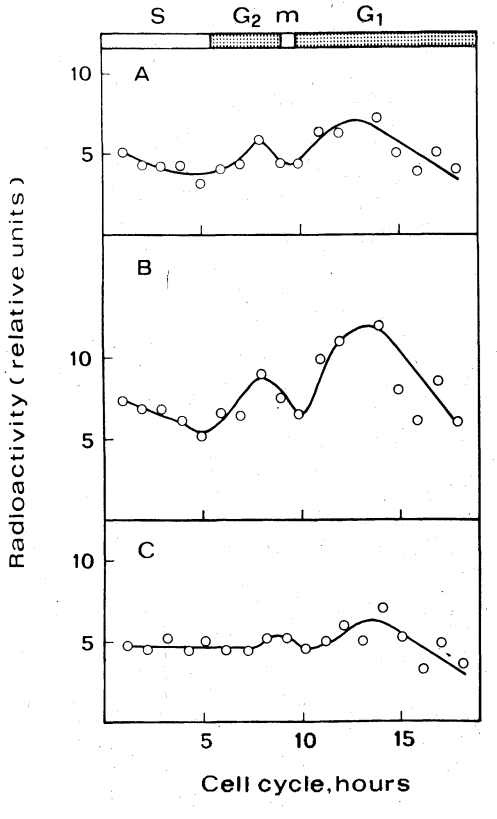


Fig. 4. – *Time course of the radioactivity associated with three groups of proteins separated from the SDS-soluble fraction by SDS-gel electrophoresis.* The surface areas corresponding to fractions 0–6 (A), 7–25 (B) and 27–55 (C) of the radioactive profiles obtained at intervals of one hour throughout the *HeLa* cell cycle (Fig. 3) were expressed in terms of paper weight and reported as a function of time.

3.2 – *Rate of synthesis of different protein fractions.*

The initial rate of synthesis of the separated *HeLa* cell proteins was considered to be proportional to the amount of ^{3}H-leucine incorporated within the 3-minute pulse (see Methods). The patterns of radioactivity obtained for the saline-soluble proteins (Fig. 1) indicate that they are not labelled to the same extent in the different phases of the cell cycle. Maximal labelling occurs in G_2 and G_1 (shaded areas), as previously reported [4]. On the whole, the radioactivity of the gel fractions is distinctly higher in the young G_1–cell than in the premitotic G_2-cell. This behaviour is most marked in the gel region containing medium-size proteins. Proteins of smaller molecular weight appear to be scantly labelled at any point of the cycle. These observations are made more evident by plotting the radioactivity of different protein groups as a function of the cell cycle (Fig. 2). While distinct oscillations of the level of radioactivity are present in all three groups, the oscillations are clearly more evident in the group of medium-size proteins.

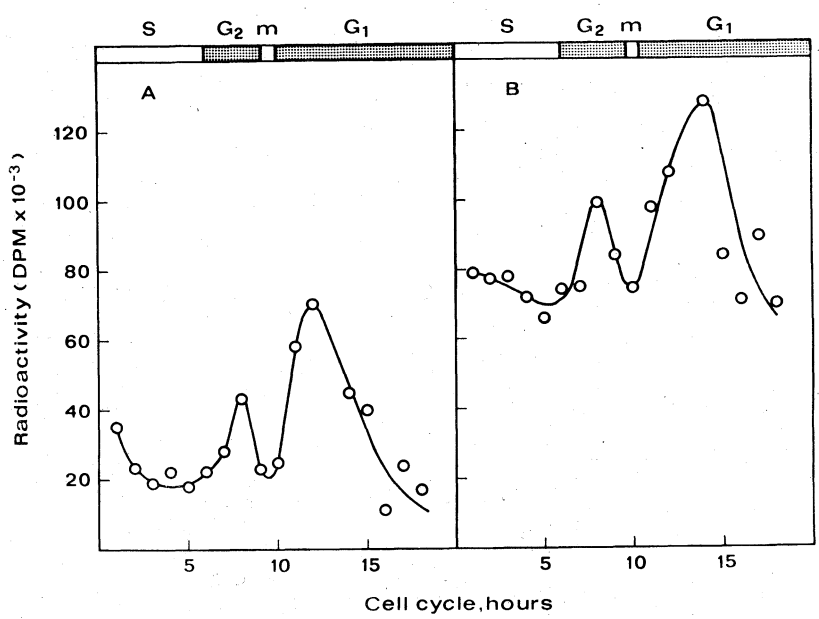


Fig. 5. – *Time course of the total radioactivity of saline-soluble (A) and SDS-soluble (B) proteins separated from HeLa cells at different times during the cycle.* Each point corresponds to the total radioactivity recovered from SDS-gels whose radioactive profiles are shown in Figs. 1 and 3, respectively.

