Dugaiczyk, A., & Woo, S. L. C. (1979) *Recent Prog. Horm. Res. 35*, 1–46.
Proudfoot, N. J., & Brownlee, G. G. (1976) *Nature (London) 263*, 211–214.
Reeves, R. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 709–722.
Riggs, A. D. (1975) *Cytogenet. Cell Genet. 14*, 9–25.
Roop, D. R., Tsai, M.-J., & O'Malley, B. W. (1980) *Cell (Cambridge, Mass.) 19*, 63–68.
Royal, A., Garapin, A., Cami, B., Perrin, F., Mandel, J. L., LeMeur, M., Bregegegre, F., Gannon, F., LePennec, J. P., Chambon, P., & Kourilsky, P. (1979) *Nature (London) 279*, 125–132.
Shepherd, J. H., Mulvihill, E. R., Thomas, P. S., & Palmiter, R. D. (1980) *J. Cell Biol. 87*, 142–151.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.
Stalder, J., Larsen, A., Engel, J. D., Dolan, M., Groudine, M., & Weintraub, H. (1980a) *Cell (Cambridge, Mass.) 20*, 451–460.
Stalder, J., Groudine, M., Dodgson, J. B., Engel, J. D., & Weintraub, H. (1980b) *Cell (Cambridge, Mass.) 19*, 973–980.
Storb, U., Arp, B., & Wilson, R. (1981) *Nature (London) 294*, 90–92.
Swaneck, G. E., Nordstrom, J. L., Kreuzaler, F., Tsai, M.-J., & O'Malley, B. W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1049–1053.
Tsai, M. J., Ting, A., Nordstrom, J., Zimmer, W., & O'-Malley, B. W. (1980) *Cell (Cambridge, Mass.) 22*, 219–230.
Vanderbilt, J. N., Bloom, K. S., & Anderson, J. N. (1982) *J. Biol. Chem. 257*, 13009–13017.
Weintraub, H., & Groudine, M. (1976) *Science (Washington, D.C.) 193*, 848–858.
Weintraub, H., Larsen, A., & Groudine, M. (1981) *Cell (Cambridge, Mass.) 24*, 333–344.
Wetmur, J. G., & Davidson, N. (1968) *J. Mol. Biol. 31*, 349–370.
Woo, S. L. C., Beattie, W. G., Catterall, J. F., Dugaiczyk, A., Staden, R., Brownlee, G. G., & O'Malley, B. W. (1981) *Biochemistry 20*, 6437–6446.
Wu, C. (1980) *Nature (London) 286*, 854–860.
Wu, C., & Gilbert, W. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1577–1580.
Wu, C., Wong, Y.-C., & Elgin, S. C. R. (1979) *Cell (Cambridge, Mass.) 16*, 807–814.

H1 Histone Kinases from Nuclei of *Physarum polycephalum*†

Timothy C. Chambers,*,‡ Thomas A. Langan, Harry R. Matthews,§ and E. Morton Bradbury§

ABSTRACT: Nuclear H1 histone kinase activity from *Physarum* plasmodia was separated into three major components by DEAE-cellulose chromatography. The enzyme fractions were termed kinase R, kinase A, and kinase B and were characterized by investigating (i) their dependence on cAMP, (ii) the effect of the heat-stable inhibitor of cAMP-dependent protein kinase, (iii) the sites in calf H1 histone phosphorylated by each kinase, and (iv) the location of incorporation of phosphate in *Physarum* H1 histone catalyzed by each kinase in vitro. All three kinases were unaffected by the addition of 1 μM cAMP to the assay mixture although inhibition of the activities of kinase A and kinase B was observed in the presence of the protein kinase inhibitor. Kinase A phosphorylated mainly serine-37 in calf H1, and we conclude that the enzyme is analogous to the catalytic subunit of cAMP-dependent protein kinase. Kinase B phosphorylated multiple sites mainly in the N-terminal half of calf H1 but was distinct from kinase A in its phosphorylation of *Physarum* H1. It is not known whether there exists a mammalian equivalent of kinase B. Kinase R was unaffected by the protein kinase inhibitor and phosphorylated multiple sites in both the N-terminal and C-terminal halves of calf H1. These sites included those identified as being phosphorylated by mammalian growth-associated H1 histone kinase (kinase GR), suggesting that kinase R is analogous to mammalian kinase GR. The identification of kinase GR in *Physarum* nuclei indicates an evolutionary stability of both enzyme specificity and the structures surrounding growth-associated phosphorylation sites in H1 histones. Conservation of these features suggests that the multiple phosphorylation reactions occurring on H1 histones are essential to mechanisms which modulate chromatin structure. We also report that *Physarum* H1 is digested by chymotrypsin in an analogous fashion to calf H1.

Early studies of H1[1] histone phosphorylation during the naturally synchronous cell cycle in *Physarum polycephalum* demonstrated a large increase in H1 phosphate content during the progression from late G2 phase to mitosis (Bradbury et al., 1973). Bradbury et al. proposed that this modification of H1 was involved in the initiation of chromosome condensation. Extensive phosphorylation of H1 has also been observed in artificially synchronized mammalian cell systems, including CHO (Gurley et al., 1973, 1978), rat hepatoma (Langan et al., 1980), and HeLa (Ajiro et al., 1981), suggesting that H1 phosphorylation is a universally occurring process directly involved in chromosome condensation and

† From the Biophysics Laboratories, Portsmouth Polytechnic, Portsmouth, PO1 2DT, England (T.C.C., H.R.M., and E.M.B.), and the Department of Pharmacology, University of Colorado Health Sciences Center, Denver, Colorado 80262 (T.A.L.). *Received January 7, 1982; revised manuscript received June 10, 1982.* This investigation was supported by a grant from the Science Research Council of Great Britain and by National Institutes of Health Grant CA-12877 from the National Cancer Institute.

‡ Present address: Department of Pharmacology, University of Colorado Health Sciences Center, Denver, CO 80262.

§ Present address: Department of Biological Chemistry, School of Medicine, University of California, Davis, CA 95616.

[1] Abbreviations: H1, histone 1; CHO, chinese hamster ovary; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Cl_3CCOOH, trichloroacetic acid; cAMP, adenosine cyclic 3′,5′-phosphate; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/83/0422-0030$01.50/0

perhaps other nuclear functions [for review, see Matthews (1980)].

