CYCLIC 3′,5′-AMP-DEPENDENT PROTEIN KINASE: ITS SENSITIVITY TOWARDS ACID-PRECIPITATION AND AMMONIUM SULPHATE FRACTIONATION

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ABSTRACT

1. Acid precipitation and ammonium sulphate fractionation are commonly used for the purification of protein kinase (ATP : protein phosphotransferase, EC 2.7.1.37). Both these procedures alter the cyclic AMP-dependence of the enzyme when applied to mouse liver cytosol. Acid precipitation also results in a low and variable recovery of enzyme activity.

2. Mouse liver cytosol was resolved into two peaks of kinase activity (I and II) by DEAE–cellulose chromatography.

3. Acid treatment (pH 4·8) strongly reduced the activity of the moderately cyclic AMP-dependent protein kinase II; prolonged treatment also affected the highly cyclic AMP-dependent protein kinase I.

4. Ammonium sulphate fractionation reduced the activity of protein kinase I and also lowered the cyclic AMP-dependence of the enzyme.

Cyclic 3′,5′-AMP (cAMP)-stimulated phosphorylation of protein substrates has been established as a major mechanism of cAMP action in the cell (Langan, 1973). cAMP-dependent protein kinase (ATP : protein phosphotransferase, EC 2.7.1.37), the enzyme mediating this effect, has therefore been studied in considerable detail. The enzyme has been extensively purified, and the physicochemical properties of the holoenzyme(s) (dissociable by cAMP), and its subunits investigated (Gill & Garren, 1971; Corbin, Brostrøm, King & Krebs, 1972; Tao & Hackett, 1973).

We have studied the action of hormones on tissue activity and fractionation pattern of protein kinase in the genital tissues of the mouse (Døskeland, Kvinnsland & Ueland, 1974). In our first attempts to obtain a protein kinase profile by DEAE–cellulose chromatography we followed the widely used procedure of Miyamoto, using acid-precipitation (pH 4·8) and ammonium sulphate fractionation prior to DEAE-cellulose chromatography (Gilman, 1970; Haddox, Nicol & Goldberg, 1973; Sands, Meyer & Rickenberg, 1973; Dastugue, Tichonicky & Kruh, 1973; van Leemput-Coutrez, Camus & Christophe, 1973). As we were unable to achieve satisfactory reproducibility and observed low recoveries of enzyme activity, we have investigated separately the effect of acid and ammonium sulphate on protein kinase preparations derived from mouse liver.

MATERIALS AND METHODS

Animals

Randomly bred, female, virgin NMRI-mice were obtained from Statens institutt for folkehelse, Oslo, Norway. They were fed a standard pellet diet and given water *ad lib.*

Chemicals

[^{3}H]cyclic 3′,5′-AMP (30 Ci/mmole), and [^{32}P]ATP (approx. 16 Ci/mmole when delivered) were from The Radiochemical Centre, Amersham, Bucks., U.K. [^{32}P]ATP was used within four weeks after delivery. Whole calf thymus histone, cyclic 3′,5′-AMP, ATP, and ethyleneglycolbis-(β-amino-ethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) were obtained from Sigma. Sucrose (AnalaR) was from BDH Chemicals Ltd., Poole, Dorset, U.K. Other chemicals were of analytical grade, from Merck, Darmstadt, W. Germany.

Assay of Protein Kinase Activity

Incubations were routinely performed at 30° C for 5 minutes. The solution to be assayed (100 μl.)

was mixed with 50 μl. of a solution of 15 mM Hepes buffer (pH 7.0), 0·9 mM EGTA, 0·1 mM EDTA, and 15 mM $Mg(CH_3COO)_2$, containing 100 μg. histone, 625 pmoles non-radioactive ATP, 0·25 μCi [^{32}P]ATP, and (when added) cAMP to give a final concentration of 4 μM in the incubation mixture. The total incubation volume was 150 μl.

The reaction was terminated (Reimann, Walsh & Krebs, 1971) by pipetting 100 μl. of the incubation mixture onto a Whatman 3MM filter disc, 2·5 cm. in diameter, presoaked in an aqueous solution of 10 mM $Na_4P_2O_4$ and 10 mM Na_3PO_4. After three washes in cold 5% trichloroacetic acid (with phosphates), the filters were transferred to 1/1 ethanol/acetone for 30 minutes, dried, and put into scintillation vials containing PPO (5 g./l.), and POPOP (0·3 g./l.) in toluene. As the radioactivity remained tightly bound to the filters, the scintillation vials with the fluors could be re-used several times without detectable increase in background counts.