The radioactivity profiles obtained with the SDS-soluble proteins also indicate the existence of two peaks occurring in G_1 and G_2, respectively. The G_1 peak is distinctly larger than the G_2 maximum (shaded areas of Fig. 3).

However, while the G_2 maximum coincides with that of saline-soluble proteins (Fig. 1), the maximum occurring in G_1 takes place with approximately two hours delay with regard to the homologous peak noted in the saline-soluble proteins. At variance with most other proteins, the protein bands which presumably correspond to the histones (Pl. I) appear to be preferentially labelled during the S-phase [15–17]. The variations in the level of radioactivity of the SDS-soluble proteins become more evident when the gel fractions are grouped into classes analogous to those adopted for saline-soluble proteins (Fig. 4). Their overall behaviour is similar to that described for saline-soluble proteins (Fig. 2), even to the extent that the amplitude of the oscillations is

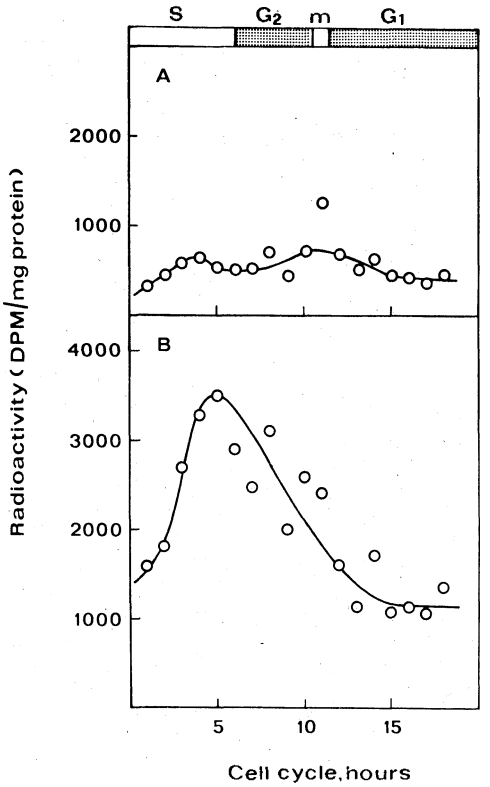


Fig. 6. – *Time course of the initial rate of methylation of saline-soluble (A) and SDS-soluble (B) proteins extracted from HeLa cells at different times during the cycle.* The synchronized cells from a 1–*l* suspension (see Methods) were allowed to complete a full mitotic cycle, from the first to the second S-phase. At hourly intervals from the " recycled " onset of DNA synthesis, 25×10^6 cells were withdrawn from the culture, harvested and washed with minimum essential medium without serum. They were resuspended in 25 ml of the same solution containing 2 % amino acid-free calf serum and labelled with 750 μCi ^{3}H-methyl-S-adenosyl-L-methionine (12.6 Ci/mmole) for 30 min at 37 °C while being stirred and supplied with a continuous flow of 5 % CO_2 in air. Labelled cells were washed three times with a large volume of Hank's salt solution and frozen. Protein fractionation into a saline-soluble and an SDS-soluble fraction was carried out as outlined in Methods.

most marked for medium-size proteins. On the other hand, the G_1 peak occurs with a delay of two hours and is not as prevalent as that in G_2, as in the case of saline-soluble proteins. A further difference concerns the behaviour of the proteins of small molecular weight which appear to lack the increase in G_2 and to show a relatively minor oscillation in G_1.

The time course of the total radioactivity recovered in the fractions of saline-soluble and SDS-soluble proteins is shown in Fig. 5. The occurrence of two periods of maximal labelling in G_2 and G_1 is clearly confirmed. In both fractions, the G_1 peak is considerably larger than the peak present in G_2. In the case of SDS-soluble proteins the G_1 peak occurs with approximately two hours delay as compared to the homologous peak present in saline-soluble proteins. Since all gels were charged with the same amount of protein, the total radioactivity recovered in each gel provides a measure of specific radioactivity and allows comparison of different protein fractions. From this point of view it is striking that the SDS-soluble proteins are markedly more radioactive (from 2 to 3.5-fold) than the saline-soluble proteins. The difference becomes even more evident outside the peaks in G_2 and G_1.

3.3 – *Protein methylation.*

Labelling *HeLa* cell proteins throughout the mitotic cycle with ^{3}H-methyl-S-adenosyl-L-methionine revealed the occurrence of a differential rate of methylation between saline-soluble and SDS-soluble proteins (Fig. 6). While the level of methylation remained relatively low and essentially unchanged in the fraction of saline-soluble proteins, SDS-soluble proteins became methylated to a considerably larger extent, especially during the S-phase. During the same phase the rate of incorporation of ^{3}H-leucine finds its minimum [4].

