Changes in Histone Phosphorylation and Associated Early Metabolic Events in Pig Lymphocyte Cultures Transformed by Phytohaemagglutinin or 6-*N*,2'-*O*-Dibutyryladenosine 3':5'-Cyclic Monophosphate

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1. Pig lymphocytes were transformed by dibutyryl cyclic AMP (6-*N*,2'-*O*-dibutyryladenosine 3':5'-cyclic monophosphate) at concentrations of 0.01–0.1 μM . The pattern of incorporation of label from [5- ^3H]uridine and [6- ^3H]thymidine into RNA and DNA respectively was identical with that obtained with unpurified phytohaemagglutinin. 2. Chlorpromazine (0.1 μM) prevented the stimulation of [5- ^3H]uridine incorporation into RNA by phytohaemagglutinin, but only slightly lowered the lymphocyte response to dibutyryl cyclic AMP. 3. An increase in the size and specific radioactivity of the intracellular P_i pool was found immediately after stimulation by both phytohaemagglutinin and dibutyryl cyclic AMP. This was followed after some 30 min by a rise in the specific radioactivity and concentration of ATP. 4. There was an immediate increase in the specific radioactivity of phosphate groups of histones; by about 45 min after stimulation only the histones remaining after extraction of histone fraction F1 continued to incorporate ^{32}P from [^{32}P] P_i . 5. Histone kinase activity increased in the first 30 min after stimulation; subsequently histone F1 kinase activity decreased, but activity with the other histones as substrate continued to increase for a further 30 min. Kinase activation was effected by cyclic AMP (adenosine 3':5'-cyclic monophosphate). 6. Histone phosphatase activity behaved similarly to that of the kinase.

A number of micro-modifications to nuclear proteins are now believed to occur where changes in the pattern of genetic transcription have been initiated. Kleinsmith, Allfrey & Mirsky (1966) reported an increased incorporation of [^{32}P] P_i into nuclear phosphoprotein after stimulation by phytohaemagglutinin. Pogo, Allfrey & Mirsky (1966) showed that an increase in acetate uptake into histones occurs during the same time-period; an increased acetylation of histones may commonly be associated with new RNA synthesis (Allfrey, 1970). Phosphorylation of histones other than fraction F1 has been reported in liver 1 h after partial hepatectomy (Ord & Stocken, 1969) and also in regenerating pancreas (Fitzgerald, Marsh, Ord & Stocken, 1970).

Earlier experiments with pig lymphocytes transformed by phytohaemagglutinin had shown (Cross & Ord, 1970) that a rise in net phosphate content did not occur in histone F1 or the other histones until the period of DNA synthesis, although an increase in [^{32}P] P_i uptake could be detected immediately after stimulation. Histone phosphorylation is energy-dependent, and a histone phospho-

kinase that utilizes ATP as substrate has been identified (Langan & Smith, 1967). It was decided therefore to examine the specific radioactivity of P_i and ATP immediately after stimulation in the presence of [^{32}P] P_i , to test whether the latter could be related to changes in the specific radioactivities of the phosphate groups on histones. The activities of histone kinase and phosphatase (Meisler & Langan, 1969) were also assayed.

Langan (1968) has established that histone phosphokinase can be activated by cyclic AMP (adenosine 3':5'-cyclic monophosphate). Actinomycin did not prevent the immediate increase in phosphate content of histones other than fraction F1 in regenerating liver (Ord & Stocken, 1969), suggesting that activation of the existing kinase might be occurring. The possibility that mitogens (Oppenheim, 1968) transform lymphocytes by binding to their membrane (Kay, 1969; Borberg, Yesner, Gesner & Silber, 1968) and produce a change in membrane permeability (Mueller & Fisher, 1968) also suggests that, *inter alia*, stimulation of adenylate cyclase might be involved.

