Histone Phosphatase and Cyclic Nucleotide-Stimulated Protein Kinase from Human Lymphocytes

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(Received 1 March 1972)

1. Extracts of human peripheral blood lymphocytes contained a histone phosphatase that catalysed the release of P_i from phosphorylated whole thymus histone. 2. Stimulation of the phosphatase was obtained by concentrations of KCl and NaCl of up to 75 mM, and by $MgCl_2$; $CaCl_2$ inhibited the enzymic activity. 3. In the absence of $MgCl_2$, phosphoenolpyruvate inhibited histone phosphatase activity; this inhibition could be partially reversed by adding $MgCl_2$ to assays. 4. Lymphocyte extracts contained a protein kinase activity which was maximally stimulated by $1\ \mu M$ -cyclic AMP (adenosine 3':5'-cyclic monophosphate) or by 0.1 mM-cyclic GMP (guanosine 3':5'-cyclic monophosphate). 5. Incubation of the enzyme with histone in the absence of ATP or $MgCl_2$ resulted in the dissociation of the enzyme into a lower-molecular-weight species that was not stimulated by cyclic AMP. This effect could be prevented if ATP and $MgCl_2$ were present in reaction mixtures before histone and enzyme were allowed to interact. 6. Cyclic AMP also dissociated the kinase into a lower-molecular-weight species. 7. In the presence of $1\ \mu M$ -AMP, half-maximal activities were obtained with 0.92 mM- $MgCl_2$, 6.0 μM -ATP and 0.23 mg of whole thymus histone/ml.

Stimulation of lymphocytes by the mitogen phytohaemagglutinin initiates a complex series of biochemical changes. Early changes (within 1 h) include increased phosphorylation of acidic nuclear proteins (Kleinsmith *et al.*, 1966) and of histones (Cross & Ord, 1971). Smith *et al.* (1971) reported that, in close analogy with the action of many hormones, phytohaemagglutinin activated human lymphocyte adenylate cyclase and induced an increase in intracellular cyclic AMP (adenosine 3':5'-cyclic monophosphate) within 1 min of stimulation. Cross & Ord (1971) have further shown that pig lymphocytes contain a cyclic AMP-stimulated histone kinase; this kinase was converted into a cyclic AMP-independent enzyme shortly after stimulation by phytohaemagglutinin. These observations strongly suggest that activation of a protein kinase by cyclic AMP occurs soon after phytohaemagglutinin stimulation.

The present paper describes some of the properties of a histone phosphatase and a cyclic nucleotide-stimulated protein kinase in a soluble extract from human lymphocytes.

Experimental

Substrates, activators and inhibitors

$[\gamma\text{-}^{32}P]\text{ATP}$ was prepared as described by Glynn & Chappell (1964). $[\gamma\text{-}^{32}P]\text{GTP}$ was prepared in a similar way, but the GTP was purified by chromatography in isobutyric acid – conc. NH_3 (sp.gr. 0.88) – water (57:4:39, by vol.). Unlabelled ATP, ADP, AMP,

phosphoenolpyruvate, cyclic AMP and cyclic GMP (guanosine 3':5'-cyclic monophosphate) were obtained from Sigma Chemical Co., St. Louis, U.S.A.

Whole calf thymus histone (type IIA), lysine-rich histone (type III), arginine-rich histone (type IV), protamine and bovine serum albumin (Cohn fraction V) were purchased from Sigma Chemical Co.