The protein kinase responsible for this large scale phosphorylation of H1 was first described by Lake & Salzman (1972) in CHO cells and has subsequently been detected in many dividing cells (Schlepper & Knippers, 1975; Langan, 1978b). Termed the growth-associated H1 histone kinase (kinase GR), the enzyme in all systems studied has been found to be cAMP independent, bound to chromatin, elevated in specific activity from interphase to mitosis, and specific for H1, phosphorylating the same sites in vitro and in vivo (Lake, 1973; Langan, 1978a). In *Physarum*, a 15-fold increase in nuclear histone kinase activity from S phase to mitosis has been observed (Bradbury et al., 1974). The increase is mainly due to activation or transport of the kinase and not to its synthesis (Mitchelson et al., 1978), and in Novikoff hepatoma cells there is evidence for activation of kinase GR (Zeilig & Langan, 1980).

Physarum macroplasmodia have a naturally synchronous cell cycle which gives them a unique advantage for cell cycle studies, particularly in G2 phase (Dove & Rusch, 1980; Aldrich & Daniel, 1982). We report here a study of three H1 histone kinase fractions isolated from *Physarum* nuclei. The findings indicate that in addition to a kinase with the characteristics of a catalytic subunit of cAMP-dependent protein kinase and a kinase with properties somewhat different from those of previously reported histone kinases, *Physarum* nuclei contain a kinase with properties analogous to those of mammalian growth-associated H1 histone kinase.

Experimental Procedures

DEAE-cellulose (DE-52) was obtained from Whatman Biochemicals and adenosine 5′-[γ-^{32}P]triphosphate from the Radiochemical Centre.

Cell Culture, Isolation of Nuclei, and Extraction and Fractionation of Nuclear Histone Kinases. All operations subsequent to cell harvesting were performed at 4 °C. *Physarum* microplasmodia (strain axi) were maintained in submerged shaken cultures (Daniel & Baldwin, 1964) and nuclei isolated from 5 L of exponentially growing culture by the method of Mohberg & Rusch (1971), with Mg^{2+} substituting for Ca^{2+} as described previously (Mitchelson et al., 1978). The nuclear pellet was suspended in 10 mM Tris-HCl, pH 7.2, 0.5 M NaCl, 1 mM EGTA, and 1 mM DTT and sonicated at an amplitude of 16 μm in 6 × 5-s bursts at 1-min intervals. The sonicated suspension was then centrifuged at 200000*g* for 3 h to remove nuclear debris. The supernatant (later termed the 0.5 M NaCl extract) was brought to 20% saturation with ammonium sulfate and the precipitate removed by centrifugation at 38000*g* for 30 min. The ammonium sulfate concentration of the supernatant was then raised to 65% saturation and after stirring for 30 min the precipitate collected by centrifugation as above. The precipitate was dissolved in 3 mL of dialysis buffer, 50 mM Tris-HCl, pH 7.5, 25% glycerol, and 1 mM DTT, and dialyzed against 200 volumes of the same buffer overnight. The inactive precipitate which formed during dialysis was removed by centrifugation at 38000*g* for 1 h and the supernatant applied to a 0.9 × 14 cm column of DEAE-cellulose equilibrated with dialysis buffer. Elution with dialysis buffer at 1.4 mL/h was continued until the absorbance at 280 nm of the effluent had returned to the background level, and then a linear gradient of 0–0.25 M NaCl, total volume of 50 mL in dialysis buffer, was applied. Aliquots of fractions were assayed for histone kinase activity as described below.

Histone Kinase Assays. Histone kinase activities were determined by using the phosphocellulose paper binding method (Witt & Roskoski, 1975). Incubation mixtures of 125 μL contained 80 mM Tris-HCl, pH 7.5, 16 mM $MgCl_2$, 125 μg of calf thymus H1, 1 mM [γ-^{32}P]ATP (specific activity 20–50 Ci/mol), and 5–50 μL of enzyme. After 1 h at 30 °C, reactions were terminated by the addition of 14 μL of 50% w/v Cl_3CCOOH in 5 mM sodium pyrophosphate, and enzyme protein was sedimented by centrifugation at 10000*g* for 5 min. Aliquots (125 μL) of supernatants were spotted onto 4 × 3 cm strips of Whatman P81 phosphocellulose paper. The strips were washed 5 times in 5 mM sodium pyrophosphate, twice in water, and twice in acetone, dried, and counted in 5 mL of scintillation fluid in a liquid scintillation spectrometer. Activity is expressed in units/mL where 1 unit is the amount of enzyme which catalyzes the transfer of 1 pmol of phosphate into 125 μg of H1 in 30 min. Blank values were obtained from the sum of the values of two control reactions for each sample, one excluding histone and one excluding enzyme protein.

Preparation of Physarum H1. Whole histone was prepared from *Physarum* microplasmodia by the method of Mohberg & Rusch (1969) and fractionated by gel filtration on a 3 × 140 cm column of Bio-Gel P60 (Corbett et al., 1977). The *Physarum* H1 which eluted in the void volume was further purified by Bio-Rex 70 ion-exchange chromatography using the gradient method developed by Kinkade & Cole (1966).

In Vitro Phosphorylation of H1. Phosphorylation and isolation from reaction mixtures of calf thymus H1 or *Physarum* H1 was carried out as previously described (Langan, 1978b), using [γ-^{32}P]ATP of specific activity 50–120 Ci/mol and 1 mg of H1 per reaction. The level of incorporation of phosphate ranged from 0.5 to 7.0 nmol/mg of H1, equivalent approximately to 0.01–0.14 mol of phosphate/mol of H1. Thus, for the duration of each phosphorylation reaction, potential phosphorylation sites were always present in excess. Such mildly phosphorylated preparations were therefore suitable for comparing different phosphorylation sites and reflected the rate of phosphorylation of different sites in the H1 histone by the kinases utilized. *Physarum* H1 was dephosphorylated with alkaline phosphatase (Balhorn et al., 1971; Corbett et al., 1977), with subsequent separation of the phosphatase and H1 by Cl_3CCOOH precipitation, before being used for in vitro phosphorylation. Aliquots of peak fractions of *Physarum* H1 histone kinases, after separation on DEAE-cellulose, were used as a source of enyzme in phosphorylation reactions. Samples of calf thymus H1 phosphorylated by characterized mammalian H1 histone kinases were also prepared, as described previously (Langan, 1978b), and used as markers for known phosphorylation sites during phosphopeptide analysis.

Chymotryptic Digestion of H1. Calf thymus H1 or *Physarum* H1, either phosphorylated or unphosphorylated, was dissolved in 50 mM Tris-HCl, pH 8, at 1 mg/mL and digested with 2.2 μg of chymotrypsin/mg of H1 at 26 °C. Aliquots were terminated by acidification at appropriate intervals and electrophoresed on acid–urea gels.

Gel Electrophoresis. H1 chymotryptic fragments were analyzed by acid–urea–15% acrylamide gels according to the method of Panyim & Chalkley (1969). Gels were cast in 1.5 mm thick slabs or 0.7 × 9 cm tubes. For ^{32}P location, slab gels were autoradiographed by exposure to X-ray film at −20 °C, and bands from tube gels were cut out, dissolved in 30% H_2O_2, and counted in scintillation fluid.