Assay of cAMP Binding

We followed essentially the Gilman (1970) procedure. The preparation to be assayed (200 μl.) was mixed with 10 μl. of a solution containing 7 pmoles of [^{3}H]cyclic 3′,5′-AMP, and incubated for six hours at 0° C. After the Millipore filters, used to separate bound from unbound cAMP, had been dried (which facilitated dissolution of the filters), they were dissolved in 1 ml. Cellosolve (Gurr). Finally, 10 ml. Unisolve (Koch-Light) was added to the scintillation vial.

Protein Estimation

The method of Klungsøyr (1969) was used. Before assay all samples were desalted by filtering through small columns packed with Sephadex G-25 (Pharmacia) and equilibrated with 15 mM Hepes buffer, pH 7·0. Aliquots of the desalted solution were taken both for protein determination and protein kinase assay. The desalting was necessary because Tris, 2-mercaptoethanol (Klungsøyr, 1969), EDTA, and EGTA interfere with the protein determination. Concentrations of EDTA and EGTA as low as 0·03 and 0·01 mM respectively, caused serious interference.

Procedure for Preparation of Protein Kinase

The animals were killed by cervical dislocation and the organs (liver, uteri) quickly removed and put into ice-cold homogenization buffer, in which it was cut into pieces. The homogenization buffer was 10 mM Tris–HCl (pH 7·5 at 22° C; pH 7·9 at 0° C) containing 4 mM EDTA, 6 mM 2-mercaptoethanol, and 0·27 M sucrose. Homogenization was in a Thomas type C homogenizer with 5 strokes at a pestle speed of 465 rev./minute.

To obtain the cytosol fraction, a 24,000 *g* supernatant was spun for 1 hour at 120,000 *g* in the 460 rotor of a B-65 International ultracentrifuge.

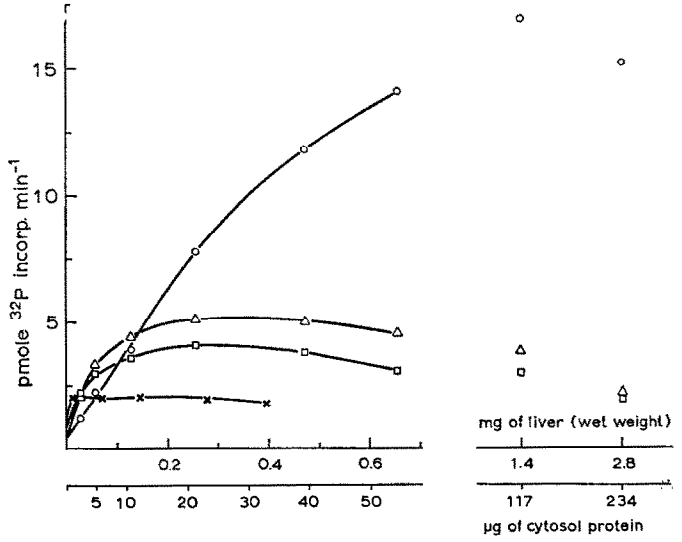


FIG. 1.—Effect of sedimentation of crude homogenate (from mouse liver) on the protein kinase assay. Crude homogenate (×) was sedimented in the SS-34 rotor of a Sorvall RC-2 centrifuge at 2000 *g* (□) and 12,000 *g* (△) for 10 minutes. Ultracentrifugation was in the B-65 International centrifuge at 120,000 *g* (○) for 1 hour. Activity was measured with cAMP present in the assay buffer. Data shown are the mean of determinations in duplicate: the background is not subtracted.

Before being applied to DEAE–cellulose columns, cytosol preparations (untreated or exposed to acid or $(NH_4)_2SO_4$) were dialyzed against 50 volumes of 10 mM Tris buffer pH 7·5 with 4 mM EDTA and 6 mM 2-mercaptoethanol, with two changes of dialyzing buffer.

RESULTS

Studies on Optimal Conditions for Protein Kinase Estimation

Experiments were performed to determine reliable assay conditions for protein kinase. The details are given in the legends to Figs. 1–3. A centrifugation step was found necessary to achieve linearity of protein kinase activity vs concentration of tissue extracts (Fig. 1).