MacManus & Whitfield (1969) have shown that cyclic AMP and dibutyryl cyclic AMP (6-*N*,2'-*O*-dibutyryladenosine 3':5'-cyclic monophosphate) stimulate DNA synthesis and mitosis in rat thymus lymphocyte cultures. The possibility that dibutyryl cyclic AMP might produce the same changes as does phytohaemagglutinin in pig lymphocyte cultures was therefore investigated.

METHODS

Lymphocyte cultures. Pig blood was collected from the slaughterhouse in sterile bottles containing heparin. A 6% (w/v) dextran solution (Dextraven; Fisons Pharmaceuticals Ltd., Loughborough, Leics., U.K.) diluted 1:1 with medium 199 (Flow Laboratories Ltd., Irvine, Ayrshire, U.K.) was added to the blood in the ratio blood/dextran solution 2:1 (v/v). After 1 h at 37°C the upper dextran layer containing leucocytes and plasma was poured off the agglutinated erythrocyte sediment. The cells were spun down at 1000*g* for 10 min. The cell precipitate was resuspended in medium, counted by using a haemocytometer and transferred to culture flasks. Cultures (Forsdyke, 1968) were set up at a lymphocyte concentration of 5×10^6 cells/ml in 50 ml portions; other leucocytes contributed a further 5% and erythrocytes about 12% of the population. Silicone-stoppered 210 ml glass centrifuge pots were used as culture vessels. The cells were grown in medium 199 with added penicillin (50 units/ml), streptomycin (50 µg/ml) and fungicide, amphotericin B (3 µg/ml). The medium was supplemented with 5% (v/v) of foetal calf serum (Flow Laboratories). Culture pots were gassed with air+CO₂ (95+5) and incubated at 37°C. The lymphocytes were cultured at 37°C for 24 h before phytohaemagglutinin or dibutyryl cyclic AMP was added. During this period many of the contaminating cells die, as shown by the eosin exclusion test, leaving a population enriched in viable small lymphocytes. Neither erythrocytes nor other leucocytes were found to respond to the mitogenic factor in phytohaemagglutinin.

For harvesting of cells the cultures were placed on ice and those lymphocytes adhering to the glass as a result of the agglutinating factors in phytohaemagglutinin were detached with a plastic spatula. The cells were centrifuged rapidly out of suspension and washed twice with 0.9% NaCl in 50 mm-tris-HCl buffer, pH 7.2. The washed cells were resuspended in a hypo-osmotic medium containing (final concentrations) 0.1 M-KCl, 2.0 mM-MgCl₂, 0.1 mM-ZnSO₄ and 10 mM-tris-HCl buffer, pH 7.1. All subsequent operations were carried out at 4°C in the cold-room.

The cells were left for 10 min and then homogenized on ice with a small glass-ball-type homogenizer of 0.2 mm radial clearance. About 70 strokes were required before the nuclei appeared clean under phase contrast. The homogenate was spun at 300*g*. The nuclear precipitate was resuspended in 8% (w/v) sucrose containing (final concentrations) 50 mM-KCl, 0.05 mM-ZnSO₄, 2.0 mM-MgCl₂ and 5 mM-tris-HCl buffer, pH 7.1, and layered on to 10 ml of 30% (w/v) sucrose containing (final concentrations) 50 mM-KCl, 0.05 mM-ZnSO₄, 2.0 mM-MgCl₂ and 5 mM-tris-HCl buffer, pH 7.1. After centrifugation

for 10 min at 800*g* the nuclei had sedimented as a pellet, the dead cells and debris remaining at the sucrose interface. The nuclei were resuspended in sucrose as described above and counted, by using a haemocytometer.