Preparation of cAMP-Dependent Protein Kinase Inhibitor. The heat-stable inhibitor of cAMP-dependent protein kinase was prepared from a homogenate of rabbit skeletal muscle by heat treatment at 95 °C, DEAE-cellulose chromatography,

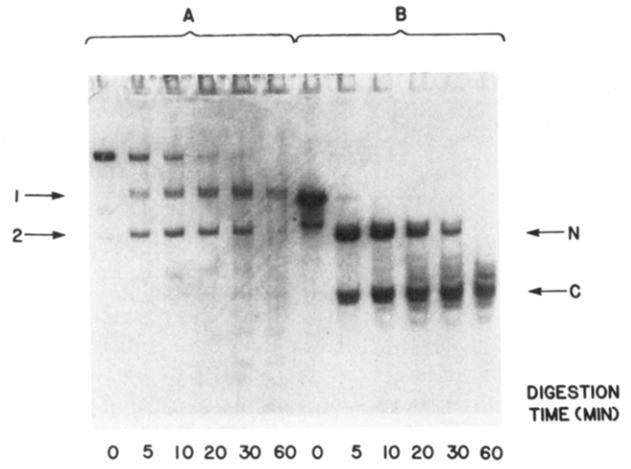

FIGURE 1: Comparative time-course chymotryptic digestion of *Physarum* H1 and calf thymus H1. The products of the digestions of *Physarum* H1 (A) and calf thymus H1 (B), removed at the times indicated, were analyzed by acid–urea gel electrophoresis. Conditions of digestion and electrophoresis were described under Experimental Procedures. Shown by arrows are the migration positions of fragment 1 (1) and fragment 2 (2) of *Physarum* H1 and the N-terminal fragment (N) and C-terminal fragment (C) of calf thymus H1.

and gel filtration on Sephadex G-100, as described previously (Cohen et al., 1977).

Determination of Carboxyl-Terminal Residues. For determination of carboxyl-terminal amino acid residues in *Physarum* H1 and its chymotryptic cleavage products, samples (0.15–0.60 mg) were dissolved in 0.3 mL of 1% $NaHCO_3$ and treated at 35 °C with a combination of carboxypeptidase A and carboxypeptidase B (Sigma; diisopropylphosphofluoridate treated). The substrate to enzyme ratios were 100:1. At intervals up to 1 h, aliquots were removed, and analysis was performed with a Rank-Hilger automatic amino acid analyzer.

Results

Physarum H1 Histone. Physarum H1 was isolated from microplasmodia as described under Experimental Procedures. The amino acid composition was determined and found to agree with previous determinations (Mohberg & Rusch, 1969; Tyrsin et al., 1977; Corbett, 1979; Chambers, 1980; Fischer & Laemmli, 1980; Matthews & Bradbury, 1982).

Chymotryptic digestion of calf thymus H1 results in initial cleavage at the peptide bond C terminal to phenylalanine-106, in the globular domain of the molecule. The resultant C-terminal fragment (residues 107–212) is very basic and migrates with a mobility much greater than that of the N-terminal fragment during electrophoresis in acid–urea–polyacrylamide gels (Bradbury et al., 1975). Figure 1 shows analysis on such a gel of the products of chymotryptic digestions of H1 histone from *Physarum* and calf thymus. The calf thymus H1 used in this particular experiment was contaminated slightly with histone H2B, but this did not affect the electrophoretic pattern of peptides derived from H1. *Physarum* H1 is also cleaved initially into two fragments, one of which, designated fragment 2, migrates with a very similar mobility to that of the N-terminal fragment of calf thymus H1. A preparative scale chymotryptic digestion of histone H1 from *Physarum* was carried out under conditions chosen to optimize the yield of the two major fragments. The products were separated by gel filtration on Sephadex G-100 and shown to be reasonably pure by gel electrophoresis (Figure 2). *Physarum* H1 and the separated fragments were each digested with carboxypeptidases A and B as described under Experimental Procedures. Lysine and alanine were released from

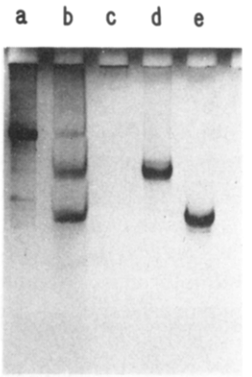

FIGURE 2: Preparation of *Physarum* H1 chymotryptic fragments. *Physarum* H1, 2.5 mg in 1 mL of 50 mM Tris-HCl (pH 8), was cleaved by incubation with 5 μg of chymotrypsin for 10 min at 26 °C and the digest applied to a 0.6 × 150 cm column of Sephadex G-100 and eluted with 0.02 N HCl. Three peaks, detected by absorbance at 218 nm, were eluted. Peptides were recovered from pooled fractions by dialysis against water and lyophilization and analyzed by acid–urea gel electrophoresis. (a) *Physarum* H1; (b) *Physarum* H1 after chymotryptic cleavage; (c) void volume peak from G-100 column; (d) second peak from G-100 column (fragment 1); (e) third peak from G-100 column (fragment 2).

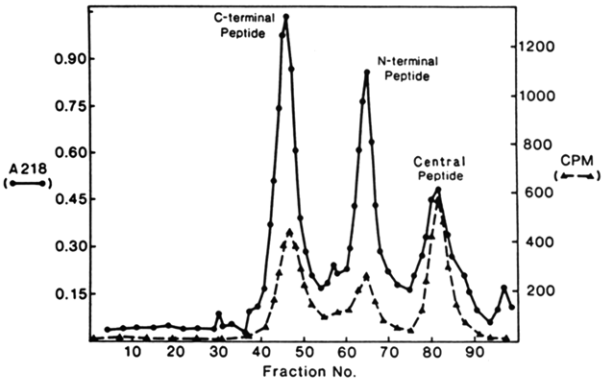

FIGURE 3: Sephadex G-100 chromatography of chymotryptic peptides derived from calf thymus H1 phosphorylated by *Physarum* histone kinases. A 0.5 M NaCl extract of *Physarum* nuclei was used to phosphorylate 0.5 mg of calf thymus H1. The isolated H1, 3.6 × 10^4 cpm, was added to 3 mg of unphosphorylated carrier H1 and digested with 5 μg of chymotrypsin for 1 h at 37 °C. Products were separated by gel filtration in a 0.9 × 200 cm column of Sephadex G-100 eluted with 0.02 N HCl at a flow rate of 2.8 mL/h. Fractions of 1.4 mL were collected, and radioactivity in 0.25-mL aliquots was determined. In order of elution, the three peptides are residues 107–212, residues 1–~60, and residues ~61–106 (Bustin & Cole, 1970; Langan, 1978a). Peak tubes were pooled, and HCl was removed by repeated rotary evaporation.

both *Physarum* H1 and fragment 1, indicating that fragment 1 is the C-terminal fragment. Phenylalanine, glycine, and an additional amino acid, probably serine or threonine, were released from fragment 2. The time course of release of these amino acids was determined, but their order in the sequence could not be deduced with certainty.