The data of Fig. 2 show that the action of exogenously added heat-stable inhibitor diminished with dilution of the mixture of tissue extract and inhibitor. Obviously, exogenous (and presumably endogenous) inhibitor no longer influenced the protein kinase assay when linearity with respect to protein kinase concentration had been achieved.

Linearity of kinase activity with respect to time does not eliminate the influence of inhibitor on the assays, as shown in the inset of Fig. 2. To determine the protein kinase activity in a given tissue extract (Fig. 3) a series of dilutions was chosen so that at least four dilutions, when assayed in the presence of cAMP, were in the linear part of the plot. The angle coefficient of the linear part of the plot was taken as the protein kinase activity. With this method, three consecutive estimations of a cytosol preparation routinely varied within a range of 5%. At high dilutions the protein kinase activity in the absence of cAMP increased relative to the activity measured in the presence of cAMP. We believe this to be due to dissociation of

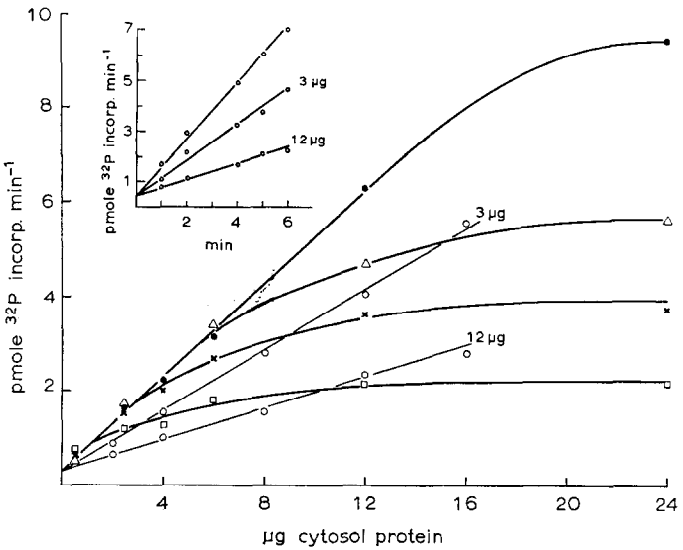


Fig. 2.—Effect of exogenous inhibitor on protein kinase activity. To 24 μg. cytosol protein from mouse uterus was added 0 μg. (●), 4·5 μg. (△), 9 μg. (×), and 27 μg. (□) of an inhibitor preparation. The inhibitor was prepared from rabbit skeletal muscle through the steps of heating and trichloroacetic acid-precipitation (essentially as described by Walsh, Ashby, Gonzalez, Calkins, Fischer & Krebs, 1971), and was passed through a Sephadex G-25 column to remove trichloroacetic acid. The cytosol/inhibitor mixtures were co-diluted in Hepes buffer. The straight lines (○) represent the results of an experiment in which a fixed amount of inhibitor was added to a range of concentrations of uterine cytosol. The amount of inhibitor added is indicated in the figure.

Inset: 12 μg. cytosol–protein was incubated for the periods of time indicated; without inhibitor, with 3 μg. inhibitor, and 12 μg. inhibitor. All data shown are the mean of duplicate determinations, not corrected for background.

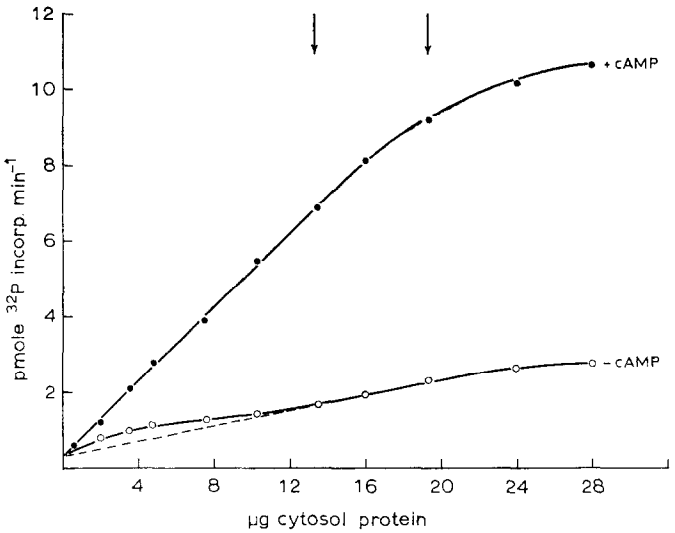


FIG. 3.—Plot for estimation of protein kinase activity. Each dilution of liver–cytosol was assayed in duplicate with and without added cAMP. The same general profile was obtained with cytosol preparations from other tissues investigated (uterus, endometrium, vaginal epithelium from mouse). Estimations of activity in the absence of cAMP were based on determinations in the contraction range indicated by the arrows.