Preparation of $[\text{}^{32}P]\text{histone}$

Incubations with pig brain protein kinase and $[\gamma\text{-}^{32}P]\text{ATP}$ were done as described by Murray & Froschio (1971), but with 2 mg of whole calf thymus histone replacing microtubular protein. After incubation for 4 h at 37°C , the reaction mixture was chilled and 0.1 ml of 75% (w/v) trichloroacetic acid was added. After centrifugation at 4°C , the supernatant was mixed with 2 ml of cold 75% (w/v) trichloroacetic acid (final concn. 25%) and centrifuged at 10000g for 10 min to sediment the histones. The precipitate was washed twice in cold 25% (w/v) trichloroacetic acid and finally redissolved in 2 ml of water. After dialysis against 500 vol. of water (with one change) at 4°C overnight, the dialysis residue was briefly centrifuged and stored at -15°C . A typical preparation contained 4.2×10^6 c.p.m./mg of protein. Most of the radioactivity (>95%) was alkali-labile (0.1 M-NaOH at 100°C for 15 min) and stable in acid (0.1 M-HCl at 100°C for 15 min). After partial acid hydrolysis (2 M-HCl at 100°C for 4 h) and electrophoresis (4 h, 25 V/cm) in formic acid–acetic acid–water (1:4:45, by vol., pH 1.9) both P_i and esterified phosphate were

detected; of the residual esterified ^{32}P 43% migrated with the *O*-phosphoserine marker and 57% was not hydrolysed and remained on the origin. No radioactivity could be detected in the *O*-phosphothreonine position. After electrophoresis in urea gels as described by Panyim & Chalkley (1969) 68% of the radioactivity was associated with the major histone bands.

Assay of histone phosphatase

The assay procedure was based on that described by Meisler & Langan (1969). Reaction mixtures contained 10 μmol of tris-HCl (pH 7.5), 2 μmol of β -mercaptoethanol, 10 μl of [^{32}P]histone and extract in a total volume of 0.2 ml. After incubation at 37°C for 30 min, the reactions were stopped by adding 0.5 ml of 5 mM-silicotungstic acid in 0.0025 M- H_2SO_4 and chilled in ice. After addition of 50 μg of whole thymus histone, assay tubes were kept at 4°C for 30 min to allow complete precipitation of histone, and then centrifuged at 5000g for 10 min. The radioactivity in 0.2 ml portions of the supernatant was measured by liquid-scintillation counting in Bray's (1960) solution. Control assays containing no extract were done with each experiment; quantitative precipitation of the [^{32}P]histone was obtained in all cases.

Preparation of lymphocytes

Human peripheral blood was obtained from donors with a history (pre-1952) of jaundice, but who were Australia-antigen negative. Blood (500 ml) was collected into a sterile, heparinized bottle. After addition of 100 ml of 6% (w/v) dextran (Macrodex; Pharmacia, Uppsala, Sweden) in phosphate-buffered saline (0.146 M-NaCl, 10 mM-sodium phosphate, pH 7.3) the blood was allowed to settle for 1–2 h at 37°C. The lymphocyte-rich serum-dextran layer was removed and centrifuged at 300g for 10 min. The sedimented cells were resuspended in 30 ml of autologous plasma and incubated for 30 min on a cotton-wool column (30 cm $\times$ 1.5 cm), surrounded by a water jacket kept at 37°C. The column was eluted with autologous plasma at 37°C and the resulting cell suspension was centrifuged at 300g for 10 min; most of the phagocytic cells and platelets were retained by the cotton-wool column. The cells were resuspended in phosphate-buffered saline and layered over 9 ml of a solution containing 1.8 ml of 50% (w/v) sodium diatrizoate (Hypaque; Winthrop Laboratories, Sydney, Australia), 6.35 ml of 6% (w/v) Ficoll (Sigma Chemical Co.) in water and 0.85 ml of phosphate-buffered saline (Mendelson *et al.*, 1971), in a 30 ml centrifuge tube. After centrifuging at 1600g for 10 min the lymphocyte-rich interface layer was removed by aspiration and the cells were washed once in phosphate-buffered saline. The isolated lymphocyte fraction consisted of

75–87% small lymphocytes, 0–4% granulocytes and erythrocytes and 11–22% platelets.