4. Discussion

The results presented in this paper confirm and extend previous data from this laboratory [4] concerning the occurrence of higher rates of protein synthesis in synchronized *HeLa* cells during the G_1 and G_2-phases. The oscillations occur with similar amplitudes in saline-soluble and SDS-soluble proteins whose radioactivities reach a minimum in the M and S-phases. The peak in G_1 is considerably higher than that in G_2 in both protein fractions, as previously reported for nascent peptide chains [4]. A distinct difference between the two fractions regards the time of occurrence of the G_1 peak which appears with a delay of two hours in the SDS-soluble proteins as compared to the saline-soluble proteins.

In general, protein size does not seem to play a major role in conditioning the rate of synthesis during the cell cycle, as judged from the occurrence of similar oscillations in proteins of large, medium and small molecular weights. Some quantitative differences do exist, however, in that medium-size saline-soluble proteins have a more pronounced oscillation in G_1 than saline-soluble

proteins of larger and smaller sizes and SDS-soluble proteins of all sizes. On the other extreme are the small-size SDS-soluble proteins whose rate of synthesis remains essentially unaffected by the cell cycle. The physiological role of these differences is still not clear. In any case they do not seem to be adequate in explaining the quantitative discrepancies which exist between polysome content and size and rate of peptide synthesis in G_1 and G_2 [4].

An additional striking difference between saline-soluble and SDS-soluble proteins concerns their respective rate of methylation. A much higher rate prevails in the latter fraction which shows a peak of labelling in the S-phase. A similar increase in methylation is known to occur in the same phase with regard to DNA and RNA [8, 9].

It is likely that a significant fraction of protein methylation concerns the histones, whose synthesis also takes place in the S-phase [15–17]. In this group of proteins, the delay between formation and methylation should be reduced to a minimum. On the other hand, if other proteins synthesized during the G_1 and G_2-phases are subjected to a similar process of methylation, the delay between synthesis and methylation would be expected to become one of a few hours for molecules made during the G_1-phase and of several hours for proteins synthesized during the G_2-phase. In the latter case proteins would pass through mitosis and undergo an almost full cell cycle before being methylated. Further work is needed to clarify this point.

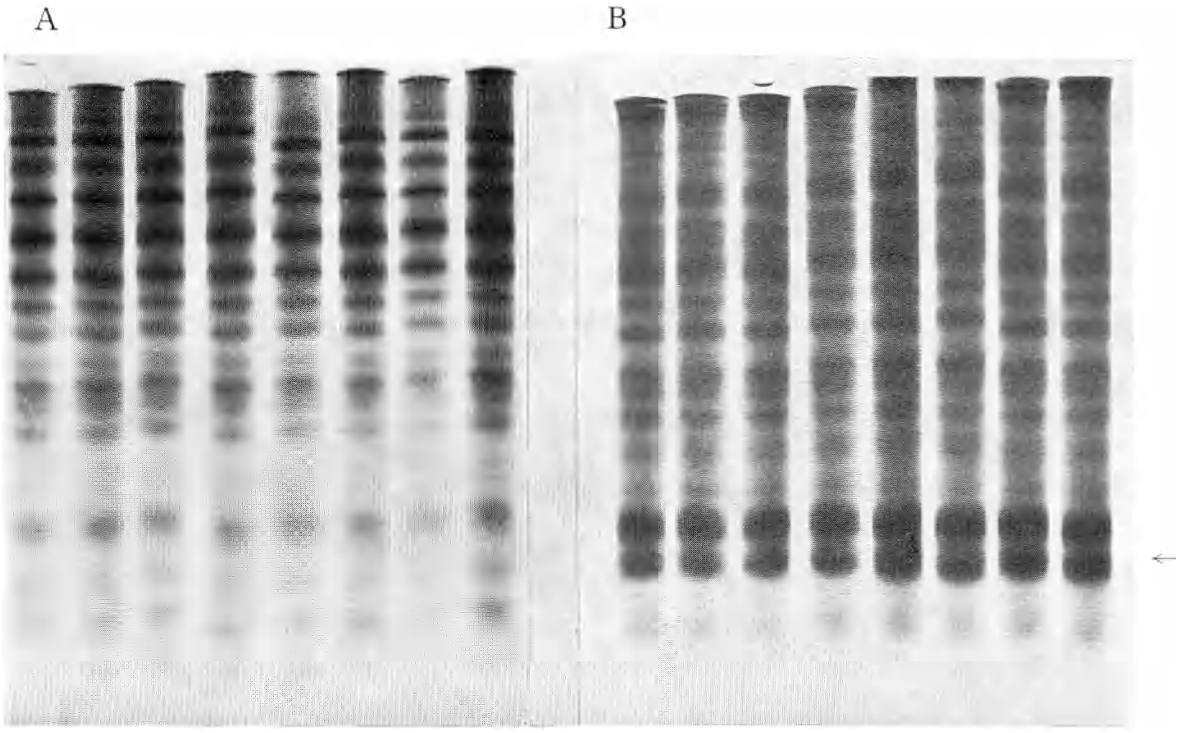
With regard to the possibility that certain groups of proteins may be made at specific periods of the cell cycle, no major differences have been revealed in this study. Nonetheless, despite the relatively limited resolution afforded by the electrophoretic method of separation on SDS-gels, some quantitative differences have been discerned in the behaviour of some protein classes. In particular, saline-soluble medium-size proteins have been found to show large amplitude oscillations in synthetic rate, while SDS-soluble small-size proteins maintain a constant rate of synthesis throughout the cycle. This suggests that the increase in synthetic rate present in G_1 and G_2 may not involve all proteins indiscriminately.