Table I.—EFFECT OF ACID-PRECIPITATION AND $(NH_4)_2SO_4$ FRACTIONATION ON HISTONE PHOSPHORYLATION AND cAMP-BINDING BY CYTOSOL

PREPARATION ASSAYED	pmoles phosphate incorporated/minute per mg. liver (wet weight)		PHOSPHATE INCORPORATION (% of cytosol)		pmoles cAMP bound/mg. liver (wet weight)	pmoles cAMP bound (% of cytosol)
	−cAMP	+cAMP	−cAMP	+cAMP		
Cytosol	5·1	29·5	100	100	0·28	100
$(NH_4)_2SO_4$ precipitate	9·4	23·4	184	80	0·23	82
Acid supernatant (10+30 minutes)	0·82	8·6	16	29	Not measured	
Acid supernatant (10+60 minutes)	0·56	3·5	11	12	0·077	27

Results of a representative experiment are shown.

Mouse liver cytosol was divided in three portions, one of which (cytosol) was kept unaltered at 2° C. $(NH_4)_2SO_4$ (32·5 g./100 ml. cytosol) was added to the second portion under continuous slow stirring.

After 45 minutes the precipitate was collected by centrifugation at 24,000 *g* for 15 minutes and redissolved in Tris buffer to the original volume. Recovery of protein was 52·4%.

The third cytosol portion was brought to pH 4·84 (measured at 0° C) by the slow addition of 0·5 M acetic acid. After 10 minutes incubation at 0° C, the precipitate was removed by centrifugation at 27,000 *g* av. for 25 minutes. The acid was removed by passing the supernatant through a desalting column (Sephadex G-25) equilibrated with Hepes buffer. The results were the same when the acid supernatant was neutralized with 1 M phosphate buffer, pH 7·2, prior to desalting.

To obtain the results indicated in the Table for acid supernatant (10+60 minutes), part of the acid supernatant was left for 30 minutes at 2° C before being neutralized. Recovery of protein was 50·4% for the cytosol exposed to extended acid treatment.

The values are the means of duplicate determinations. Estimation of protein kinase activity was performed as above.

the holoenzyme into free catalytic and regulatory subunits (Brostrøm, Reimann, Walsh & Krebs, 1970). The estimation of basal protein kinase activity therefore had to be based on assays in the concentration range immediately before the activity in the presence of cAMP levelled off. The range of three consecutive measurements of basal protein kinase activity was routinely less than 20%, but a range of up to 40% was noted.

Differential Effect of Acid and Ammonium Sulphate Fractionation on Protein Kinase Species Analyzed by DEAE-Cellulose Chromatography

Untreated cytosol was resolved into two peaks of protein kinase activity on DEAE–cellulose chromatography (Fig. 4a). Protein kinase I is extremely cAMP-dependent (15–20-fold stimulated), while protein kinase II is moderately (approx. 3·5-fold) stimulated (Fig. 4a).

Ammonium sulphate fractionation (Table I) caused a slight reduction of total protein kinase activity measured in the presence of cAMP, and a considerable increase in basal activity, giving a markedly reduced cAMP-dependence.

By DEAE-cellulose chromatography (Fig. 4b) this is shown to be the result of a reduction of protein kinase I and a lowered degree of stimulation (approx. 2·2-fold) of protein kinase II.

The puzzling increase in cAMP-dependence of total protein kinase activity (Table I) brought about by acid precipitation results from a selective removal of the moderately cAMP-dependent protein kinase II, while protein kinase I seems unaffected (Fig. 4c). However, prolonged acid treatment (Fig. 4d) also reduced the degree of stimulation of protein kinase I.

When acid precipitation and $(NH_4)_2SO_4$ fractionation were combined (Fig. 4e), both protein kinase I and protein kinase II showed reduced activity.

