Changes in the Phosphorylation and Thiol Content of Histones in Phytohaemagglutinin-Stimulated Lymphocytes

By MARGARET E. CROSS and MARGERY G. ORD

Department of Biochemistry, University of Oxford, Oxford OX1 3QU, U.K.

(Received 18 March 1970)

Acetylation (Pogo, Pogo, Allfrey & Mirsky, 1968), methylation (Tidwell, Allfrey & Mirsky, 1968) and phosphorylation (Ord & Stocken, 1967) are well-established modifications of histone structure that may occur at various stages of the cell cycle. Oxidoreduction changes in thiol groups of histones have also been reported in HeLa-cell cultures at metaphase (A. Sadgopal & J. Bonner, personal communication, and in fertilized sea urchins, but interpretation of changes in the latter system is complicated by uncertainties about the nature of the proteins involved (Ord & Stocken, 1970). To look at histone modification in relation to specific nuclear states a synchronous system, homogeneous in cell population, with histones similar to those of calf thymus, is required. Phytohaemagglutinin-stimulated small lymphocytes from peripheral pig blood (Forsdyke, 1968) were used as an approximation to this system. Changes in phosphorylation and thiol/disulphide ratios were examined.

Histone modification was examined during the initial peak of RNA synthesis, in the first hour after phytohaemagglutinin stimulation, and during DNA synthesis, which occurs 45–85 h later. Cultures were set up according to the procedure of Pogo, Allfrey & Mirsky (1966). After 24 h incubation most blood cells except small lymphocytes die when kept in culture, and a purified small-lymphocyte population could therefore be obtained. Because of variations in the time-scale for different cultures all results were related to a stimulation ratio. Cultures were 'pulsed' with [^3H]uridine or [^3H]thymidine for 30 min and the ratio of the specific radioactivity of RNA or DNA in stimulated cultures to that in controls was taken as an index of nucleic acid synthesis.

Nuclei were isolated by homogenization from cells disrupted by a hypo-osmotic medium and separated from cytoplasmic debris on a sucrose density gradient. After the nuclei had been washed, histone F1 was extracted with 5% (w/v) HClO_4 , reprecipitated and dissolved in water (method 1 of Johns, 1964). The remaining histones were extracted with 250 mM-HCl. Histone fractions were characterized by gel electrophoresis (Reisfield, Lewis & Williams, 1962) and amino acid analysis (Butler, 1964). Net phosphate (Bartlett, 1959),

thiol (Todd & Gronow, 1969) and reduced disulphide in the fractions were determined. As a check on the location of these modifications, cultures were exposed to [^{32}P]phosphate for 30 min and the radioactivity of the histone band obtained after gel electrophoresis was measured. In other experiments the histones remaining after extraction of histone F1 were labelled *in vitro* with [^{14}C]N-ethylmaleimide and the radioactivity of the histone bands was again determined after electrophoresis (Ord & Stocken, 1970).

These electrophoretic experiments confirmed that the redox state of thiol in histone F3 is altered during phytohaemagglutinin stimulation, and that phosphorylation occurs on histone F1 and on proteins in the histones extracted subsequently with 250 mM-HCl, which migrate in a band corresponding to the positions of histones F3 and F2b. As a check on isotope incorporation in the initial period of RNA synthesis, a [^{14}C]acetate pulse was given to a set of cultures and the incorporation of acetate into the histone fractions at different times after stimulation was measured (Pogo *et al.* 1966).

The data (Table 1) show that no net increase in histone F1 phosphate occurs during the initial period of RNA synthesis. Similarly the thiol/thiol+disulphide ratio remains constant. There is, however, an incorporation of ^{32}P radioactivity into both histone F1 and total histone minus histone F1 in the stimulated cultures during this time. The kinetics of incorporation are similar, showing a rapid increase in radioactivity in the first 15 min after stimulation. The [^{14}C]acetate experiment confirmed the result of Pogo *et al.* (1966) in this system; no acetate incorporation occurred in the histone F1 fraction, but [^{14}C]acetate incorporation into total histone minus histone F1 was increased during the first 20–30 min after stimulation.

During DNA synthesis net phosphorylation of histone F1 increases two- to three-fold. There is a corresponding increase in ^{32}P incorporation. The other histones show a small increase in net phosphate and ^{32}P radioactivity over this period. The redox state is reversed, rising from 25 to 80% thiol over the peak of DNA synthesis and reverting to a more oxidized state as the cells enter mitosis.

The increase in net phosphate, with attendant

Table 1. *Histone changes in phytohaemagglutinin-stimulated pig lymphocyte cultures*

The stimulation ratio is the ratio of the specific radioactivity of nucleic acid in cultures stimulated with phytohaemagglutinin to that in control cultures. [^{14}C]Acetate is expressed as $\mu\text{p.m.}/\text{mg}$ of protein. No change was detected in the labelling of histone F1 during the period of RNA stimulation. Phosphate content is expressed as nmol/mg of protein. [^{32}P]Phosphate is expressed as $10^{-2} \times \text{c.p.m.}/\text{mg}$ of protein in bands obtained after electrophoresis of the histones on polyacrylamide gel (Reisfeld *et al.* 1962). Control values were taken as 1.0 and results are expressed relative to this value. The error in this method is high ($\pm 15\%$) because of gel quenching of ^{32}P counts. During the period of RNA stimulation the cultures were given a continuous label of [^{32}P]phosphate starting 30 min before stimulation. The values for the period of DNA synthesis were obtained with a 30 min pulse of [^{32}P]phosphate. The % SH values represent the ratio $100 \times \text{SH}/(\text{SH} + \text{S-S})$. The SH + S-S content remained constant throughout ($81 \pm 9 \text{ nmol}$ of SH + S-S/mg of protein). Control values are given as means $\pm$ s.e.m. The numbers of determinations are given in parentheses.