Extraction of histones. Nuclei were centrifuged out of the sucrose and resuspended in medium containing (final concentrations) 0.14 M-NaCl, 3 mM-MgCl₂ and 5 mM-tris-HCl buffer, pH 7.1, for 15 min. The pellet, after centrifugation at 300*g*, was washed twice with 3 mM-MgCl₂ in 10 mM-tris-HCl buffer, pH 7.1, to remove nuclear ribosomal material (Frenster, Allfrey & Mirsky, 1960). The pellet was given a further wash with 1 mM-HCl. Histone fraction F1 was extracted in 5% (w/v) perchloric acid (Johns, 1964), precipitated in 20% (w/v) trichloroacetic acid and redissolved in water. The remaining histone fractions were precipitated similarly from the 250 mM-HCl extract and redissolved in 250 mM-HCl.

Characterization of histones. Histone fractions were characterized electrophoretically from their band migration on polyacrylamide gels. The method of Reisfeld, Lewis & Williams (1962) was used. Amino acid analysis of the fractions was performed. Protein samples were hydrolysed with 6 M-HCl for 21 h at 110°C by the method of Moore & Stein (1963). A Locarte amino acid analyser, with a single column for measurement of acidic, neutral and basic amino acids, was used for the analyses.

Preparation of cytoplasmic and nuclear extracts for histone kinase and phosphatase assays. Nuclei were prepared in 0.25 M-sucrose containing (final concentrations) 5 mM-MgCl₂ and 5 mM-tris-HCl buffer, pH 7.2. The cells were homogenized and the resuspended nuclei layered on to a sucrose density gradient as described above. The nuclear pellet was resuspended in 0.9% NaCl in 10 mM-tris-HCl buffer, pH 7.4. Portions of this nuclear suspension were used for the assays. To obtain the cytoplasmic enzymes, the supernatants from the initial 300*g* centrifugation of the nuclear homogenate were centrifuged at 100 000*g* for 1 h. Enzyme activity remained in the supernatant.

Kinase assay. Kinase activity was assayed in an incubation volume of 1.5 ml. The reaction mixture contained 1 mg of sheep thymus histone F1, or 1 mg of total histone after extraction of histone F1, 0.5 µmol of [γ -³²P]ATP (approx. 24.1 Ci/mol), 50 µmol of tris-HCl buffer, pH 7.4, 5 µmol of MgCl₂ and 0.3–0.6 ml of enzyme. The system was incubated at 37°C for 20 min, and the reaction terminated by the addition of trichloroacetic acid [final concentration 25% (w/v)]. The precipitate was extracted twice with 250 mM-HCl. The pooled extracts, containing histone F1, or the remaining histones after extraction of histone F1, were washed five times by precipitation with 25% (w/v) trichloroacetic acid and re-extraction with 250 mM-HCl. The radioactivities of the final 250 mM-HCl extracts were counted. Enzyme activity was expressed as mCi of [³²P]P_i incorporated/g of histone recovered per g of DNA. In control assays 1 mg of histone was not added until after the reaction had been stopped by adding trichloroacetic acid. Kinase activities at different times after stimulation were expressed as enzyme activity/µg of DNA in the original cell or nuclear preparation.

Phosphatase assay. Phosphatase was assayed under similar conditions to the kinase, but with the omission of ATP from the incubation medium. These conditions are

not optimum for histone phosphatase (Meisler & Langan, 1969), but the assay results are more immediately comparable with those for the kinase. The substrate was 1 mg of ^{32}P -labelled sheep thymus histone F1 or total histone after extraction of histone F1. The substrate was prepared by incubating the histone with $[\gamma\text{-}^{32}\text{P}]$ ATP and a highly active preparation of Novikoff-tumour histone kinase. The resulting histone F1 had a specific radioactivity of 0.076 mCi/g and the remaining histones a specific radioactivity of 0.044 mCi/g.

The phosphatase reaction was terminated by adding trichloroacetic acid [final concentration 25% (w/v)]. The precipitate was washed twice with 25% (w/v) trichloroacetic acid, and the supernatants were pooled. The radioactivity of the $[\text{}^{32}\text{P}]\text{P}_i$ released from the histone by the phosphatase was counted on these extracts.

