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Cyclic 3′,5′-AMP-Stimulated and Non-stimulated Phosphorylation of Protein Fractions from Rat-liver Cell Sap on Incubation with (γ-^{32}P)ATP

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ABSTRACT

Rat-liver cell sap protein was separated into four fractions by pH 5.5 precipitation and ammonium sulfate fractionation. On incubation with (^{32}P)ATP, protein of two of the fractions was phosphorylated, showing the presence of protein kinase activity and endogenous protein substrates. The phosphorylation of one of the fractions was markedly stimulated by cyclic 3′,5′-AMP. Strong evidence was obtained that this phosphate-incorporating material was neither active phosphorylase, phosphorylase kinase nor glycogen synthetase. The two phosphate-incorporating fractions were chromatographed on DEAE-cellulose with stepwise elution. Several subfractions contained material which was phosphorylated on incubation with (^{32}P)ATP. The phosphorylation of two subfractions was greatly stimulated by cyclic 3′,5′-AMP. By polyacrylamide gel electrophoresis in detergent of (^{32}P)ATP-incubated material from these fractions, three major ^{32}P-labelled components with estimated molecular weights of 62 000, 55 000 and 47 000 were demonstrated. It is supposed that these components are derived from enzymes or other proteins with functions regulated by phosphorylation–dephosphorylation reactions.

INTRODUCTION

Rat-liver cell sap contains protein which is phosphorylated on serine and threonine residues on incubation of the cell sap with (^{32}P)ATP (8, 17). In the presence of cyclic 3′,5′-AMP a considerable increase of the phosphorylation occurs. Strong evidence has been obtained that most of the phosphorylation is catalyzed by protein kinase activity, and is not due to the formation of intermediate phosphoryl enzymes (17).

In mammalian tissues there appear to be two types of protein kinases which differ according to whether or not they are dependent on cyclic 3′,5′-AMP (13). They also seem to differ with regard to substrate specificity (13). Accordingly, proteins phosphorylated in protein kinase reactions can probably be divided into two groups depending on whether or not their phosphorylation is stimulated by cyclic 3′,5′-AMP. Thus, the function of at least the former group of phosphoproteins should be related to hormone action, as has been clearly demonstrated for phosphorylase kinase (EC 2.7.1.38), phosphorylase (EC 2.4.1.1), and glycogen synthetase (EC 2.4.1.11) (13, 21). A regulatory phosphorylation which is not stimulated by cyclic 3′,5′-AMP has recently been reported for rat-liver acetyl-CoA carboxylase (EC 6.4.1.2) (1).

It seemed to be of value to separate the rat-liver cell sap proteins which become labelled with ^{32}P on incubation with (^{32}P)ATP in order to obtain information on the number of phosphoproteins present and to find out whether cyclic 3′,5′-AMP specifically increases the phosphorylation of some proteins; and if so, to examine the relationship between these latter proteins and the aforementioned enzymes which are phosphorylated under the influence of cyclic 3′,5′-AMP.

During a preliminary purification of cyclic 3′,5′-AMP-dependent protein kinase from rat liver, essentially in accordance with Kuo & Greengard (14), we found that endogenous protein substrate was co-purified with the enzyme during the initial steps (pH 5.5 precipitation, ammonium sulfate fractionation and DEAE-cellulose chromatography).

In the present paper, an account is given of experiments in which protein fractions from rat-liver cell sap became labelled with ^{32}P on incubation with (^{32}P)ATP. The labelled proteins were

Abbreviations: SerP, phosphorylserine; ThrP, phosphoryltreonine.

studied by polyacrylamide gel electrophoresis in detergent. The phosphorylation of three major components found was greatly stimulated by cyclic 3′,5′-AMP.

EXPERIMENTAL

(^{32}P)ATP synthesis

(γ-^{32}P)ATP was prepared as described earlier (6). Its specific radioactivity after mixing with unlabelled ATP was 10 000–60 000 cpm·nmole^{-1}.

Analytical methods

The radioactivity of ^{32}P-labelled phosphate was measured either on dried aliquots in aluminium cups as previously described (7), or by determining its Cerenkov radiation (12), using an Intertechnique SL30 liquid scintillation spectrometer. Orthophosphate was determined according to Martin & Doty (19). ^{32}P-labelled orthophosphate was assayed by measuring the radioactivity of the organic phase. Protein concentration was estimated by the biuret method (15) with bovine serum albumin as a standard or by measuring the absorbance at 280 nm using a Zeiss PMQ II spectrophotometer, assuming the absorbance of 1·cm^{-1} to correspond to 1 mg of protein/ml.

Phosphorylase activity was determined according to Hedrick & Fischer (11). One mM AMP was included in the substrate solutions. On estimating phosphorylase *b* the substrate contained 0.7 M Na_2SO_4. Glycogen synthetase activity was assayed by the method of Leloir & Goldemberg (16). One enzyme unit is defined as the amount of enzyme that transforms one μmole of substrate per min under the conditions used.