Phosphopeptide Analysis of Calf Thymus H1 Phosphorylated by Physarum Nuclear Histone Kinases. Calf thymus H1 was phosphorylated by using unfractionated *Physarum* nuclear histone kinases (0.5 M NaCl extract of nuclei) and [γ-^{32}P]ATP. Phosphate incorporation was 2 nmol/mg of H1. H1 was isolated from the reaction mixture, diluted with unlabeled carrier H1, and digested with chymotrypsin to an extent sufficient to generate three fragments: residues 1–~60, residues ~61–106, and residues 107–212 (Bustin & Cole, 1970; Langan, 1978a). The fragments were separated by gel filtration on Sephadex G-100 and [^{32}P]phosphate was found in each fragment (Figure 3). The N-terminal fragment was

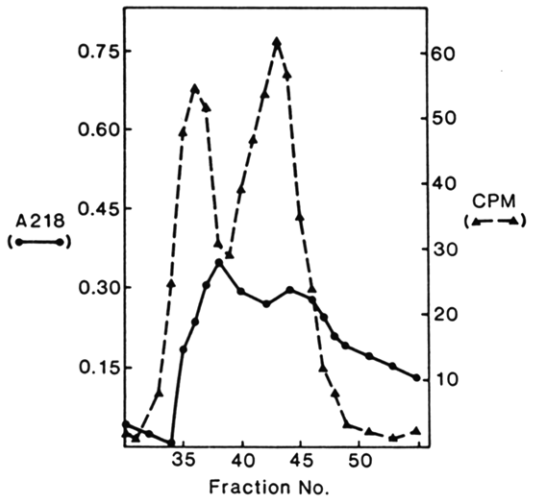

FIGURE 4: Sephadex G-50 chromatography of a tryptic digest of the N-terminal peptide from calf thymus H1 phosphorylated by *Physarum* histone kinases. The N-terminal peptide (second peak in Figure 3) was dissolved in 0.5 mL of 0.1 M NH_4HCO_3 (pH 6.5) and digested with 25 μg of trypsin for 1 h at 37 °C. The digest was applied to a 0.9 × 150 cm column of Sephadex G-50 and eluted with 0.02 N HCl at a flow rate of 7.5 mL/h. Fractions of 1.87 mL were collected, and radioactivity in 0.25-mL aliquots was determined.

digested with trypsin, and the peptides were fractionated by Sephadex G-50 gel filtration as shown in Figure 4. Figure 4 shows radioactivity in a peak which elutes earlier than the unlabeled carrier peptides. This phenomenon has been well characterized and is indicative of phosphorylation of threonine-16 since the presence of a phosphate group at this position inhibits trypsin cleavage at lysine-15, thus generating a larger peptide (Langan, 1978a). Threonine-16 has been recognized as the major N-terminal growth-associated phosphorylation site in mammalian H1 histones (Langan, 1978a). ^{32}P was also observed in a second peak at a position where several smaller peptides, including the peptide containing serine-37, elute. Serine-37 is the site of phosphorylation in H1 catalyzed by cAMP-dependent protein kinase (Langan, 1971). The C-terminal fragment from Figure 3 was also digested with trypsin, and the peptides were purified by high-voltage paper electrophoresis at pH 7.9. The major radioactive band was eluted and further separated by high-voltage paper electrophoresis at pH 1.9 into three bands which coelectrophoresed with marker H1 peptides containing the three major C-terminal growth-associated phosphorylation sites, threonine-136, threonine-153, and serine-180 (Langan, 1978a). Figure 5 shows the autoradiograph of the electrophoretic separation at low pH. The relative proportion of label in the three phosphopeptides was similar when Ehrlich ascites kinase GR or unfractionated *Physarum* kinases were used.

Fractionation of Physarum Nuclear Histone Kinases. Figure 6 shows the elution profile of *Physarum* nuclear H1 histone kinase activity from DEAE-cellulose. Three major peaks, eluting as an unbound run-through component, termed kinase R, and two components, termed kinase A and kinase B eluting at 0.05 and 0.14 M NaCl, respectively, were routinely observed, in agreement with the results of a previous study (Hardie et al., 1976). The greater complexities of the chromatogram, evidenced in Figure 6 by a trailing shoulder in kinase R, a peak of activity preceding kinase A, and a leading shoulder in kinase B, were not reproducibly obtained, and no attempt was made to further increase resolution on the ion exchanger. The kinases were rather unstable, particularly in buffers of low ionic strength, and attempts at recovering the activities from column fractions by ammonium sulfate

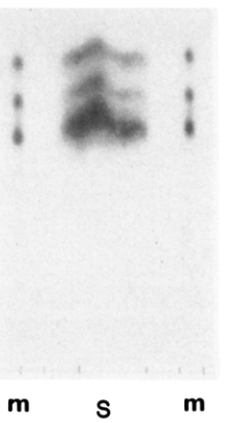

FIGURE 5: Paper electrophoretic separation of tryptic phosphopeptides derived from the C-terminal fragment of calf thymus H1 phosphorylated by *Physarum* histone kinases. The C-terminal fragment (first peak in Figure 3) was dissolved in 0.15 mL of 0.067 M NH_4HCO_3 (pH 8) and digested with 25 μg of trypsin for 1 h at 37 °C. The resultant peptides, after initial purification by paper electrophoresis at pH 7.9, were subjected to paper electrophoresis at pH 1.9 under conditions previously described (Langan, 1978a). Sample peptides (S) were applied to an origin in the center and were coelectrophoresed with marker peptides (m) derived from the C-terminal fragment of calf thymus H1 which had been phosphorylated by Ehrlich ascites kinase GR. Migration was from the anode (bottom) to the cathode (top), and phosphopeptides were located by autoradiography. The phosphorylation site in the three phosphopeptides is threonine-153 (upper band), serine-180 (center band), and threonine-136 (lower band).