Enzyme activity was expressed as mCi of $[\text{}^{32}\text{P}]\text{P}_i$ released from 1 g of histone/g of DNA in the original cell or nuclear preparations.

Determination of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used.

Determination of phosphate. The total phosphorus content of histone preparations was measured by the method of Bartlett (1959), but with 60% (w/v) HClO_4 instead of 5M- H_2SO_4 , the H_2O_2 being omitted.

Counting of ^{32}P radioactivity in gel bands. Gels were sliced and the protein bands were dissolved in 60% (w/v) H_2O_2 by incubation at 37°C for about 5 h. Radioactivity in the extracts was measured as Čerenkov radiation, counts of the samples in water being taken on the tritium channel of a Beckman CMP 200 liquid-scintillation counter. Counting of ^{32}P radioactivity by this method is about 30% efficient.

Extraction and determination of nucleic acid. DNA and RNA were extracted by the method of Schmidt & Thannhauser (1945) as described by Munro & Fleck (1966). RNA was determined by a modification of the original method described by Hurlbert, Schmitz, Brumm & Potter (1954). The sensitivity was improved by increasing the proportion of reagent from 66.6% to 75% of the final assay mixture. DNA was measured by the diphenylamine method of Burton (1956).

Extraction of ATP and P_i . P_i , ATP and other low-molecular-weight materials were extracted from part of each culture sample with 5% (w/v) trichloroacetic acid. This was removed by extensive extraction with ether, and the ether was finally removed by bubbling air through the sample. ATP and P_i were then determined in the extract.

Determination of P_i . The method of Berenblum & Chain (1938) was used. The quantities were scaled down to give a final aqueous volume of 2.0 ml, to which 1.8 ml of 2-methylpropan-1-ol was added. Radioactivity was counted on a sample of the final extract before the addition of SnCl_2 .

Determination of ATP. ATP was measured by the luciferin-luciferase assay (Holton, 1959).

Incorporation of radioisotopes and measurement of radioactivity. Nucleic acid synthesis was measured by determining the incorporation of $[\text{5-}^3\text{H}]\text{uridine}$ (19.4 Ci/mmol) and $[\text{6-}^3\text{H}]\text{thymidine}$ (22 Ci/mmol) into RNA and DNA respectively. Cultures were exposed to the tritiated precursor (1 $\mu\text{Ci/ml}$) 30 min before the cells were harvested. Where initial polydisperse RNA synthesis was measured

a continuous label was given 30 min before stimulation. Cultures were also exposed to $[\text{}^{32}\text{P}]\text{P}_i$ (5 $\mu\text{Ci/ml}$) 30 min before the cells were harvested.

Incorporation was measured, by using a Beckman liquid-scintillation counter, by counting the radioactivity of RNA and DNA extracts (0.02 ml) in scintillation fluid containing 0.8% (w/v) 5-(4-biphenyl)-2-(4-*tert*-butylphenyl)-1-oxa-3,4-diazole and 8% (w/v) naphthalene in dioxan. Measured counts of ^3H incorporation into nucleic acids were never less than 1000 c.p.m.; ^{32}P counts in histones were not less than 500 c.p.m. The efficiency of tritium counting was about 50%. $[\text{}^{32}\text{P}]\text{P}_i$ incorporation was measured either as Čerenkov radiation or on the ^{32}P channel of the Beckman liquid-scintillation counter with a toluene-Triton X-100-based scintillant described by Patterson & Greene (1965).

Materials. Phytohaemagglutinin (reagent grade; Wellcome Research Laboratories, Beckenham, Kent, U.K.) was reconstituted by adding 5 ml of sterile water/vial, and 0.5 ml of this solution was added to 50 ml of culture medium containing 5×10^6 lymphocytes/ml. The sodium salt of dibutyl cyclic AMP was obtained from C. F. Boehringer and Soehne G.m.b.H. (Mannheim, Germany), chlorpromazine (Largactil) from May and Baker Ltd. (Dagenham, Essex, U.K.) and theophylline from British Drug Houses Ltd. (Poole, Dorset, U.K.). All radioactive compounds were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.).

