*RNA SYNTHESIS AND HISTONE ACETYLATION DURING THE COURSE OF GENE ACTIVATION IN LYMPHOCYTES**

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The experiments to be described are concerned with the control of chromosomal function in the differentiated cells of higher organisms. They deal, in particular, with the metabolism of histones during gene activation in cells stimulated to enlarge and divide.

Special attention has been paid to the phenomenon of histone acetylation, since earlier experiments had suggested that acetylation of the basic proteins associated with DNA may signal changes in DNA-histone interactions and in the template activity of the chromatin in RNA synthesis.[1-3] This premise, based originally on experiments showing a positive correlation between histone acetylation and RNA synthesis in chromatin subfractions derived from isolated nuclei[2] and on observations indicating that chemically acetylated histones were not very effective as inhibitors of calf thymus or *E. coli* RNA polymerases,[1] has now been tested in a particularly apt system, the human lymphocyte responding to stimulation by phytohemagglutinin (PHA).

It is well known that human lymphocytes maintained in tissue culture rarely go on to divide; indeed they have usually been considered to be the end cell in the pathway of differentiation. However, in the presence of phytohemagglutinin (a protein fraction derived from the red kidney bean), a striking transformation occurs; the cells increase their metabolic activity, enlarge, and then divide. This dramatic proliferative response of the small lymphocyte has been widely studied since its discovery about 6 years ago,[4, 5] and the results of many investigations since then have recently been summarized.[6] It is now clear that the "transformation" of lymphocytes by PHA involves an increased synthesis of ribonucleic acids[7-9] and protein[10] and thus may be regarded as an instance of extensive gene activation. Experiments summarized below support the view that previously inactive genetic loci are called into play and show that the increased activity of the chromosomes can be detected at very early times, long before the cells enlarge and go into mitosis.

What happens to the histones when lymphocyte chromosomes are "triggered" in this way? In what follows we will describe some tracer studies of RNA, DNA, and protein synthesis and of histone acetylation in different types of lymphocytes in the presence and absence of phytohemagglutinin.

Conditions have been discovered which make it possible to show that changes in the metabolism of the chromatin take place within minutes after PHA is added to white cell suspensions. In small lymphocytes the acetylation of some histones occurs at a greatly increased rate, and it appears to precede the increase in the rate of nuclear RNA synthesis. On the other hand, the addition of phytohemagglutinin to polymorphonuclear leucocytes leads to a decrease in RNA synthesis and the acetylation of histones is curtailed.

Materials and Methods.—Tissue culture procedures for isotopic labeling experiments: Human heparinized blood from normal donors was allowed to sediment for 1–2 hr at 37°. The plasma

and white cell fraction was transferred to centrifuge tubes and spun down at 1000 × *g* for 10 min. The pellets were resuspended in Eagle's minimal essential medium (MEM)[11] in the presence of heparin (1 mg/ml). Polymorphonuclear leucocytes were then removed by low-speed centrifugation (100 × *g* for 2 min). The supernatant phase contains about 90% small lymphocytes and 10% polymorphonuclear cells. These lymphocytes were dispersed in MEM containing 20% inactivated newborn calf serum, glutamine, and antibiotics, to give a final concentration of 2–5 × 10^6 cells per ml. A similar fractionation was carried out on a larger scale for the preparation of polymorphonuclear leucocytes and lymphocytes from horse blood. Equine lymphocytes are also "transformed" by PHA.

We have observed that cell response to phytohemagglutinin is best observed after 20 hr in culture at 37°. At that time a phytohemagglutinin solution (Difco Preparation P) was added at a concentration of 0.1 ml per 5.0 ml of cell suspension. The cells were incubated at 37°, without shaking, in the presence of the desired isotopic precursors: uridine-2-C^{14}, specific activity 26.7 mc/mmole, 0.5 μc per ml; thymidine-2-C^{14}, sp. act. 30 mc/mmole, 1 μc per ml; DL-alanine-1-C^{14}, sp. act. 8.1 mc/mmole, 1 μc per ml; DL-alanine-methyl-H^3, sp. act. 332 mc/mmole, 10 or 20 μc per ml; sodium acetate-2-C^{14}, sp. act. 16.7 or 23 mc/mmole, 5 μc per ml; and sodium acetate-methyl-H^3, sp. act. 50 or 210 mc/mmole, 20 μc per ml. The effects of actinomycin D and puromycin-HCl were tested at a concentration of 10 μg per ml of cell suspension containing 5 × 10^6 cells per ml.