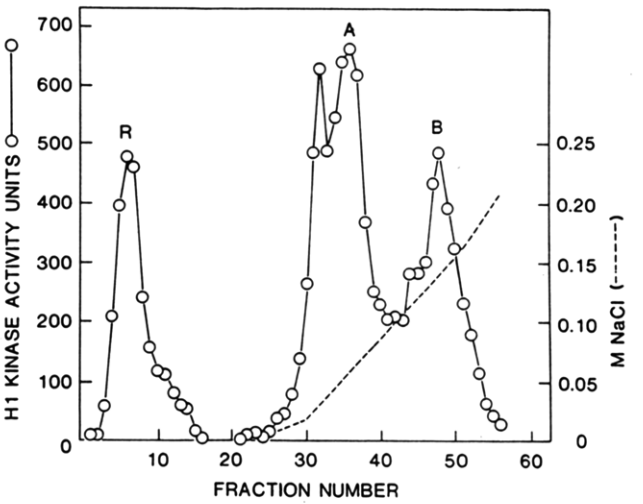

FIGURE 6: DEAE-cellulose chromatography of *Physarum* nuclear histone kinases. Chromatographic conditions were described under Experimental Procedures. Fractions of 1 mL were collected, and histone kinase activity in 50-μL aliquots was determined.

precipitation resulted in poor yields. Aliquots of the peak fraction of each of kinase R, kinase A, and kinase B were utilized in the following experiments in order to avoid this problem and, more importantly, to minimize the possibility of cross-contamination. Thus the three kinase preparations, although completely separated from each other, were not necessarily homogeneous in terms of possible additional H1 kinase content.

Effect of cAMP and Protein Kinase Inhibitor. Cyclic AMP at a concentration of 1 μM had no major effect on the activity of any of the three *Physarum* kinase preparations when assayed with calf thymus H1 as substrate. This was not due to the degradation of cAMP during the incubation [M. Bertulis, unpublished results, quoted in Matthews (1980)]. The heat-stable inhibitor of cAMP-dependent protein kinase, however, was effective in inhibiting the activities of both kinase

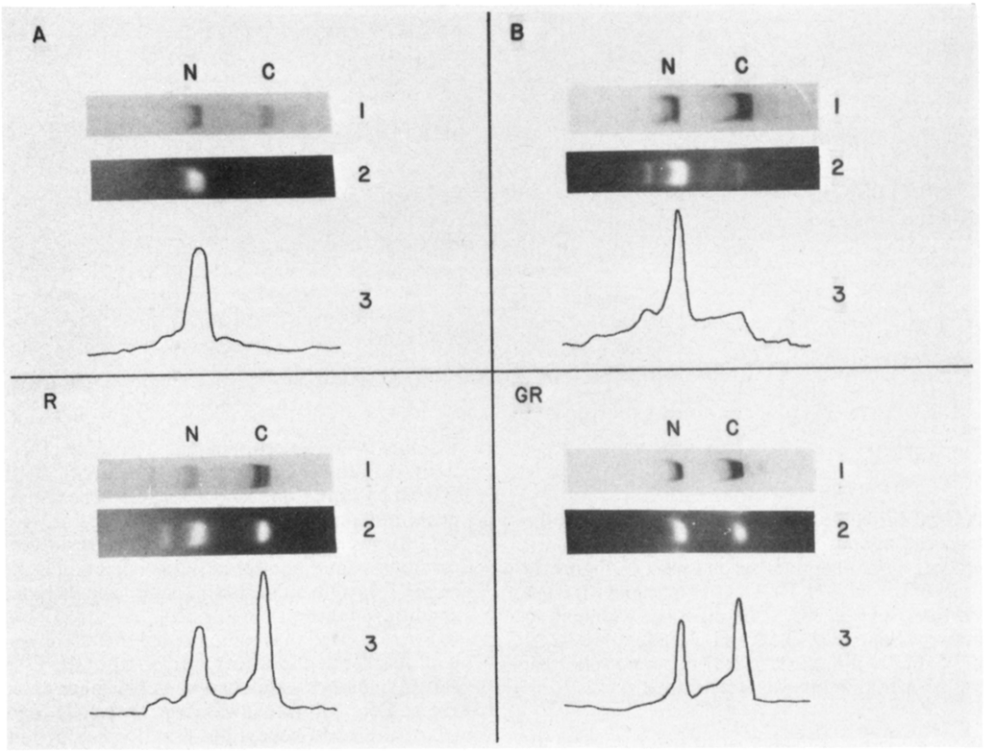

FIGURE 7: Gel electrophoresis of the N-terminal and C-terminal fragments of phosphorylated calf thymus H1. Preparations of calf thymus H1, phosphorylated with [γ-^{32}P]ATP and either *Physarum* kinase A (panel A), *Physarum* kinase B (panel B), *Physarum* kinase R (panel R), or Ehrlich ascites kinase GR (panel GR), were cleaved at phenylalanine-106 with chymotrypsin and the N-terminal (N) and C-terminal (C) fragments separated by acid–urea gel electrophoresis. Migration was from left to right. Gels were analyzed by (1) amido black stain, (2) ^{32}P autoradiography, and (3) microdensitometer scan of autoradiographic film.

Table I: Effect of the Heat-Stable Inhibitor of cAMP-Dependent Protein Kinase on the Activities of *Physarum* Nuclear Histone Kinases

	^{32}P incorporation[a] (cpm)			kinase activity (units/mL)	
histone kinase	−inhibitor[c]	+inhibitor[c]	blank[b] (cpm)	−inhibitor[c]	+inhibitor[c]
kinase R	17429	15623	5243	1151	980
kinase A	8151	4160	4166	381	0
kinase B	4277	2437	2389	181	4

[a] ^{32}P incorporation determined under standard assay conditions with calf thymus H1 as substrate. [b] Blank values were obtained from assay mixtures incubated in the absence of both H1 and inhibitor. [c] The quantity of inhibitor added was sufficient to inhibit 50% of the activity of 0.28 unit (Cohen et al., 1977) of mammalian cAMP-dependent protein kinase, equivalent to 8400 units of *Physarum* kinase activity. Reaction mixtures of kinase R, kinase A, and kinase B contained 57, 19, and 9 units of activity, respectively.

A and kinase B but did not significantly affect kinase R (Table I).

Calf Thymus H1 Phosphorylated by Physarum H1 Histone Kinases. Calf thymus H1 was phosphorylated with [γ-^{32}P]-ATP and either kinase A, kinase B, kinase R, or Ehrlich ascites kinase GR. The levels of phosphate incorporation, per milligram of H1, were 4.2, 1.5, 7.5, and 1.8 nmol, respectively. The phosphorylated H1 molecules were reisolated and digested with chymotrypsin under conditions where the molecule was cut once, at phenylalanine-106, as already shown in Figure 1. The N-terminal and C-terminal fragments were separated by acid–urea–polyacrylamide gel electrophoresis, and the gels were stained and photographed (Figure 7). It is apparent that each stained C-terminal fragment migrates as a double band. This effect has been observed previously and is presumed to be due to heterogeneity in this region of H1 (Sherod et al., 1974; Bradbury et al., 1975; Cole, 1977). Phosphorylation was detected by autoradiography, and Figure 7 shows that *Physarum* kinase R phosphorylated residues in both the N-terminal and C-terminal fragments like Ehrlich ascites kinase GR, while *Physarum* kinase A only phosphorylated residues in the N-terminal fragment and *Physarum* kinase B mainly phosphorylated residues in the N-terminal fragment. The autoradiographs were scanned with a microdensitometer, and the scans, also shown in Figure 7, confirm the similar pattern of phosphorylation by *Physarum* kinase R and Ehrlich ascites kinase GR.