Preparation of cell-free extracts from lymphocytes

Cells prepared as described above were suspended in 0.25 M-sucrose–20 mM-tris-HCl buffer (pH 7.0) (approx. 25×10^6 cells/ml of extraction buffer), frozen in liquid N_2 and thawed at room temperature. The process of freezing and thawing was repeated twice and the broken-cell suspension was centrifuged at 10000g for 10 min. Extracts centrifuged for 1 h at 100000g contained the same amounts of histone phosphatase and protein kinase activity. Endogenous phosphorylation in the 10000g or 100000g extracts in the absence of added histone was less than 1% of the phosphorylation in the presence of histone. The supernatant was dialysed overnight at 4°C against approx. 500 vol. of 20 mM-tris-HCl buffer (pH 7.0) with one change. The dialysis residue was stored at –15°C. Protein was determined as described by Lowry *et al.* (1951) using bovine serum albumin (fraction V) as a standard; extracts contained between 0.31 and 1.8 mg of protein/ml.

Assay of protein kinase

Assay mixtures contained 10 μmol of tris-HCl buffer (pH 7.5), 2 μmol of MgCl_2 , 1.2–2 nmol of [γ - ^{32}P]ATP (specific radioactivity between 0.1 and 1.0 mCi/ μmol), 100 μg of whole calf thymus histone and extract (6–35 μg of protein) in a final volume of 0.2 ml, and were incubated for 10 min at 37°C as a routine. The protein kinase reactions were stopped by adding 50 μl of a solution of bovine serum albumin in 100 mM-EDTA (50 mg/ml); 100 μl portions were then pipetted on to discs (2.3 cm diameter) of Whatman 50 paper and allowed to dry at room temperature. After washing as described by Murray & Froscio (1971), radioactivity associated with the discs was measured by liquid-scintillation counting. Most of the radioactive product (>98%) was labile in alkali and stable in acid when tested as described above for the preparation of phosphorylated histone. After partial acid hydrolysis both P_i and esterified phosphate were detected; of the residual esterified ^{32}P 58% was not hydrolysed, 41% was associated with *O*-phosphoserine and 1% with *O*-phosphothreonine. The phosphorylated product could be absorbed on CM-cellulose but not on DEAE-cellulose, and after gel electrophoresis (Panyim & Chalkley, 1969) 62% of the activity was associated with the major histone bands.

Sucrose-gradient centrifugation

Sucrose gradients (5–20%; 4.8 ml) containing 50 mM-tris-HCl buffer (pH 7.5) were centrifuged at

5°C for 16 h at 75000g in a MSE Superspeed 65 centrifuge. Fractions (7 drops) were collected and 25 μ l portions assayed for protein kinase activity. These kinase assays contained the same concentrations of reactants as described above, but in a final volume of 100 μ l. After the reaction had been stopped with 25 μ l of the bovine serum albumin-EDTA solution, 100 μ l portions were applied to the paper discs and subjected to the acid-washing procedure.

Yeast alcohol dehydrogenase (0.5 mg), horse heart cytochrome *c* (0.5 mg) and catalase (0.2 mg) were used as standards for molecular-weight estimations.

Calculation of kinetic parameters

The results were fitted to the Michaelis-Menten equation by using the method of least squares.

Assay for cyclic AMP-binding protein

The assay relied on the adsorption of the binding protein to Millipore filters, as described by Gilman (1970).

Results

Purity of lymphocyte preparations

To estimate the contribution of contaminating platelets (see the Experimental section) to the enzymic activities measured, purified preparations of both platelets and lymphocytes were obtained from the same sample of blood. Platelets were purified by sucrose-gradient centrifugation (Perper *et al.*, 1968) and extracts were prepared as described for the lymphocytes.

In the presence of 1 μ M-cyclic AMP, extracts from lymphocytes and platelets had protein kinase specific activities of 437 and 466 pmol/min per mg of protein respectively, and had histone phosphatase specific activities of 25000 and 18400 c.p.m./min per mg of protein respectively.

Thus in view of the smaller platelet volume, it is not likely that the maximum contamination with platelets observed (22%) would contribute more than 5% of the measured kinase and phosphatase activities. Nevertheless it is important to recognize that the observed activities were derived from a mixed population of cells.

General properties of lymphocyte histone phosphatase

Phosphatase activity did not require the addition of a bivalent cation, and was not inhibited by the addition of 1 mM-EDTA. The removal of mercapto-ethanol from reaction mixtures resulted in a 16% decrease in activity. Phosphate release from [³²P]-histone was linear for at least 1 h at 37°C; as a routine assays were performed for 30 min.