Isolation of lymphocyte nuclei: Following incubation, the nuclei were isolated by a modified citric acid procedure.[12] The cells were washed twice with MEM, resuspended in 0.01 *M* citric acid, and homogenized at 6000 rpm for 2 min in a small blendor (Micro-Omnimixer; Ivan Sorvall, Inc.). Cell breakage was monitored by examination under the light microscope. The nuclei were sedimented at 1000 × *g* for 6 min. The nuclear pellet was washed three times more with 0.01 *M* citric acid before extraction of the histones.

Extraction and electrophoretic purification of the histones: The nuclei were washed with 88% ethanol–0.01 *N* HCl in order to remove a tryptophan-containing protein fraction. They were then resuspended in cold 0.2 *N* HCl for 20 min to extract the histones. The suspension was centrifuged at 1000 × *g* for 10 min and the residue re-extracted as before. The extracts were combined and the histones precipitated by the addition of 10 vol of acetone. The histone precipitate was collected, washed with acetone and ether, and dried.

Electrophoresis on cellulose polyacetate was employed in order to purify the histones from nonbasic proteins. The histone fraction was redissolved in 0.05 *M* veronal buffer, pH 9.0, containing 4 *M* urea. The solution was applied as a thin band on Sepraphore III strips (Gelman Instrument Co.), and the strips were subjected to a field of 12 v/cm for 60 min. At that time the strips were fixed by immersion in saturated picric acid, washed with buffer, stained with Fast Green at pH 9, and then destained.[13] Two well-defined histone bands appear: a narrow, faster-moving fraction (band I) consists of lysine-rich histones (lys/arg ratio: 14/1),[13] while a slower, broader band (band II) includes the "arginine-rich" histone fractions. The strips were cut lengthwise, and the histone concentrations in the bands on one half of the strip were determined colorimetrically.[13] Corresponding segments from the other half of each strip were dissolved in Bray's scintillation mixture[14] for measurement of histone C^{14}- or H^3-activity. The samples were counted in a Nuclear-Chicago scintillation counter at an efficiency of 75% for C^{14} and 4% for tritium-labeled samples.

Analytical methods: RNA was extracted in 0.3 *N* KOH (3 hr at 37°) and its concentration determined by the two-wavelength UV absorption method of Fleck and Munro.[15] DNA was extracted in hot 0.5 *N* perchloric acid and measured by the Burton procedure.[16] Protein content was determined by the biuret method.[17]

Results.—Evidence for gene activation in PHA-stimulated lymphocytes: The addition of phytohemagglutinin to lymphocyte suspensions leads to an increase in the rates of ribonucleic acid and protein synthesis. These processes can be followed by the incorporation of uridine-2-C^{14} and alanine-1-C^{14}, respectively. The increase in RNA synthesis is indicated in Figure 1*A*, which compares the time courses of uridine-C^{14} incorporation in control and PHA-stimulated cells. The figure also shows that ribonucleic acid synthesis in this system is blocked by the addition of

actinomycin D (10 μg per ml). This result, which can be taken as an indication of the DNA-dependence of RNA synthesis,[18, 19] is also of significance in considering protein synthesis in PHA-stimulated cells.