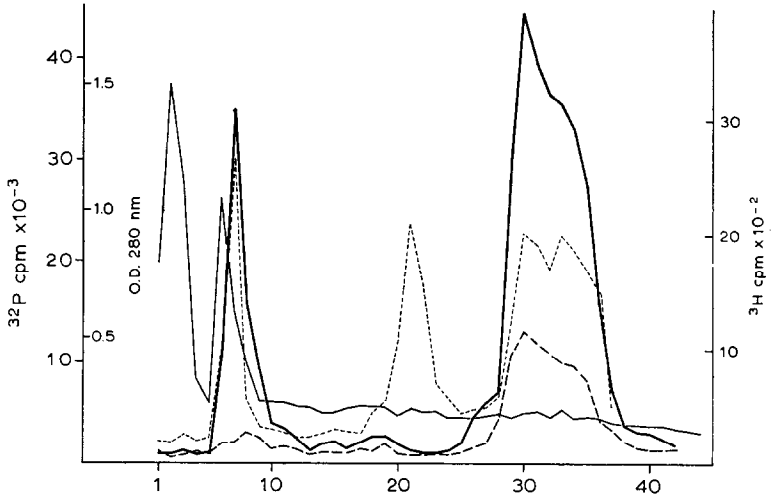
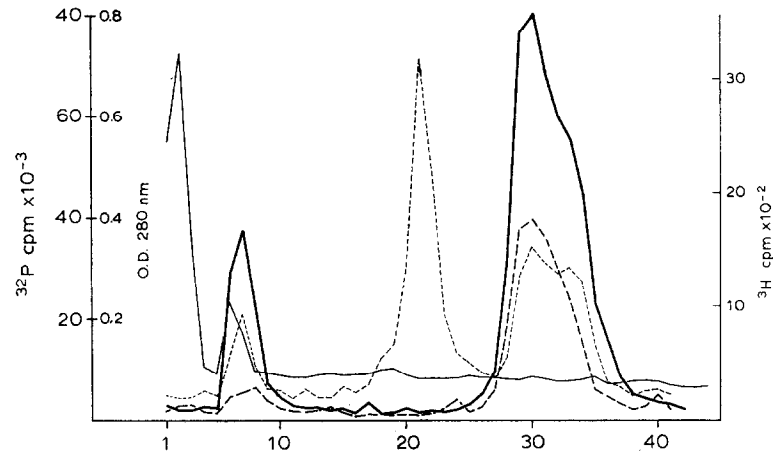
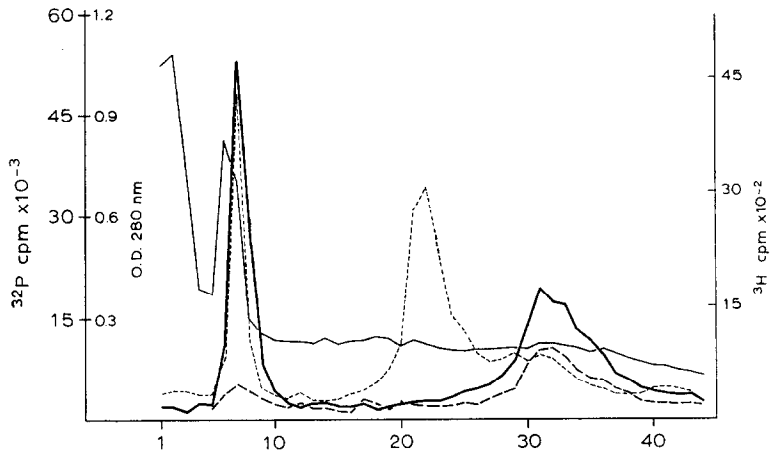


Fig. 4.—DEAE–cellulose chromatography of acid-treated, $(NH_4)_2SO_4$-treated, and untreated cytosol. Samples containing less than 40 mg. of protein were applied to DEAE–cellulose columns (0·9 × 15 cm.) packed with DE-52 (Whatman) and equilibrated with 10 mM Tris–HCl pH 7·5, 4 mM EDTA, 6 mM 2-mercaptoethanol. Elution was with a linear gradient of 0–0·25 M KCl. (0·75 M KCl did not elute additional kinase or binding activities). 50 fractions of 5 ml. were collected. Before assay for protein kinase activity, samples were desalted by passing through Sephadex G-25 columns equilibrated with 15 mM Hepes pH 7·0. (Column dimensions were adjusted to give minimal and reproducible dilution of the samples.) For assay of cAMP binding, samples were taken directly from the eluted fractions. At least two chromatographic runs were done with each preparation. Fine solid line: O.D. 280 nm. Thick solid line: Protein kinase activity in the presence of 4 μM cAMP. Dashed line: Activity without added cAMP. Dotted line: cAMP bound.