Stimulation ratio	RNA stimulation period 0-1 h					DNA stimulation period 45-85 h					
	Histone F1			Total histone-histone F1		Stimulation ratio	Histone F1		Total histone-histone F1		
	³¹ P	³² P	[¹⁴ C]Acetate	³¹ P	³² P		³¹ P	³² P	³¹ P	% SH	
Control	34 ± 6 (5)	1.0	1.0	39 ± 6 (5)	1.0	Control	40 ± 7 (2)	1.0	34 ± 4 (2)	1.0	27 ± 7 (2)
1.03	—	1.40	—	—	1.10						
1.1	36 (3)	1.60	1.30	43 (2)	1.90	1.3	41 (2)	2.0	43 (2)	1.1	42 (2)
1.2	39 (1)	3.20	—	39 (1)	3.10	1.8	63 (2)	—	—	—	59 (2)
1.3	39 (1)	4.80	1.45	48 (1)	3.90	2.0	60 (5)	4.0	47 (3)	1.8	62 (4)
1.8	28 (1)	—	1.55	41 (1)	—	2.7	105 (2)	—	—	—	74 (2)
2.2	31 (1)	—	—	35 (2)	—	3.0	95 (1)	6.0	49 (1)	1.47	77 (1)
3.1	41 (2)	4.80	—	36 (1)	3.60	2.3	92 (2)	—	44 (1)	—	74 (1)
						1.3	51 (3)	—	36 (3)	—	49 (2)

increase in radioactivity, in histone F1 during DNA synthesis is in keeping with results in other systems such as regenerating liver (Ord & Stocken, 1969). The increase in the proportion of thiol groups in histone F3 during replication is consistent with the decreased proportion of chromatin in the condensed state at this time, and can be correlated also with changes in redox state of cell proteins during the cell cycle (Mauro, Grasso & Tolmach, 1969).

No increase in phosphate content was observed during the initial period of RNA synthesis, but the immediate increase in ^{32}P radioactivity suggests that the rate of turnover of histone phosphate is greatly accelerated. Ord & Stocken (1969) have found an increase in phosphate content of histones other than histone F1 in the first 2h of liver regeneration, and on the basis of ^{32}P -incorporation studies in liver Langan (1969) has suggested that histone phosphorylation could be a mechanism by which certain hormones, such as glucagon, may induce RNA synthesis. Increased incorporation of ^{32}P may not necessarily be accompanied by detectable increase in phosphate content (see Langan, 1969). The extent of the genome involved in new nucleic acid synthesis may be relevant, as well as the ATP pool and activation of histone kinases (Pawse, Ord & Stocken, 1969) or phosphatases. These latter factors require further investigation in the lymphocyte system.

M. E. C. is grateful to the Medical Research Council for a research studentship. We also thank the British Empire Cancer Campaign for financial support.

- Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 466.
Butler, J. A. V. (1963). In *The Nucleohistones*, p. 36. Ed. by Bonner, J. & Ts'o, P.O.P. San Francisco: Holden-Day Inc.
Forsdyke, D. R. (1968). *Biochem. J.* **108**, 297.
Johns, E. W. (1964). *Biochem. J.* **92**, 55.
Langan, T. A. (1969). *Fedn Proc. Fedn Am. Soc. exp. Biol.* **28**, 1899.
Mauro, F., Grasso, A. & Tolmach, L. J. (1969). *Biophys. J.* **9**, 1377.
Ord, M. G. & Stocken, L. A. (1967). *Biochem. J.* **102**, 631.
Ord, M. G. & Stocken, L. A. (1969). *Biochem. J.* **112**, 81.
Ord, M. G. & Stocken, L. A. (1970). *Biochem. J.* **116**, 415.
Pawse, A. R., Ord, M. G. & Stocken, L. A. (1969). *Biochem. J.* **114**, 54 p.
Pogo, B. G. T., Allfrey, V. G. & Mirsky, A. E. (1966). *Proc. natn. Acad. Sci. U.S.A.* **55**, 805.
Pogo, B. G. T., Pogo, A. O., Allfrey, V. G., & Mirsky, A. E. (1968). *Proc. natn. Acad. Sci. U.S.A.* **59**, 1337.
Reisfield, R. A., Lewis, U. J. & Williams, D. E. (1967). *Nature, Lond.*, **195**, 281.
Tidwell, T., Allfrey, V. G. & Mirsky, A. E. (1968). *J. biol. Chem.* **243**, 707.
Todd, P. & Gronow, M. (1969). *Analyt. Biochem.* **28**, 369.