RESULTS

Stimulation of lymphocytes by phytohaemagglutinin is a complex phenomenon. In an endeavour to by-pass membrane participation in the transformation, dibutyl cyclic AMP was substituted for phytohaemagglutinin, and nucleic acid synthesis in the cultures was followed. Three periods of nucleic acid synthesis were examined: initial polydisperse-RNA synthesis immediately after stimulation (0–1 h) (Pogo *et al.* 1966; Forsdyke, 1968), RNA synthesis, initiated 4–5 h later (Monjardino & MacGillivray, 1968), and eventual DNA synthesis, which takes place after 2–3 days (Loeb, Agarwal &

Table 1. *Stimulation of pig lymphocytes by different concentrations of dibutyl cyclic AMP*

Concn. of dibutyl cyclic AMP (μM)	Stimulation ratio
0	1.00
10.01	1.00
1.00	1.20
0.10	1.94
0.01	1.91
0.001	1.00
Phytohaemagglutinin	1.90–2.00

Table 2. *Transformation of pig lymphocytes by dibutyryl cyclic AMP: effects on RNA and DNA synthesis*

The conditions of culture, exposure to labelled precursors and concentrations of phytohaemagglutinin used are described in the Methods section. The concentration of dibutyryl cyclic AMP in the medium was 0.01 μM . The stimulation ratios for dibutyryl cyclic AMP represent the means of four experiments, with a range of $\pm 9\%$.

Administration of labelled precursor	Time after stimulation	Stimulation ratio	
		For dibutyryl cyclic AMP	For phytohaemagglutinin
[5- ³ H]Uridine at -30 min	10 min	1.20	1.10-1.25
	20 min	1.40	1.25-1.55
	30 min	1.90	1.50-2.30
	60 min	2.80	2.30-3.10
[5- ³ H]Uridine 30 min before cells were harvested	4.5 h	2.10	1.80-2.40
[6- ³ H]Thymidine 30 min before cells were harvested	48 h	1.20	1.20
	72 h	3.10	2.00-3.50
	79 h	1.80	1.60-2.30
	86 h	1.20	1.20-1.40

Woodside, 1968). In the pig lymphocyte system here described the peak of DNA synthesis was reached 72 h after stimulation.

RNA synthesis in the first hour after stimulation was examined in cultures to which different concentrations of dibutyryl cyclic AMP had been added. It was found that polydisperse-RNA synthesis was stimulated only in the concentration range 0.01-1 μM -dibutyryl cyclic AMP. Results (Table 1) are expressed as a ratio of the specific radioactivity of RNA or DNA in stimulated cultures to that in controls for a given experiment. This was necessary because of variations between culture preparations from different batches of pig blood. In the presence of phytohaemagglutinin, stimulating concentrations of dibutyryl cyclic AMP produced no enhancement of the RNA synthesis observed. At concentrations above 0.01 mM dibutyryl cyclic AMP inhibited the lymphocyte response to phytohaemagglutinin. The critical dependence on the concentration of dibutyryl cyclic AMP used may explain results of previous workers (Hirschhorn, Grossman & Weissmann, 1970; Novogrodsky & Katchalski, 1970) who failed to find transformation induced by dibutyryl cyclic AMP.

Lymphocytes were treated with 0.1 μM -dibutyryl cyclic AMP and the characteristic phases of nucleic acid synthesis during transformation were compared with those obtained by treatment with phytohaemagglutinin. In each case the results with 0.1 μM -dibutyryl cyclic AMP were similar to those with phytohaemagglutinin (Table 2). Microscopic examination of the cells revealed an increase in cell size and an increased occurrence of mitosis and division in both sets of cultures.