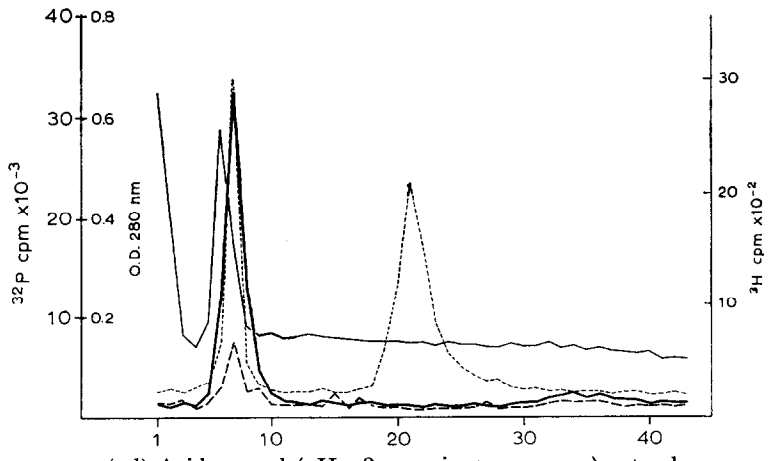
(4a) Cytosol pretreated only with dialysis.



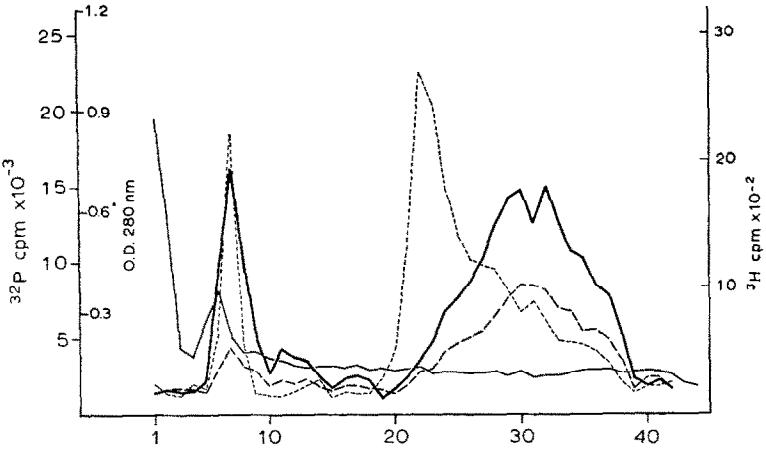
(4b) $(NH_4)_2SO_4$ precipitate of cytosol.



(4c) Acid-treated (pH 4·8, 40 minute exposure) cytosol.



(4d) Acid-treated (pH 4·8, 70 minute exposure) cytosol.



(4e) Acid (pH 4·8)-treated and $(NH_4)_2SO_4$-precipitated 27,000 *g* supernatant. In this experiment, homogenization was performed in 4 mM EDTA pH 7·0 in a Warren-type blendor (full speed 2 minutes), and starting material was 27,000 *g* supernatant instead of cytosol (Miyamoto *et al.*, 1969).

While reproducibility was satisfactory with untreated cytosol and $(NH_4)_2SO_4$-treated cytosol, it was unsatisfactory with acid treatment alone or in combination with $(NH_4)_2SO_4$ fractionation. We have found the timing for acid-precipitation to be critical (Table I, Figs. 4c, d). In the procedure of Miyamoto *et al.* (1969) a 10-minute acid precipitation (pH 4·8) is followed by a 30-minute high-speed centrifugation before neutralization, giving a 40-minute total acid exposure. As shown in Table I an increase in overall acid exposure from 40 minutes to 70 minutes causes serious changes of recovery and cAMP-dependence of protein kinase and may explain the poor reproducibility we have observed with acid precipitation. Obviously, delay in removing tubes from the centrifuge or delayed neutralization will influence the result of acid precipitation.

Gel Chromatography of Cytosol

When gel-filtration (Sepharose 4B or Sephadex G-200) was used as the only purification step prior to DEAE–cellulose chromatography, the recovery of kinase activity and binding activity was between 90 and 100% (when all fractions containing activity were pooled and tested). The degree of stimulation by cAMP was not altered. This material chromatographed exactly like dialyzed cytosol on DEAE–cellulose.