Fig. 1. Effect of KCl on the activity of lymphocyte histone phosphatase

For experimental details see the text.

Fig. 2. Inhibition of lymphocyte histone phosphatase by phosphoenolpyruvate

Assays were done in the presence of 5 mM-MgCl₂ (Δ), 5 mM-CaCl₂ (▲), 5 mM-CaCl₂ plus 5 mM-MgCl₂ (●), or in the absence of added bivalent cations (○).

A variety of uni- and bi-valent cations modified enzymic activity to some extent. Concentrations of KCl up to about 57 mM had some stimulatory effect;

higher concentrations were inhibitory (see Fig. 1). Similar effects were observed with NaCl. Mg^{2+} was a weak stimulator and Ca^{2+} an inhibitor of enzymic activity; 5 mM- $MgCl_2$ stimulated the phosphatase by 26% and 5 mM- $CaCl_2$ caused 53% inhibition. Phosphoenolpyruvate was the most effective inhibitor of a variety of phosphate esters tested. The effect of various concentrations of phosphoenolpyruvate in the presence and absence of Mg^{2+} and Ca^{2+} is shown in Fig. 2. Addition of Mg^{2+} partially reversed the inhibitory effects of both phosphoenolpyruvate and Ca^{2+} . Under the same assay conditions, but in the absence of both Ca^{2+} and Mg^{2+} , 5 mM-phospho-L-serine, acetyl phosphate or pyruvate inhibited the phosphatase reaction by 54, 25 and 39% respectively.

A range of cyclic AMP concentrations (0.1 μM –1 mM) had no effect on the activity of lymphocyte histone phosphatase. In the presence of Mg^{2+} little inhibition (<6%) was obtained with 2 mM-ATP, -ADP or -AMP. However, in the absence of Mg^{2+} ATP was a powerful inhibitor of the enzyme; 2 mM-ATP caused 95% inhibition of phosphate release.

Histone phosphatase had maximum activity at about pH 7.8, and showed 60 and 73% of this activity at pH 7.2 and 8.5 respectively.

General properties of lymphocyte protein kinase

Activation by cyclic AMP and cyclic GMP. As shown in Fig. 3, lymphocyte protein kinase could be activated by both cyclic AMP and cyclic GMP. Cyclic AMP produced a definite stimulation at 10 nM, with a broad maximum between 0.1 and 10 μM , whereas cyclic GMP provided some stimulation at 0.1 μM and gave a maximum response at about 0.1 mM. Experiments done on the same day with the same batch of extract indicated that there were no significant differences in the maximum velocities obtainable with both cyclic nucleotides.

Substrate specificity. A number of proteins were phosphorylated in the presence of ATP, $MgCl_2$ and soluble extract from human lymphocytes (see Table 1).

However, the total activities and the percentage stimulation by cyclic AMP varied widely.

Requirement for bivalent cations. As for other kinases, lymphocyte protein kinase had an absolute dependence on a bivalent cation. A small residual activity was observed in the absence of added cation (about 5% of the maximal activity), but this was completely abolished by the addition of 1 mM-EDTA.

Fig. 3. Effect of cyclic AMP (○) and cyclic GMP (●) concentration on the activity of lymphocyte protein kinase

For experimental details see the text. Relative activity: percentage of activity in the absence of added cyclic nucleotide.

Table 1. Substrate specificity of lymphocyte protein kinase

Assays were done as described in the Experimental section; each protein was tested at a concentration of 100 μg /assay.

Substrate	Activity (pmol/min per assay)		Stimulation by cyclic AMP (%)
	Without cyclic AMP	With 1 μM -cyclic AMP	
Whole thymus histone	1.66	5.70	243
Thymus histone (lysine rich)	0.18	1.21	572
Thymus histone (arginine rich)	0.36	1.89	425
Casein	3.73	4.16	12
Protamine	1.34	2.98	122
Bovine serum albumin	0.06	0.13	117

Table 2. *Bivalent-cation requirement of lymphocyte protein kinase*

Assays were done as described in the Experimental section; cations were added to give a final concentration of 5 mM. Fe^{2+} was tested as the sulphate; all other cations were tested as chlorides.