Phosphopeptide analysis of a preparative quantity of calf thymus H1 phosphorylated by *Physarum* kinase A was carried out in order to identify the N-terminal phosphorylation site(s). Phosphate incorporation was 1.1 nmol/mg of H1. The N-terminal peptide (residues 1–~60), obtained by chymotryptic digestion of the phosphorylated H1 and Sephadex G-100 gel filtration, was digested with trypsin, and peptides were analyzed by Sephadex G-50 gel filtration and pH 7.9 high-voltage paper electrophoresis. In both systems, the major radioactive component had the properties of the tryptic peptide of H1 containing serine-37. However, certain N-terminal tryptic peptides of H1 which contain uncharacterized phosphorylation sites also elute from Sephadex G-50 and migrate at pH 7.9 during paper electrophoresis in a similar position to the peptide containing serine-37. For definitive analysis, the peptide derived from calf thymus H1 phosphorylated by kinase A was subjected to cellulose thin-layer chromatography under previously described conditions (Langan, 1978a). This demonstrated the presence of a single phosphopeptide with a mobility which closely paralleled that of a marker phosphopeptide derived from H1 phosphorylated at serine-37 by mammalian cAMP-dependent protein kinase, and this was clearly distinct from the mobilities of peptides containing growth-associated phosphorylation sites.

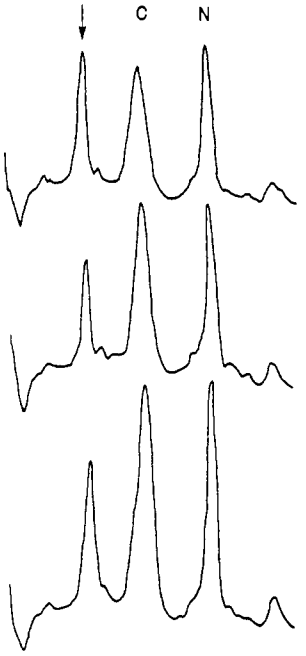

FIGURE 8: Gel electrophoresis of partial chymotryptic digestion of phosphorylated *Physarum* H1. Preparations of *Physarum* H1 phosphorylated by kinase R (top scan), kinase A (center scan), and kinase B (lower scan) were each digested with chymotrypsin and the products separated by acid–urea gel electrophoresis in tubes. Gels were scanned with a Joyce-Loebl Polyfrac gel scanner. Migration was from left to right with the position of intact *Physarum* H1 arrowed. Bands corresponding to the C-terminal fragment (C) and N-terminal fragment (N) were cut out for ^{32}P determination (see Table II) as described under Experimental Procedures.

Physarum H1 Phosphorylation. *Physarum* H1 was dephosphorylated with alkaline phosphatase and then phosphorylated with [γ-^{32}P]ATP and either kinase R, kinase A, or kinase B, as described under Experimental Procedures. Each incubation contained a predetermined amount of kinase activity toward calf thymus H1. *Physarum* H1 was isolated from each reaction mixture and dissolved in 50 mM Tris-HCl, pH 8, and an aliquot was counted in scintillation fluid to determine ^{32}P incorporation. This demonstrated that kinase R phosphorylated *Physarum* H1 to the same extent as calf thymus H1 but kinase A and kinase B phosphorylated *Physarum* H1 to a lesser extent. Each phosphorylated H1 was digested with chymotrypsin, and the products were separated by electrophoresis in acid–urea–polyacrylamide tube gels. Microdensitometer scans of the stained gels, shown in Figure 8, indicated that the conditions of chymotryptic digestion were such that the major products were undigested H1, the C-terminal fragment, and the N-terminal fragment, with some additional product formation. Since the additional products were present in minor proportion, the assumption could be made that in each gel the C-terminal and N-terminal fragments were present in approximately equimolar quantities. This assumption was tested by measuring the areas beneath the scans, and it was shown that the relative proportion of the two fragments was very similar for each gel. The bands from each gel corresponding to the N-terminal and C-terminal fragments were cut out and counted. Table II gives the ^{32}P radioactivity found in each fragment band and compares it with the equivalent data for calf thymus H1 derived from Figure 7. It is apparent that kinase A and kinase B show a reduced phosphorylation of *Physarum* H1 in the N-terminal region and an increased phosphorylation in the C-terminal region compared with calf thymus H1 but kinase R shows little difference toward *Physarum* and calf thymus H1 molecules.

Table II: Distribution of Phosphate between the Two Major Chymotryptic Fragments of *Physarum* H1 and Calf Thymus H1 after Phosphorylation with *Physarum* Nuclear Histone Kinases

	phosphate distribution			
	Physarum H1 fragments[a]		calf thymus H1 fragments[b]	
histone kinase	N-terminal [cpm (%)]	C-terminal [cpm (%)]	N-terminal (%)	C-terminal (%)
kinase R	197 (43)	266 (57)	41	59
kinase A	51 (53)	46 (47)	93	7
kinase B	55 (39)	87 (61)	80	20

[a] *Physarum* data obtained by counting fragment bands from tube gels shown in Figure 8. Indicated in parentheses are the values expressed as percentages. [b] Calf thymus data obtained by estimating the area beneath the microdensitometer peaks shown in Figure 7.