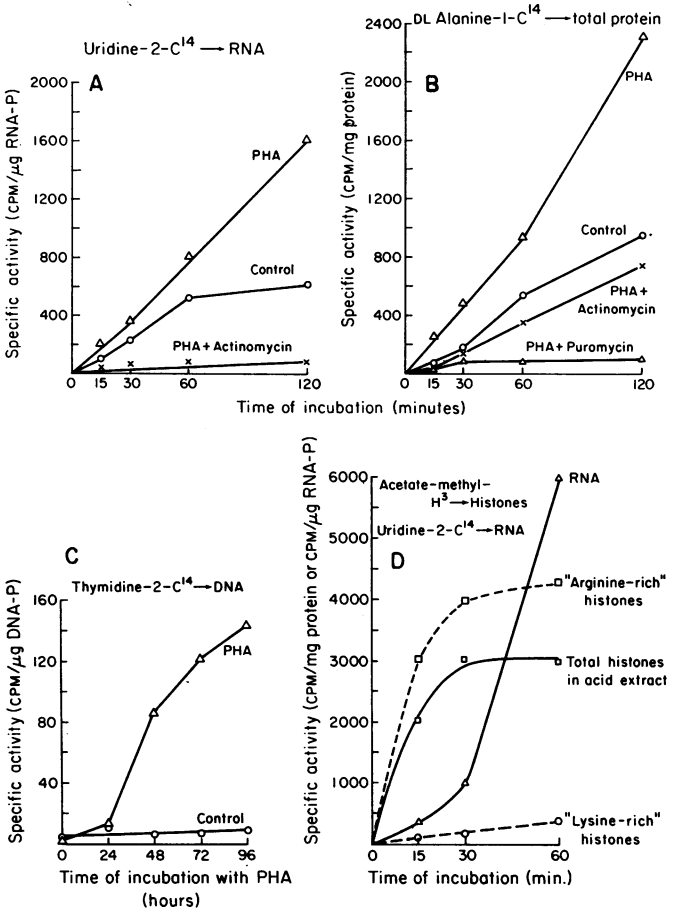

FIG. 1.—Effects of phytohemagglutinin on RNA, DNA, and protein synthesis and on histone acetylation in human peripheral lymphocytes in culture. (*A*) Time course of RNA synthesis in control and PHA-treated cells showing inhibitory effect of actinomycin D. (*B*) Time course of protein synthesis showing stimulation by PHA, inhibition by puromycin, and suppression by actinomycin of the PHA-induced surge in protein synthetic activity. (*C*) Time course of DNA synthesis in PHA-treated lymphocytes. (*D*) Time course of acetylation of total histone and of histone fractions purified by electrophoresis compared with the time course of RNA synthesis in the same cell population.

The addition of actinomycin D blocks the *increase* in protein labeling usually obtained in cells responding to phytohemagglutinin (Fig. 1*B*). This result is consistent with the view that the early increase in protein synthesis reflects a prior increase in the synthesis of new RNA "messengers." Actinomycin D, by blocking DNA-template function in RNA synthesis, thus inhibits the formation of new proteins associated with the proliferative response. The "transformation" of the

TABLE 1

INCORPORATION OF ALANINE-1-C^{14} AND ACETATE-(METHYL)-H^3 INTO HISTONES OF NORMAL AND PHA-STIMULATED LYMPHOCYTES

	Conditions of Experiment				Uptake into Histones* of			
					Control Cells		PHA-Treated Cells	
	Exposure to PHA	Period of incubation (min)	Precursor	Precursor concentration (μc/ml)	Band I ($\mu\mu$moles)	Band II ($\mu\mu$moles)	Band I ($\mu\mu$moles)	Band II ($\mu\mu$moles)
(*A*)	Immediate	60	DL-alanine-1-C^{14}	1.0	Trace	0.002	0.001	0.012
	"	60	" " "	10.0	Trace	0.011	Trace	0.017
			Na acetate-(methyl)-H^3	20.0	0.029	0.125	0.160	0.490
(*B*)	24 Hr	60	DL-alanine-1-C^{14}	1.0	0.029	0.050	0.044	0.115
	" "	60	Na acetate-(methyl)-H^3	20.0	0.044	0.123	0.062	0.410

* Histone fractions purified by electrophoresis at pH 9, counting 2 major bands: band I ("lysine-rich"), av. mol wt 16,000; band II ("arginine-rich"), av. mol wt 13,000. Uptake expressed as $\mu\mu$moles of precursor incorporated per 100 $\mu\mu$moles of histone.