Cation	Activity (pmol/min per assay)		Stimulation by cyclic AMP (%)
	Without cyclic AMP	With 1 μM -cyclic AMP	
Mg^{2+}	1.8	6.9	283
Co^{2+}	1.8	5.1	183
Mn^{2+}	1.2	1.8	50
Fe^{2+}	0.9	0.88	-2

Maximum activity was obtained with about 5 mM- MgCl_2 , and concentrations higher than 10 mM had some inhibitory effect. The concentrations of MgCl_2 required to give half-maximal activity were 0.81 ± 0.19 mM in the absence of cyclic AMP and 0.93 ± 0.28 in the presence of 1 μM -cyclic AMP; maximum velocities were 3.7 ± 0.2 and 11.6 ± 1.0 pmol/min per assay respectively.

Some other bivalent cations were able to replace Mg^{2+} to some extent (see Table 2). Essentially no activity was obtained with 5 mM- Cd^{2+} , - Zn^{2+} and - Ca^{2+} (tested as chlorides) or with 5 mM- Cu^{2+} (tested as sulphate). In the presence of 5 mM- MgCl_2 addition of 5 mM- CaCl_2 resulted in about 68% inhibition in both the absence and the presence of 1 μM -cyclic AMP.

Inhibition by KCl. Lymphocyte protein kinase was inhibited by KCl (see Fig. 4). The concentration of KCl resulting in 50% inhibition of kinase activity was 30 and 34 mM in the absence and presence of cyclic AMP respectively. In view of the reported K^+ content (approx. 120 mM) of human lymphocytes (Lichtman & Weed, 1969) the K^+ effect may have physiological importance.

Effect of ATP concentration. The effect of varying the concentration of ATP in the presence of 10 mM- MgCl_2 was measured in both the presence and the absence of cyclic AMP. The ATP concentration required to give half-maximal activities was 6.7 ± 2.1 and 6.0 ± 0.97 μM in the absence and presence of 1 μM -cyclic AMP respectively; maximum velocities were 30.6 ± 2.2 and 71 ± 2.5 pmol/min per assay respectively.

$[\gamma\text{-}^{32}\text{P}]\text{GTP}$ could not act as the phosphoryl donor in the lymphocyte protein kinase reaction. Under standard assay conditions in the absence of cyclic nucleotides, GTP resulted in less than 2% of the activity with ATP; this activity was not stimulated by the addition of either 0.1 mM- or 1 μM -cyclic GMP or cyclic AMP.

Effect of histone concentration. The histone concentration required to give half-maximal activities was 0.46 ± 0.16 and 0.23 ± 0.07 mg/ml in the absence

Fig. 4. *Inhibition of lymphocyte protein kinase by KCl*

Assays were done in the absence (●) and in the presence (○) of 1 μM -cyclic AMP.

and presence of 1 μM -cyclic AMP respectively. The maximum velocity in the presence of cyclic AMP was 2.5-fold that in its absence.

Dissociation of protein kinase by histone and by cyclic AMP

Preliminary experiments established that when histone kinase assays were started by the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, cyclic AMP caused only a small and variable stimulation of kinase activity. The order of addition of reagents proved to be of crucial importance, and effects of starting the assay with individual components of the assay system are summarized in Table 3. It is clear that if histone and extract were allowed to interact in the absence of either ATP or

Table 3. *Effect of order of addition of reagents on cyclic AMP stimulation of protein kinase*

Assays were done as described in the Experimental section, but were started by addition of the indicated component. All components except the one being tested were preincubated for 10 min at 37°C before the reaction was started.

Reaction started with	Activity (pmol/min per assay)		
	Without cyclic AMP	With 1 μ M-cyclic AMP	Stimulation by cyclic AMP (%)
MgCl ₂	3.32	3.55	7
ATP	2.45	3.67	50
Extract	0.85	4.06	378
Histone	0.91	3.21	253

MgCl₂, very little stimulation of protein kinase activity was obtained on the addition of cyclic AMP. In the experiments reported in Table 3, histone and extract were allowed to preincubate for 10 min before the addition of either ATP or MgCl₂. However, assays in which the time of preincubation was varied indicated that the development of histone-induced cyclic AMP independence was complete within 1 min.