Acknowledgements. The skilful assistance of Mr. C. Buono is gratefully appreciated. This work was partly supported by Programma Finalizzato Virus CNR (No. 130.950).

REFERENCES

[1] J.M. MITCHISON (1971) – « The Biology of the Cell Cycle », Cambridge Univ. Press, Cambridge.
[2] O. I. EPIFANOVA (1973) – « The Cell Cycle: Problems of Regulation », Nauka, Moscow.
[3] E. ROBBINS and M. D. SCHARFF (1967) – « J. Cell Biol. », *34*, 684–686.
[4] T. EREMENKO and P. VOLPE (1975) – « Eur. J. Biochem. », *52*, 203–210.
[5] T. EREMENKO, T. MENNA and P. VOLPE (1979) – « Microbiologica », *2*, 137–145.
[6] T. EREMENKO, T. MENNA and P. VOLPE (1978) – « 12th FEBS–Meeting », Dresden, Abstr. 355.
[7] A. D. PAPAPHILIS, (1973) – « J. Theor. Biol. », *38* 613–625.

[8] D. GERACI, T. EREMENKO, R. COCCHIARA, A. GRANIERI. E. SCARANO and P. VOLPE, (1974) – « Biochem. Biophys. Res. Commun. », *57*, 353-358.
[9] P. VOLPE (1976) in « Horizons in Biochemistry and Biophysics » Vol. 2 (Quagliariello E., Palmieri F. and Singer, T. P., eds) Addison–Wesley, Reading, pp. 285–340.
[10] P. VOLPE and T. EREMENKO, (1970) – « Eur. J. Biochem. », *12*, 195–200.
[11] K. A. PIEZ, L. LEVINTOW, V. I. OYAMA and H. EAGLE (1960) – « Nature » *188*, 59–61.
[12] P. VOLPE and T. EREMENKO (1973) – in « Methods in Cell Biology » Vol. 6 (Prescott, D. M. ed), pp. 113–126 Acad. Press, New York.
[13] K. WEBER and M. OSBORN (1969) – « J. Biol. Chem. », *244*, 4406–4412.
[14] O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDAL (1951) – « J. Biol. Chem. », *193*, 265–275.
[15] J. SPALDING, K. KAJIWARA and G. MUELLER (1966) – « Proc. Nat. Acad. Sci. US », *56*, 1535-1542.
[16] E. ROBBINS and T. W. BORUN (1967) – « Proc Nat. Acad. Sci. US », *57*, 409–416.
[17] G. S. STEIN and T. W. BORUN (1972) – « J. Cell Biol. », *52*, 293–307.



Electrophoretic separation of saline-soluble (A) and SDS-soluble (B) proteins obtained at hourly intervals from synchronized HeLa cell cultures (see Methods). The synchronized cells from a 2-*l* suspension were allowed to complete a full mitotic cycle, from the first to the second S-phase. Starting from the "recycled" onset of DNA synthesis, 5×10^7 cells were withdrawn every hour, washed with Hank's salt solution, resuspended in 5 ml of the same medium and labelled with 20 μCi ^{3}H-leucine (35.5 Ci/mmole) for 3 min at 37 °C with stirring. The labelled cells were washed three times with a large volume of Hank's salt solution and frozen. Saline-soluble and SDS-soluble proteins were prepared from each sample as outlined in Methods. Each gel was charged with 200 μg protein. Electrophoresis was carried out on SDS-gels according to the method of Weber and Osborn [13]. The direction of migration was downwards. Only eight gels taken at random from the 18 different cell samples examined are shown for each protein fraction. The resolution obtained in the other samples and the relative proportion of the protein bands were essentially the same as those presented.