TABLE 2

COMPARISON OF HISTONE ACETYLATION AND HISTONE SYNTHESIS IN CONTROL AND PHA-STIMULATED LYMPHOCYTES

	Conditions of Experiment			Uptake into Histones* of			
				Control Cells		PHA-Treated Cells	
Expt.	Precursor	Specific activity (mc/mmole)	Concentration (μc/ml)	Band I ($\mu\mu$moles)	Band II ($\mu\mu$moles)	Band I ($\mu\mu$moles)	Band II ($\mu\mu$moles)
1	Na acetate-(methyl)-H^3	50	20	0.019	0.127	0.110	0.247
2	" " " "	210	20	0.014	0.100	0.140	0.475
3	" " " "	210	20	0.029	0.125	0.160	0.490
4	" " " "	210	20	0.020	0.123	0.194	0.750
5	Na acetate-2-C^{14}	16.7	5	0.031	0.138	0.256	0.945
6	DL-alanine-(methyl)-H^3	332	10	0.001	0.018	0.012	0.027
7	" " " "	332	20	Trace	0.005	0.010	0.020
8	DL-alanine-1-C^{14}	8.1	1	Trace	0.002	0.001	0.012
9	" " "	8.1	1	Trace	0.005	Trace	0.006
10	" " "	8.1	1	Trace	0.017	Trace	0.011

Cells incubated 1 hr at 37° immediately after addition of PHA.
* Histones purified by electrophoresis at pH 9. Incorporation of precursor expressed as $\mu\mu$moles per 100 $\mu\mu$moles histone.

lymphocyte appears to involve an activation of previously repressed genetic loci. More direct tests for the synthesis of "new" messenger-RNA's are now in progress.

Effects of puromycin: Figure 1*B* also shows that puromycin blocks the uptake of alanine-1-C^{14} into cell proteins almost completely. An inhibition of amino acid uptake into the proteins of the nucleus is also observed (Table 3).

DNA synthesis in PHA-stimulated cells: It should be stressed that the events described above are "early" events and that they occur before the cells enlarge and long before the synthesis of new DNA is initiated; the time scale is in minutes in one case and hours in the other. The time course of DNA synthesis in control and PHA-stimulated cells is indicated by the incorporation of thymidine-2-C^{14}. The results summarized in Figure 1*C* show that little DNA synthesis occurs in the first 24 hr after adding PHA; at this time the cells have been in culture for 44 hr. This result is in accord with the findings of other workers using somewhat different culture conditions.[7, 20, 21]

The delay in the initiation of new DNA synthesis has a parallel in histone synthesis. Studies of the incorporation of alanine-1-C^{14} or alanine-methyl-H^3 into histone fractions purified by electrophoresis show that histone labeling is negligible in the first hour after adding PHA (Table 1*A*) but becomes appreciable after 24 hr exposure to the plant protein (Table 1*B*).

TABLE 3

COMPARATIVE EFFECTS OF PUROMYCIN ON PROTEIN SYNTHESIS AND HISTONE ACETYLATION IN PHA-STIMULATED LYMPHOCYTES

Expt.	Puromycin concentration (μg/ml)	Specific Activity of Histones in Control cells (cpm/mg)	Specific Activity of Histones in PHA-treated cells (cpm/mg)	Specific Activity of Residual Nuclear Proteins* Control cells (cpm/mg)	Specific Activity of Residual Nuclear Proteins* PHA-treated cells (cpm/mg)
1	0	—	6000	—	920
	10	—	6900	—	617
2	0	3000	7100	835	1370
	10	—	7350	—	817
3	0	1650	4000	166	235
	10	—	4700	—	106

Cells incubated for 1 hr in the presence of Na acetate-(methyl)-H^3; 20 μc per ml.
* Radioactivity present largely as H^3-aspartic and glutamic acids derived from precursor.

Histone acetylation in lymphocyte cultures: Both normal and PHA-stimulated cells incorporate C^{14}- or H^3-acetate into their histones. Acetate so incorporated can be recovered as free acetic acid by steam distillation of the histone hydrolyzate in 6 N H_3PO_4:[1] in a typical experiment more than 10^6 cpm out of a total of 1.17×10^6 counts were recovered in the distillate. Other tests have shown that the acetate incorporated into lymphocyte histones is not released by treatment with 2M neutral hydroxylamine, a result which precludes an -S-acetyl linkage[22] and thus rules out contamination by acyl-carrier proteins (e.g., refs. 23–25). The results are in accord with the original findings of Phillips[26] that calf thymus histones contain N-terminal acetyl groups.