With Sepharose 4B (Fig. 5a) the major peak of protein kinase activity was well separated from high molecular weight material, presumably representing the fraction of membranes not sedimented in the ultracentrifuge. Separation from the heat-stable protein kinase inhibitor was incomplete (unpublished observations). With Sephadex G-200 (Fig. 5b) complete separation from the membranous material was not achieved, but separation from inhibitor was near complete. The peak of binding activity was eluted behind the peak of protein kinase activity. This suggests that the cAMP-binding material eluting between protein kinase I and protein kinase II on DEAE–cellulose chromatography has a lower Stokes radius than the major part of protein kinase enzyme. It also indicates that the cAMP-binding material unassociated with protein kinase activity is not a receptor subunit dissociated by the DEAE–cellulose (Gill & Garren, 1970), but is separated from protein kinase activity already in the cytosol.

For analytical purposes (comparing protein kinase patterns from different tissue extracts) when a modest amount of enzyme is required, acid precipitation and $(NH_4)_2SO_4$ fractionation should be avoided. Because of its lack of effect on the protein kinase, we suggest gel-filtration as a suitable purification

step prior to a high resolution separation of protein kinase activities (e.g. by chromatography or electrophoresis).

DISCUSSION

The first part of this work is concerned with defining conditions for a precise determination of protein kinase activity in tissue extracts. An ultracentrifugation step of crude homogenate is sufficient to make the protein kinase assay linear (up to 20 μg. of cytosol protein in 150 μl. incubation volume) with respect to concentration. The experiments were therefore performed on cytosol, although the relevance of the results is thereby restricted to the fraction of the cell's protein kinase which is not sedimented with

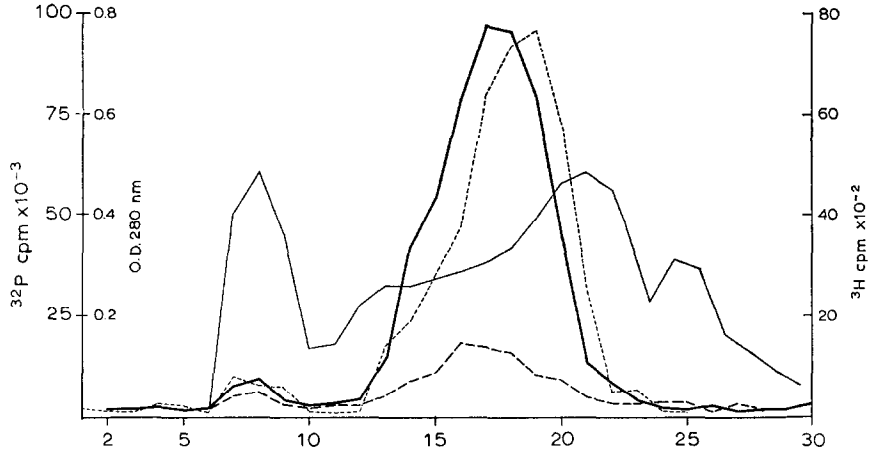
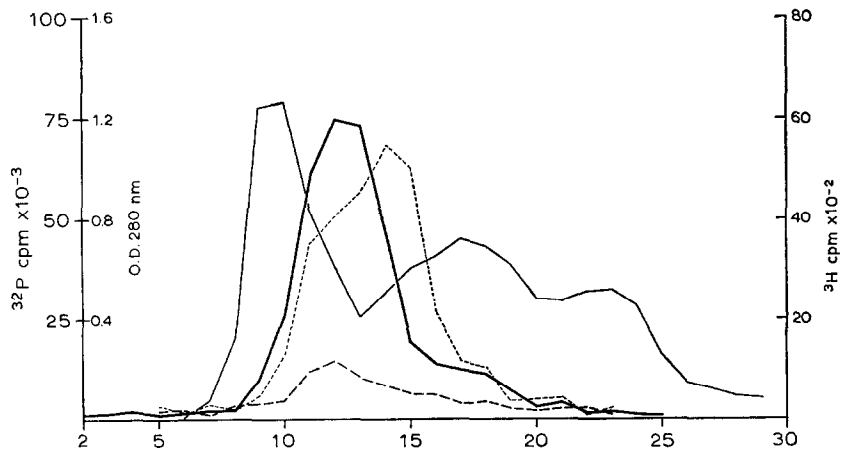


FIG. 5.—Gel-chromatography of cytosol. 5a): 5 ml. of liver-cytosol was applied to a 1·6 × 65 cm. column packed with Sepharose 4B (Pharmacia) and equilibrated with 10 mM Tris–HCl pH 7·5, 4 mM EDTA, 6 mM 2-mercaptoethanol. Fractions (4 ml.) were collected. Pressure-head was 50 cm. H_2O. Fractions were assayed as described in legend to FIG. 4.