These results suggested that histone may have been causing a dissociation of histone kinase into catalytic and regulatory subunits, as described for the bovine brain enzyme (Miyamoto *et al.*, 1971), and that this dissociation was prevented by the presence of ATP and MgCl₂. This conclusion is supported by sucrose-gradient centrifugation of lymphocyte extracts carried out in the presence of histone or of histone plus ATP and MgCl₂ (see Fig. 5). On a sucrose gradient the protein kinase activity sedimented as a single peak, which was highly dependent on the addition of cyclic AMP. The inclusion of histone in the gradient again resulted in a single peak of protein kinase activity with a lower molecular weight, and which was not stimulated by cyclic AMP (see Fig. 5). A more complex situation was observed when histone, ATP and MgCl₂ were included in the gradients. Three peaks of activity were apparent. A high-molecular-weight cyclic AMP-dependent form and a low-molecular-weight cyclic AMP-independent form corresponded to the peaks in gradients centrifuged in the absence and presence of histone respectively. The third peak of kinase activity had an intermediate molecular weight and was stimulated by cyclic AMP. The three molecular-weight species observed have similarities to those reported by Miyamoto *et al.* (1971). These workers reported two high-molecular-weight forms (mol. wts. about 140000 and 80000), which were dependent on cyclic AMP, and a low-molecular-weight species (mol. wt. about 40000), which was not stimulated by cyclic AMP.

Dissociation of protein kinase into catalytic and regulatory subunits by low concentrations of cyclic

AMP has been reported for enzymes from many tissues (Tao *et al.*, 1970; Erlichman *et al.*, 1971; Gill & Garren, 1971; Reimann *et al.*, 1971). Similar results were obtained with lymphocyte protein kinase (see Fig. 6). In the absence of cyclic AMP the enzyme mol. wt. was about 90000, and in the presence of 1 μ M-cyclic AMP, the kinase activity was associated with a component of mol. wt. about 40000. Direct assays of cyclic AMP binding were also performed on the fractions from both gradients. In both cases the binding activity was associated with the protein kinase activity peaks. This correspondence in gradients done in the presence of cyclic AMP presumably indicates that the dissociated catalytic and regulatory subunits have similar molecular weights.

Discussion

An initial aim of the present work was to determine whether the regulation of the activities of protein kinase and histone phosphatase were correlated in any way. No evidence of a sensitive control of histone phosphatase, analogous to the regulation of protein kinase by cyclic AMP, was found. Some weak stimulatory and inhibitory effects were observed with uni- and bi-valent cations and with phosphoenolpyruvate, but at present there does not seem to be any obvious way of connecting these to a control mechanism *in vivo*.

The lymphocyte protein kinase has many features in common with protein kinases isolated from other sources. Enzyme activity is stimulated by cyclic AMP and this stimulation is almost certainly a consequence of dissociation of catalytic subunits from cyclic AMP-binding regulatory subunits. In the present studies, ATP plus MgCl₂ partially protected the enzyme from dissociation by histone, and in this respect the lymphocyte protein kinase appears to differ from the brain enzyme studied by Miyamoto *et al.* (1971). Although it is not known if the effects of histone and of ATP plus MgCl₂ are important physiologically the