There is a striking contrast in the extent of histone acetylation as compared with histone synthesis in both normal and PHA-stimulated cells. Some indication of the difference is afforded by the data in Table 2, which compares the uptake of labeled acetate with that of C^{14}- or H^3-alanine under similar conditions. (Alanine was selected as a marker because it is not present in the tissue culture medium.) The results are expressed in terms of the number of $\mu\mu$moles of precursor incorporated per 100 $\mu\mu$moles of histone (taking an average molecular weight of the histones in electrophoretic band I as 16,000, and of histones in band II as 13,000).[13] It is clear that acetate uptake far exceeds alanine incorporation in both histone fractions.

The experiments summarized in Table 2 also show two other important features of histone acetylation. First, the uptake of acetate is more pronounced in the "arginine-rich" fractions. This result is in accord with our previous findings on the rapid acetylation of the *f3* histone fraction in isolated thymus lymphocyte nuclei.[1] Second, the addition of phytohemagglutinin leads to a great *increase* in the rate of histone acetylation. The increase is easily detected in the first hour following the addition of PHA. It persists and can be detected in pulse-labeling experiments 24 hr later (Table 1*B*).

An additional argument for the distinction between histone synthesis and acetylation is provided by the effects of puromycin. Histone acetylation is not affected by the addition of this inhibitor of protein synthesis (Table 3). This result is also in accord with earlier findings on isolated thymus nuclei.[1]

Correlations between histone acetylation and RNA synthesis: A comparison of histone acetylation and RNA synthesis at early times after the addition of phytohemagglutinin reveals a striking difference in the kinetics of these two processes. Figure 1*D* shows the time course of uridine-2-C^{14} incorporation into RNA. A short lag period is followed by a rapidly increasing rate of RNA labeling. The figure also

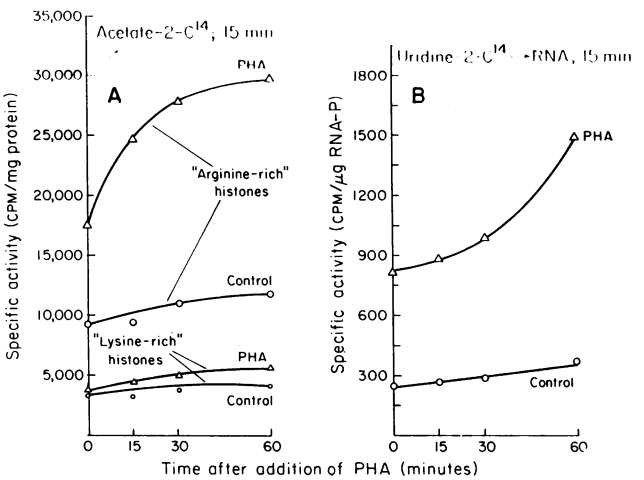

FIG. 2.—Comparative effects of PHA on histone acetylation and RNA synthesis in human lymphocytes in culture. Cells were exposed to PHA for the indicated times and then pulse-labeled for 15 min in the presence of C^{14}-acetate or C^{14}-uridine. The specific activity of the purified histone fractions is plotted against the time of exposure to PHA. Note that the increase in nuclear capacity to acetylate histones precedes the increase in the rate of nuclear RNA synthesis.

shows the extent of acetylation of the total histone and of the histone fractions purified by electrophoresis. It is evident that most of the acetylation takes place in the "arginine-rich" fraction (band II).

Especially suggestive are the kinetics of acetate incorporation: *the acetylation of the histones appears to precede the increase in nuclear RNA synthesis.*

This conclusion was verified in another series of experiments comparing the rates of histone acetylation and RNA synthesis by pulse labeling of normal and PHA-stimulated cells (Fig. 2). In these tests a uniform cell culture was divided equally: one half received a PHA supplement; the other half served as a control. Aliquots of the two cultures were withdrawn at 15-min intervals and incubated for a fixed time period (15 min) in the presence of uridine-2-C^{14} or Na acetate-2-C^{14}. The incorporation into RNA or histone was measured and plotted as a function of the time of withdrawal of the sample. As shown in Figure 2*B*, little change in RNA synthetic capacity takes place in the control cultures, while PHA-treated cells gradually augment their rates of RNA synthesis. Figure 2*A* illustrates the changes in histone acetylation during the same time interval. It can be seen that PHA-stimulated cells rapidly alter their capacity to acetylate the histones, especially those basic proteins in the "arginine-rich" fraction (band II). The increased capacity for histone acetylation becomes evident at very early times when changes in RNA synthetic capacity are less marked, but incipient.