A distinction might be made between the stimulatory effects of dibutyryl cyclic AMP and phytohaemagglutinin on the basis of the different

responses they cause in the presence of chlorpromazine. This phenothiazine drug has been reported by Wolff & Jones (1970) to inhibit membrane-bound adenylate cyclase in a number of tissues. With 0.1 μM -chlorpromazine there was a striking difference between the responses to dibutyryl cyclic AMP and to phytohaemagglutinin; the former stimulated the lymphocytes significantly, but the latter produced only 28% of the stimulation found in the absence of chlorpromazine (Table 3). With the greatest concentration of chlorpromazine (0.1 mM) used, cell membranes were damaged, but this was not apparent at lower concentrations. The inhibition by chlorpromazine was marked only if it and the stimulant were added in rapid succession and the culture flask was immediately shaken. If phytohaemagglutinin was added first, the inhibitory effect of chlorpromazine was decreased. Experiments in which the concentrations of phytohaemagglutinin and dibutyryl cyclic AMP were increased showed that binding of chlorpromazine to these compounds was not a factor in determining the extent of stimulation. If dibutyryl cyclic AMP was added to a culture to which phytohaemagglutinin and chlorpromazine had already been added the lymphocytes were stimulated to the extent expected for dibutyryl cyclic AMP in the presence of chlorpromazine.

Theophylline, which increases the concentration of cyclic AMP within the cell by inhibiting phosphodiesterase, has some stimulatory effect on lymphocytes. At 5 mM a stimulation ratio for DNA of about 1.5 was observed after 72 h, compared with 2.5-3.5 for phytohaemagglutinin. A similar mitogenic effect of caffeine was reported by MacManus & Whitfield (1969).

Cultures stimulated by either phytohaemagglutinin or dibutyryl cyclic AMP were exposed to

Table 3. *Effect of chlorpromazine on phytohaemagglutinin and dibutyryl cyclic AMP stimulation of pig lymphocytes*

[5-³H]Uridine was added at -30 min. Stimulant and chlorpromazine were added at 0 min and the cultures were examined at +60 min. Where present, the concentration of dibutyryl cyclic AMP was 0.1 μM.

Concn. of chlorpromazine (μM)	Stimulation ratio (% of that in the absence of chlorpromazine)	
	For dibutyryl cyclic AMP	For phytohaemagglutinin
	1.0	1.0
100	11.6	3.8
10.0	13.8	8.6
1.0	72.5	28.4
0.1	99.0	96.5
0.01	100	100
0

Fig. 1. Changes in intracellular P_i concentration after stimulation of lymphocytes by phytohaemagglutinin or dibutyryl cyclic AMP. Results are expressed as fractions of control (net P_i) and percentages of control (specific radioactivity of P_i). The cultures were exposed to [³²P] P_i for 30 min before examination. ○, Specific radioactivity of P_i ; Δ, net amount of P_i ; □, incorporation of [5-³H]uridine into RNA.

Fig. 2. Changes in ATP concentration after stimulation of lymphocytes by phytohaemagglutinin or dibutyryl cyclic AMP. Results are expressed as fractions of control (net ATP) and percentages of control (specific radioactivity of ATP). The cultures were exposed to [³²P]ATP for 30 min before examination. ○, Specific radioactivity of ATP; Δ, net amount of ATP; □, incorporation of [5-³H]uridine into RNA.

[³²P] P_i for 30 min, and the P_i and ATP pools were examined immediately after stimulation. There was an immediate increase in net P_i within the cell (Fig. 1). Within 30 min this had returned to control, or below control, values. The specific radioactivity of P_i similarly increased rapidly immediately after stimulation. The size and specific radioactivity of the ATP pool both increased, but not significantly until 30 min after stimulation (Fig. 2).