Discussion

Physarum H1 Histone. *Physarum* histones were first characterized by Mohberg & Rusch (1969, 1970), who reported that *Physarum* has an H1-like histone with a lower mobility on acid–urea gel electrophoresis and a lower lysine content than calf thymus H1. However, *Physarum* H1, like calf thymus H1, is soluble in 5% perchloric acid, is eluted from nuclei at lower ionic strength than the other histones, and has a high proline and alanine content. Subsequent reports have confirmed these results (Bradbury et al., 1973; Jockusch & Walker, 1974; Tyrsin et al., 1977; Corbett et al., 1977; Johnson et al., 1978; Fischer & Laemmli, 1980; Matthews & Bradbury, 1982) and data from electrophoresis in $NaDodSO_4$ gels give an approximate molecular weight of 25 000 (Jockusch & Walker, 1974; Tyrsin et al., 1977; Fischer & Laemmli, 1980; Matthews & Bradbury, 1982), significantly higher than the value of 21 000 reported for calf thymus H1 (Teller et al., 1965; Panyim & Chalkley, 1971). *Physarum* H1 behaves like calf thymus H1 on columns of Bio-Rex 70 (Corbett et al., 1977), on columns on DNA–cellulose (Fischer & Laemmli, 1980), and in its ability to aggregate with DNA (Corbett et al., 1980). We have now begun to show that *Physarum* H1 has some similarities of sequence and structure with calf thymus H1. The data of Figure 1, together with recent evidence demonstrating comigration on $NaDodSO_4$ gels and a close similarity in amino acid composition (L. Mende, J. H. Waterborg, R. Mueller, and H. R. Matthews, unpublished observations), suggest that the N-terminal chymotryptic fragments are of similar size and charge and that the phenylalanine (residue 106 in calf H1) is in a similar chymotrypsin-sensitive environment, in both histones. The C-terminal chymotryptic fragment shows heterogeneity, evidenced by broad bands, in both histones (see Figures 1, 7, and 8). However, the C-terminal fragment from *Physarum* H1 migrates much more slowly on acid–urea gels than the analogous fragment from calf thymus H1. *Physarum* H1 has a higher molecular weight and lower lysine content compared to calf thymus H1, and since the N-terminal fragments of both histones are of very similar size and charge, the data indicate that *Physarum* H1 has a C-terminal fragment which is both larger and of lower charge than that of calf thymus H1. In both histones, the N-terminal chymotryptic fragment is subject to further digestion by chymotrypsin while the C-terminal fragment is relatively stable. This indicates that large hydrophobic residues are absent from the C-terminal region. More detailed sequence studies on *Physarum* H1 are in progress.

Physarum Nuclear H1 Histone Kinases. When *Physarum* nuclear extracts were chromatographed on DEAE-cellulose

and the fractions assayed for H1 histone kinase activity, an unbound run-through component, kinase R, and two major bound components, kinase A and kinase B, were eluted reproducibly. This is in agreement with the findings of Hardie et al. (1976) although in the original report kinase R was not characterized. In the present study, additional complexity was observed in a nonreproducible fashion and, when present, was evident as a peak of activity eluting ahead of kinase A and a leading shoulder in kinase B. The variation in activity pattern profile may arise from changes in proportions of, for example, isozymes of a limited number of kinases or may represent nonreproducible resolution of additional, catalytically unique H1 histone kinases. Attempts at fractionating the two major kinases which bind to DEAE-cellulose into additional components by a number of techniques, including gel filtration, isoelectric focusing, and density gradient centrifugation, have not, however, led to further separation (Hardie et al., 1976; T. C. Chambers, unpublished observations). On the basis of the assignments made in Figure 6, experiments were undertaken to investigate the properties of the H1 histone kinase activity present in each of the preparations labeled kinase R, kinase A, and kinase B.

Physarum kinase A elutes from DEAE-cellulose at 0.05 M NaCl, is not affected by cAMP in the assay medium, and is inhibited by the heat-stable inhibitor of mammalian cAMP-dependent protein kinase. In addition, the tryptic phosphopeptide derived from calf thymus H1 phosphorylated by kinase A has gel filtration, electrophoretic, and chromatographic properties which correspond closely to the properties of the phosphopeptide derived from H1 histone phosphorylated by cAMP-dependent protein kinase. These observations strongly suggest that kinase A is a catalytic subunit of a cAMP-dependent protein kinase. *Physarum* cytoplasm contains histone kinase activity that is stimulated 8-fold by cAMP [P. Campbell, quoted in Matthews (1976)], suggesting that *Physarum* has cAMP-dependent protein kinase holoenzyme in the cytoplasm but only free catalytic subunit in the nucleus. *Physarum* kinase A in nuclei shows a large increase in activity in G2 phase (Bradbury et al., 1974; Hardie et al., 1976) which could be due to transport of catalytic subunit from cytoplasm to nucleus (Castagna et al., 1975), although this has not been demonstrated in *Physarum*.

Physarum kinase B elutes from DEAE-cellulose at 0.14 M NaCl, is not affected by cAMP in the assay medium, and is inhibited by the heat-stable inhibitor of mammalian cAMP-dependent protein kinase. Hardie et al. (1976) showed that kinase B is specific for the phosphorylation of histone H1, unlike kinase A which also phosphorylated histone H2B and protamine. In this report we have shown that *Physarum* kinase B phosphorylates calf thymus H1 mainly at a site or sites between the N terminus and phenylalanine-106. An unfractionated *Physarum* kinase preparation, in addition to phosphorylating H1 at sites in the N-terminal and C-terminal regions, phosphorylated the region between residues ~61 and 106, and this may have been due to kinase B activity. A mammalian histone kinase, HK2, has been isolated and shown to phosphorylate serine-105 in calf thymus H1 (Langan, 1978b). However, unlike *Physarum* kinase B, HK2 is not inhibited by the inhibitor of cAMP-dependent protein kinase (J. Vandepeute and T. A. Langan, unpublished observations). It remains to be established whether a relationship exists between kinase B and any mammalian histone kinase.

Physarum kinase R does not bind to DEAE-cellulose and is affected neither by cAMP nor by the inhibitor of cAMP-dependent protein kinase. Kinase R phosphorylates calf thymus H1 in both the N-terminal and C-terminal regions with a pattern of phosphate distribution which corresponds closely to that generated when calf thymus H1 is phosphorylated by Ehrlich ascites kinase GR. *Physarum* kinases A and B do not phosphorylate the C-terminal region of calf thymus H1 to a great extent, suggesting that the C-terminal phosphorylation catalyzed by unfractionated *Physarum* kinases is due to kinase R. These sites include those identified as being phosphorylated by mammalian growth-associated H1 kinase, or kinase GR (Langan, 1978a), which has been shown to catalyze the phosphorylation of H1 histone in mitotic cells (Lake, 1973).

Our studies have demonstrated the presence in *Physarum* nuclei of at least three enzymes which phosphorylate H1 in vitro and exhibit distinct properties as protein kinases. Kinase A has maximum activity in late G2 phase (Hardie et al., 1976) and is closely related to the catalytic subunit of mammalian cAMP-dependent protein kinase. However, when we consider the generalized function of this protein kinase in mammalian cells together with the fact that detectable phosphorylation of H1 at the cAMP-dependent site (serine-37) does not occur at mitosis in mammalian cells (T. A. Langan, unpublished observations), it would seem unlikely that kinase A is involved in the phosphorylation of *Physarum* H1 during mitosis. Kinase B also peaks in activity in late G2 phase and is specific for the phosphorylation of H1 in vitro (Hardie et al., 1976), but additional experiments are required to ascertain whether this enzyme phosphorylates H1 in vivo.