Thus, both types of labeling experiment (simple time course and pulse labeling) indicate that cells stimulated by phytohemagglutinin increase the acetylation of previously existing histones, and this change in the chemistry of the basic proteins of the chromosomes appears to signal a later increase in the RNA synthetic activity of the chromatin. Since the histones occur in combination with the DNA of the chromosomes, a change in chromosomal fine structure appears to have been initiated by the acetylation reaction. Such a change has been visualized in another

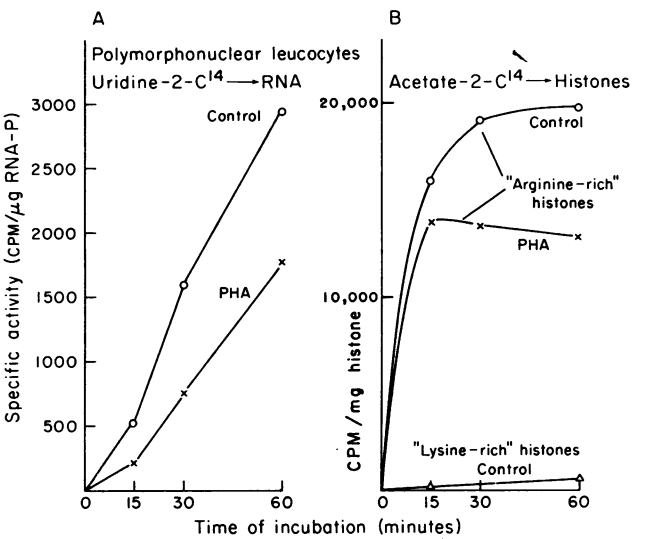

FIG. 3.—Comparative effects of PHA on RNA synthesis and histone acetylation in equine polymorphonuclear leucocytes in culture. Note that RNA synthesis is inhibited and that histone acetylation is curtailed.

way; Killander and Rigler[27] have recently reported that lymphocytes stimulated by PHA rapidly increase their capacity to bind acridine orange to DNA. The kinetics of this increase are very similar to those we have described for histone acetylation.

PHA effects on equine polymorphonuclear leucocytes: Not all cells respond to phytohemagglutinin with an increase in genetic activity. A revealing contrast in behavior is provided by equine polymorphonuclear leucocytes; these cells are not stimulated by PHA; on the contrary, RNA synthesis is considerably slowed soon after the addition of the plant protein (Fig. 3*A*). Under these conditions histone acetylation is also curtailed (Fig. 3*B*). The extent of inhibition of acetylation, as judged by a 1-hr incorporation of acetate-2-C^{14}, is in fair agreement with the reduction in RNA synthesis in the same time period. It has not yet been established whether there is a causal relationship between these two effects, or whether the decrease in histone acetylation precedes the suppression of RNA synthesis. However, some preliminary experiments have shown that acetylation of the "arginine-rich" fraction (band II) may fall to as little as 30 per cent of the control values in 15 min after addition of the PHA. Further studies of the kinetics of the decrease in acetylation and RNA synthesis are in progress, using equine polymorphonuclear cells. (It should be noted, in passing, that preliminary tests of *human* polymorphonuclear leucocytes did not reveal a corresponding inhibitory effect of PHA, but a slight stimulation of RNA synthesis and histone acetylation was observed.)

Summary.—Human and equine lymphocytes increase their rates of RNA synthesis within minutes after exposure to phytohemagglutinin (PHA). Histone acetylation (but not histone synthesis) is greatly increased in lymphocytes responding to PHA. The increase in acetylation of the basic proteins of the chromosomes appears to precede the increase in nuclear RNA synthesis.

Equine polymorphonuclear cells treated with PHA decrease their rates of RNA synthesis, and histone acetylation is curtailed.

The results are consistent with the view that histone acetylation signals a change in the fine structure of the chromatin and in the capacity of the DNA to serve as a template for RNA synthesis.

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