Changes in the pulse-labelling of histone phos-

phate were also examined during this period. Two histone fractions were examined, histone F1 and the histones remaining after extraction of histone F1. No net increase in phosphate was detectable in either fraction during this initial period of RNA synthesis. Incorporation of [³²P] P_i into the histone was checked by polyacrylamide-gel electrophoresis of the extracts, cutting out the bands after staining, and counting the ³²P radioactivity of the solubilized gel band. The specific radioactivity of both

gel bands was found to increase in the first 30 min after stimulation (Table 4), preceding any detectable rise in the specific radioactivity of the precursor ATP pool (Fig. 2). Subsequently [^{32}P]P_i continued to be incorporated into histones other than F1, the increase in specific radioactivity of this fraction now reflecting that of the ATP pool.

Histone kinase activity was examined both in nuclei and in the cytoplasm immediately after stimulation. Histone F1 and the histones remaining after extraction of histone F1 were used as substrates. The nuclei were obtained in isosmolar sucrose and there was little difference in enzyme activity between the two cell fractions. With both histone fractions there was an increase in kinase activity during the first 30 min after stimulation (Table 5). In the next 30 min histone F1 kinase activity decreased, but activity with the other

histones continued to increase until 60 min after stimulation. When cyclic AMP was added to kinase preparations from either stimulated or control cultures the enzyme was activated to the same extent (Table 5).

Histone phosphatase activity also increased immediately after stimulation, declining after 30 min (Table 6). No activation of the phosphatase by cyclic AMP was found under conditions in which the kinase is activated.

DISCUSSION

Stimulation of pig lymphocyte cultures by either phytohaemagglutinin or dibutyryl cyclic AMP resulted in an immediate increase in P_i and a slight fall in ATP concentration. After 30–40 min the specific radioactivity and net ATP content of the

Table 4. *Specific radioactivity of ATP and histone phosphate after stimulation by phytohaemagglutinin or 0.1 μM -dibutyryl cyclic AMP, after exposure to labelled precursors in the initial period of polydisperse-RNA synthesis*

[5- ^3H]Uridine was added to the cultures 30 min before stimulation and [^{32}P]P_i was added 30 min before the cells were harvested. Specific radioactivities are expressed relative to those of the unstimulated cultures. The results are the means of at least two determinations.

Approx. time after stimulation (h)	Relative specific radioactivities			
	RNA	ATP	Histones other than F1	Histone F1
0	1.0	1.0	1.00	1.00
0.75	1.45	1.25	1.89	1.59
0.8	1.5	1.6	3.36	1.13
1.0	1.6	2.0	4.44	1.13
1.5	1.9	3.9	5.22	—
2.5	2.5	5.0	—	1.31

Table 5. *Histone kinase activity in lymphocytes immediately after stimulation by phytohaemagglutinin*

The cells were stimulated and the kinase activity was measured in nuclei and cytoplasm as described in the text. Kinase activity is expressed as mCi of [^{32}P]P_i incorporated/g of histone per g of DNA. The results are the averages of three experiments, the individual values varying by $\pm 10\%$.

Time after stimulation (min)	Kinase activity			
	Cytoplasmic		Nuclear	
	Histone F1	Histones other than F1	Histone F1	Histones other than F1
0 (control)	13.6	4.3	15.2	5.3
10	16.0	5.0	16.0	7.3
20	26.5	5.2	24.4	6.5
30	25.8	5.3	22.1	8.3
60	17.8	8.5	20.6	10.3
90	13.4	4.6	15.1	6.8
Control+0.01 mM-cyclic AMP	28.5	11.3	21.3	6.8
Stimulated (30 min)+0.01 mM-cyclic AMP	28.2	11.8	22.2	6.9

Table 6. *Histone phosphatase activity in lymphocytes immediately after stimulation by phytohaemagglutinin*

The cells were stimulated and the phosphatase activity was measured in nuclei and cytoplasm as described in the text. Phosphatase activity is expressed as mCi of [32 P]P_i released/g of histone per g of DNA. The results are the averages of two experiments, the individual values varying by $\pm 8\%$.