(5b): Conditions as described in legend to FIG. 5a, except that the column was packed with Sephadex G-200 (Pharmacia) and the pressure-head was 15 cm. H_2O. Fine solid line: O.D. 280 nm. Thick solid line: protein kinase activity in the presence of 4 μM cAMP. Dashed line: activity without added cAMP. Dotted line: cAMP bound.

the subcellular particles. We have avoided the inclusion of NaF, which inhibits ATPase (Glick, 1972; Corbin, Soderling & Park, 1973) and protein phosphatase (van de Werve, van den Berghe & Hers, 1974), as the protein kinase activity is inhibited (unpublished observations; van de Werve *et al.*, 1974).

Several investigators have reported more than 100% recovery of protein kinase activity after purification of crude homogenate or cytosol (Miyamoto, Kuo & Greengard, 1969; Majumder & Turkington, 1971; Rapaport & Degroot, 1972; Spaulding & Burrow, 1972; Tao & Hackett, 1973). The high recovery has been ascribed to removal of inhibitory substances (Miyamoto *et al.*, 1969). The concentration of heat-stable inhibitor of protein kinase may vary widely in different organs (Ashby & Walsh, 1972). Differences in inhibitor content in tissue extracts might invalidate comparison of their protein kinase activities. We have found that the effect of inhibitor on protein kinase activity in uterine cytosol can be overcome by diluting the cytosol so that protein kinase activity is linear with respect to enzyme concentration (FIG. 2). Linearity of kinase activity versus concentration thus ensures that the heat-stable inhibitor does not influence the assay.

The second part deals with the effect of the commonly used procedures of acid precipitation and $(NH_4)_2SO_4$ fractionation on the mouse liver cytosol protein kinase, which can be resolved into protein kinase I (highly cAMP-dependent) and protein kinase II (moderately cAMP-dependent) by DEAE–cellulose chromatography (FIG. 4a). Acid precipitation reduces the amount of the moderately cAMP-dependent protein kinase II (FIG. 4c), which is the major protein kinase fraction in the liver cytosol. The highly cAMP-dependent protein kinase I is less affected by acid. Acid precipitation may therefore give a protein kinase preparation with an increased cAMP dependency as a result of selective reduction of one species of protein kinase. $(NH_4)_2SO_4$ precipitation regularly reduces the amount of protein kinase I and also lowers further the cAMP-dependence of protein kinase II (FIG. 4b). As more than 100% of the cAMP-independent activity is recovered after $(NH_4)_2SO_4$ precipitation (Table I), we believe that $(NH_4)_2SO_4$ treatment has dissociated the holoenzyme, giving more free catalytic subunit. We suppose that the effect of acid may be either to provide a suitable pH for lysosomal proteases (Tsung & Weissmann, 1973) or to precipitate the protein kinase. In favour of the last possibility is our finding of considerable protein kinase activity in the material pelleted after acid-precipitation (data not shown), and the observation by Gill & Garren (1971) that a protein kinase from bovine adrenal cortex appeared as a cloudy band on isoelectric focusing (isoelectric point pH 4·75). In addition to precipitation at pH 4·8, acid precipitation has been performed at pH 4·6 (Majumder & Turkington, 1971), pH 4·9 (Rapaport & Degroot, 1972; Abou-Issa, Kratowich & Mendicino, 1974; Chambaut, Leray & Hanoune, 1971), pH 5·0 (Pierre & Loeb, 1972), pH 5·5 (Reimann, Walsh & Krebs, 1971), and pH 5·9 (Gill & Garren, 1971). Although protein kinase from other sources than mouse liver may be more resistant to acid precipitation and $(NH_4)_2SO_4$ fractionation, we think precautions should be taken to ensure intactness of the protein kinase when acid precipitation and/or $(NH_4)_2SO_4$ fractionation is used as an early purification step in the process of obtaining highly purified protein kinase on which elaborate studies are made.

ACKNOWLEDGEMENT

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