Since the mammalian enzyme responsible for the extensive phosphorylation of H1 which occurs in growing cells and kinase R of *Physarum* both phosphorylate the same sites in calf thymus H1, it appears most likely that kinase R catalyzes the phosphorylation of *Physarum* H1 in vivo. The phosphorylation of the same sites in calf H1 by the *Physarum* and mammalian enzymes indicates a high degree of evolutionary conservation of both enzyme specificity and the structures surrounding phosphorylation sites in H1 histones. Conservation of these features suggests that the multiple phosphorylation reactions which take place on H1 histones are essential to mechanisms universally present in cells which are involved in the regulation of chromatin structure.

Acknowledgments

We thank Sharon Miller and Roberta Hall for their excellent technical assistance, Dr. P. G. Hartman for supplying calf thymus H1 and Professor P. Cohen, the University of Dundee, for providing the inhibitor of cAMP-dependent protein kinase.

Registry No. H1 histone kinase, 70431-11-7.

References

Ajiro, K., Borun, T. W., & Cohen, L. H. (1981) *Biochemistry 20*, 1445–1454.

Aldrich, H., & Daniel, J. W. (1982) *The Cell Biology of Physarum and Didymium*, Academic Press, New York.

Balhorn, R., Riecke, O., & Chalkley, R. (1971) *Biochemistry 10*, 3952–3959.

Bradbury, E. M., Inglis, R. J., Matthews, H. R., & Sarner, N. (1973) *Eur. J. Biochem. 33*, 131–139.

Bradbury, E. M., Inglis, R. J., & Matthews, H. R. (1974) *Nature (London) 247*, 257–261.

Bradbury, E. M., Chapman, G. E., Danby, S. E., Hartman, P. G., & Riches, P. L. (1975) *Eur. J. Biochem. 57*, 521–528.

Bustin, M., & Cole, R. D. (1970) *J. Biol. Chem. 245*, 1458–1466.

Castagna, M., Palmer, W. K., & Walsh, D. A. (1975) *Eur. J. Biochem. 55*, 193–199.

Chambers, T. C. (1980) Ph.D. Thesis, CNAA Portsmouth, England.
Cohen, P., Nimmo, G. A., & Antoniw, J. F. (1977) *Biochem. J. 162*, 435–444.
Cole, R. D. (1977) in *The Molecular Biology of the Mammalian Genetic Apparatus* (Ts'o, P., Ed.) Elsevier/North-Holland Biomedical Press, New York.
Corbett, S. (1979) Ph.D. Thesis, Portsmouth Polytechnic.
Corbett, S., Miller, S., Robinson, V. J., Matthews, H. R., & Bradbury, E. M. (1977) *Biochem. Soc. Trans. 5*, 943–946.
Corbett, S., Bradbury, E. M., & Matthews, H. R. (1980) *Exp. Cell Res. 128*, 127–132.
Daniel, J. W., & Baldwin, H. H. (1964) *Methods Cell Physiol. 1*, 9–41.
Dove, W. F., & Rusch, H. P. (1980) *Growth and Differentiation in Physarum polycephalum*, Princeton University Press, Princeton.
Fischer, S. G., & Laemmli, U. K. (1980) *Biochemistry 19*, 2240–2246.
Gurley, L. R., Walter, R. A., & Tobey, R. A. (1973) *Biochem. Biophys. Res. Commun. 50*, 744–750.
Gurley, L. R., D'Anna, J. A., Barham, S. S., Deaven, L. L., & Tobey, R. A. (1978) *Eur. J. Biochem. 84*, 1–16.
Hardie, D. G., Matthews, H. R., & Bradbury, E. M. (1976) *Eur. J. Biochem. 66*, 37–42.
Jockusch, B. M., & Walker, I. O. (1974) *Eur. J. Biochem. 48*, 417–425.
Johnson, E. M., Allfrey, V. G., Bradbury, E. M., & Matthews, H. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1116–1120.
Kinkade, J. M., & Cole, R. D. (1966) *J. Biol. Chem. 241*, 5790–5797.
Lake, R. S. (1973) *J. Cell Biol. 58*, 317–331.
Lake, R. S., & Salzman, N. P. (1972) *Biochemistry 11*, 4817–4826.
Langan, T. A. (1971) *Ann. N.Y. Acad. Sci. 185*, 166–180.
Langan, T. A. (1978a) *Methods Cell Biol. 19*, 127–142.
Langan, T. A. (1978b) *Methods Cell Biol. 19*, 143–152.
Langan, T. A., Zeilig, C. E., & Leichtling, B. (1980) in *Protein Phosphorylation and Bioregulation* (Thomas, G., Podesta, E. J., & Gordon, J., Eds.) pp 70–82, S. Karger, Basel.
Matthews, H. R. (1976) in *Eukaryotic Cell Function and Growth* (Dumont, J., & Brown, B. L., Eds.) pp 733–745, Plenum Press, New York.
Matthews, H. R. (1980) in *Protein Phosphorylation in Regulation* (Cohen, P., Ed.) pp 235–254, Elsevier/North-Holland, Amsterdam.
Matthews, H. R., & Bradbury, E. M. (1982) in *The Cell Biology of Physarum and Didymium* (Aldrich, H., & Daniel, J. W., Eds.) Academic Press, New York.
Mitchelson, K., Chambers, T., Bradbury, E. M., & Matthews, H. R. (1978) *FEBS Lett. 92*, 339–342.
Mohberg, J., & Rusch, H. P. (1969) *Arch. Biochem. Biophys. 134*, 577–589.
Mohberg, J., & Rusch, H. P. (1970) *Arch. Biochem. Biophys. 138*, 418–432.
Mohberg, J., & Rusch, H. P. (1971) *Exp. Cell Res. 66*, 305–316.
Panyim, S., & Chalkley, R. (1969) *Arch. Biochem. Biophys. 130*, 337–346.
Panyim, S., & Chalkley, R. (1971) *J. Biol. Chem. 246*, 7557–7560.
Schlepper, J., & Knippers, R. (1975) *Eur. J. Biochem. 60*, 209–220.
Sherod, D., Johnson, G., & Chalkey, R. (1974) *J. Biol. Chem. 249*, 3923–3931.
Teller, D. C., Kinkade, J. M., & Cole, R. D. (1965) *Biochem. Biophys. Res. Commun. 20*, 739–744.
Tyrsin, Yu. A., Krasheninnikov, I. A., & Tyrsina, E. G. (1977) *Biokhimiya 42*, 898–905.
Witt, J. J., & Roskoski, R. (1975) *Anal. Biochem. 66*, 253–258.
Zeilig, C. E., & Langan, T. A. (1980) *Biochem. Biophys. Res. Commun. 95*, 1372–1379.