Time after stimulation (min)	Phosphatase activity			
	Cytoplasmic		Nuclear	
	Histone F1	Histones other than F1	Histone F1	Histones other than F1
0 (control)	63	23	58	49
10	176	30	110	79
20	167	35	114	71
30	130	52	160	85
60	—	29	110	71
90	36	—	83	70

cells exposed to [32 P]P_i started to increase and this was maintained for the next 20–30 min. Since these effects were produced by both phytohaemagglutinin and dibutyryl cyclic AMP, and since the results with chlorpromazine (0.1 μ M) suggest that the action of phytohaemagglutinin, but not that of cyclic AMP, may be prevented by the presence of the drug, there is a suggestion that the increase in P_i is consequent on the metabolic effects of cyclic AMP and is not dependent on membrane changes produced by the mitogen.

In the first 30 min after stimulation, before the specific radioactivity of the precursor ATP pool was detectably increased, [32 P]P_i was incorporated into histone. These results with a 30 min exposure to [32 P]P_i are consistent with earlier findings (Cross & Ord, 1970) where label was administered continuously. The relative increases in specific radioactivity of the phosphate groups on histone F1 and the other histones were similar for the first 30 min, but subsequently a difference between histone F1 and the other histones became apparent in the pulse-labelling experiments. Whereas there was only slight incorporation of [32 P]P_i into histone F1, the relative specific radioactivity of the phosphate on the other histones increased at a rate even greater than that of the total cellular ATP pool. Kleinsmith *et al.* (1966) had concluded that increased phosphorylation of phosphoprotein in their lymphocyte cultures was not due to increased specific radioactivity of ATP intermediates after stimulation by phytohaemagglutinin.

Kinase activity paralleled the [32 P]P_i incorporation results, increasing rapidly in the first 20–30 min after stimulation, when either histone F1 or the other histones was used as substrate. After 30 min histone F1 kinase activity decreased, whereas activity with the other histones continued to increase until 60 min, subsequently falling. Phos-

phatase activity behaved similarly, the increase slightly preceding that of the kinase. With histones other than F1 as substrate, the hydrolase activity decreased after 30 min, with the kinase activity still continuing to increase; this, and the increased specific radioactivity of the ATP, account for the increased specific radioactivity of the other histones at 60 min and 90 min after stimulation. The mechanism of the rapid activation of the phosphatase after stimulation is not established. It is still uncertain whether there is one histone kinase and one histone phosphatase, or whether a number of enzymes with different specificities for histones exist. The different time-courses reported here for histone F1 and the other histones might suggest the latter. Preliminary results from this laboratory (Siebert, Ord & Stocken, 1971) are also consistent with the existence of at least two kinases.

There is a further question of the location of both enzymes. They are found both in the nucleus and the cytoplasm (Siebert *et al.* 1971), but in these experiments similar changes in activity were observed in both fractions. This might be due to the use of isosmolar solutions to isolate the nuclei.

Cyclic AMP (0.01 mM) increased the kinase activity with both groups of histones. The activity of the enzyme in cultures stimulated with phytohaemagglutinin was not further increased by cyclic AMP, suggesting that *in vivo* the enzyme is activated by endogenous nucleotide immediately after lymphocyte stimulation.

The comparable stimulation of the cultures by unpurified phytohaemagglutinin and dibutyryl cyclic AMP (0.01–0.1 μ M) is not unexpected if part of the action of the mitogen occurs through activation of adenylate cyclase. Smith, Steiner & Parker (1970) have found an early increase in cyclic AMP concentration in human peripheral

blood lymphocytes transformed by phytohaemagglutinin, but in other cases (Novogrodsky & Katchalski, 1970) no increase in cyclic AMP in transformed systems has been detected. The role of the nucleotide, or of the metabolic stimulus that it normally produces, in initiating transformation remains unresolved, but the results described here, in conjunction with the measured increase in cyclic AMP reported by Smith *et al.* (1970), would suggest that cyclic AMP activation of histone kinase is one of the early events occurring after stimulation.

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