Fig. 5. Dissociation of lymphocyte protein kinase by whole calf thymus histone

Sucrose gradients (5–20%) were set up as described in the Experimental section, containing 50 mM-tris-HCl buffer (pH 7.5) with and without 0.5 mg of whole thymus histone/ml or 0.5 mg of histone/ml plus 20 μ M-ATP and 10 mM-MgCl₂. These gradients were loaded with 0.45 ml of a solution (preincubated at 37°C for 10 min) containing 50 mM-tris-HCl buffer (pH 7.5), 0.2 ml of lymphocyte extract and either 0.2 mg of histone or 0.2 mg of histone plus 20 μ M-ATP and 10 mM-MgCl₂ respectively, and centrifuged at 75000g for 16 h. Fractions were collected and assayed for protein kinase activity as described in the Experimental section. (a) Control gradient; fractions were assayed in the absence (●) and presence (○) of 1 μ M-cyclic AMP. (b) The gradient contained 0.5 mg of histone/ml; fractions were assayed in the absence (●) or presence (○) of 1 μ M-cyclic AMP. (c) The gradient contained 0.5 mg of histone/ml plus 20 μ M-ATP and 10 mM-MgCl₂; fractions were assayed in the absence (●) and presence (○) of 1 μ M-cyclic AMP.

results do suggest that enzyme-substrate interactions can influence the association between catalytic and regulatory subunit(s) of the protein kinase. The alter-

Fig. 6. Dissociation of lymphocyte protein kinase by cyclic AMP

Sucrose gradients were set up as described in Fig. 5 with and without 1 μ M-cyclic AMP. Control samples of lymphocyte extract and samples containing 1 μ M-cyclic AMP were loaded and the gradients centrifuged as described in the Experimental section. The control gradient was assayed in the absence (●) and presence (○) of 1 μ M-cyclic AMP, and the gradient containing the cyclic nucleotide was assayed in the presence of 1 μ M-cyclic AMP (□). The third gradient contained yeast alcohol dehydrogenase, catalase and cytochrome *c* (see the Experimental section) and was used to provide standards for molecular-weight estimations.

native is to propose specific sites for binding substrates on the regulatory subunit(s).

It should be emphasized that there is no information at present about the natural substrate(s) of lymphocyte protein kinase(s). Histone has been used in the present experiments for reasons of convenience. Further attempts to implicate cyclic nucleotide-stimulated protein kinases as part of a 'cascade' phenomenon, after stimulation of cells at membrane receptor sites, will require identification of the protein substrate(s) of both the soluble kinase and that associated with the chromatin fraction (Cross & Ord, 1971).

This work was supported by grants from the University of Adelaide Anti-Cancer Foundation and the Australian

Research Grants Committee. We are grateful to Dr. R. W. Beal of the Red Cross Blood Bank, Adelaide, for providing facilities for blood collection.

References

- Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
- Cross, M. E. & Ord, M. G. (1971) *Biochem. J.* **124**, 241–248
- Erlichman, J., Hirsch, A. H. & Rosen, O. M. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 731–735
- Gill, G. N. & Garren, L. D. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 786–790
- Gilman, A. G. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 305–312
- Glynn, I. M. & Chappell, J. B. (1964) *Biochem. J.* **90**, 147–149
- Kleinsmith, L. J., Allfrey, V. G. & Mirsky, A. E. (1966) *Proc. Nat. Acad. Sci. U.S.* **55**, 1182–1189
- Lichtman, M. A. & Weed, R. I. (1969) *Blood* **34**, 645–660
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Meisler, M. H. & Langan, T. A. (1969) *J. Biol. Chem.* **244**, 4961–4968
- Mendelson, J., Skinner, A. & Kornfeld, S. (1971) *J. Clin. Invest.* **50**, 818–825
- Miyamoto, E., Petzold, G. L., Harris, J. S. & Greengard, P. (1971) *Biochem. Biophys. Res. Commun.* **44**, 305–312
- Murray, A. W. & Froschio, M. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1089–1095
- Panyim, S. & Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130**, 337–346
- Perper, R. J., Zee, T. W. & Mickelson, M. M. (1968) *J. Lab. Clin. Med.* **72**, 842–848
- Reimann, E. M., Brostrom, C. O., Corbin, J. D., King, C. A. & Krebs, E. G. (1971) *Biochem. Biophys. Res. Commun.* **42**, 187–194
- Smith, J. W., Steiner, A. L., Newberry, W. M. & Parker, C. W. (1971) *J. Clin. Invest.* **50**, 432–441
- Tao, M., Salas, M. L. & Lipmann, F. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 408–414