Phosphorylase kinase activity was estimated by incubating the kinase-containing fraction with rat-liver phosphorylase *b* (Fraction 3 prepared as described below), 2.5 mM ATP, 0.1 mM cyclic 3′,5′-AMP, 2.5 mM magnesium acetate and 50 mM Tris-acetic acid (pH 7.5) for 20 min at 30°C, using a final incubation volume of 0.1 ml. In the absence of added phosphorylase kinase the incubation mixture contained 0.3 units of phosphorylase activity when assayed in the absence of sodium sulfate. After incubation with an excess of phosphorylase kinase the corresponding figure was 1.7 units. In the assays the amount of kinase added was chosen to give a maximal increase of phosphorylase activity of 0.7 units. The kinase reaction was interrupted by dilution with 0.2–1.0 ml of 0.1 M sodium maleate-maleic acid buffer (pH 6.5). The phosphorylase activity was determined by incubating 0.1 ml of the diluted incubation mixtures for 5 min at 30°C with 0.1 ml of phosphorylase substrate solution in the absence of sodium sulfate.

One unit of kinase activity is defined as that amount of enzyme which increases the phosphorylase activity by one unit per min under the conditions used.

Histone kinase activity was determined by incubating 50 μl of enzyme (diluted with 5 mM potassium phosphate (pH 7.0)) with 150 μl of substrate solution for 5 min at 30°C. The incubation mixtures contained (final concentrations): 50 mM sodium glycerophosphate (pH 6.5), 1 mg/ml of calf thymus histone (Sigma Type II-A), 10 mM magnesium acetate, 10 mM sodium fluoride, 2 mM theophylline, 0.24 mM EGTA, 5×10^{-6} M cyclic 3′,5′-AMP, and 10^{-5} M (^{32}P)ATP (about 10^5 cpm·nmole^{-1}). The reaction was interrupted by adding 0.1 ml of 10 mM ATP in order to reduce unspecific adsorption of (^{32}P)ATP, immediately followed by 0.2 ml of bovine serum albumin (6.3 mg/ml) and 4 ml of 10% (w/v) trichloroacetic acid. After 5 min in an ice-water bath the precipitated protein was centrifuged down and the supernatant removed. The protein was dissolved in 0.5 ml of 0.2 M NaOH, followed by reprecipitation with 2 ml of 10% trichloroacetic acid. This was repeated three times. The final protein pellet was dissolved in 0.5 ml of 0.1 M NaOH and the radioactivity determined. Blank determinations in the absence of histone were performed in parallel. One unit of histone kinase activity is defined as that amount of enzyme which catalyzes the formation of one nmol of protein-bound phosphate per min.

Preparation of rat-liver cell sap

All the preparation steps were performed at 0–4°C. Livers from 250–350 g male Sprague-Dawley rats were homogenized with 5 vol. (v/w) of a solution consisting of cold 0.25 M sucrose, 50 mM Tris-acetic acid buffer (pH 7.5) and 1 mM EDTA, in a Potter-Elvehjem homogenizer. The rats were either fed *ad libitum* with a standard laboratory diet or were fasted for about 20 h. The homogenate was centrifuged at 8 500×*g* for 10 min. Cell sap was then prepared by centrifugation of the supernatant at 105 000×*g* for 60 min.

Fractionation of cell sap proteins by pH 5.5-precipitation, ammonium sulfate fractionation and DEAE-cellulose chromatography

The cell sap proteins were separated into four different fractions (Fractions 1–4) as follows: 1 M acetic acid (about 0.15 ml per g of liver) was added to the cell sap at 0°C to adjust the pH to 5.5. After 5–10 min precipitated protein was recovered by centrifugation at 15 000×*g* for 15 min and dissolved in 0.1 M potassium phosphate (pH 7.2) (1 ml/g of liver) for preparation of Fractions 1 and 2. The supernatant was adjusted at 0°C to pH 7 by adding 1 M potassium phosphate, pH 7.2 (about 0.5 ml per g of liver) and was used to prepare Fractions 3 and 4.

To the dissolved pH 5.5-precipitated proteins, as well as to the neutralized supernatant, a neutral saturated ammonium sulfate solution was added to give 50% saturation. After 15 min at 0°C the precipitated protein (Fractions 1 and 3, respectively) was recovered by centrifugation at 15 000×*g* for 15 min and each precipitate was dissolved in 0.2 ml of water per g of liver. Solid ammonium sulfate was added to each supernatant to give a final saturation of 90% and the mixtures were left for 30 min at 0°C. After centrifugation each pellet was dissolved in 0.2 ml of water/g liver. Thus, Fraction 2 was obtained from the pH 5.5 pellet and Fraction 4 from the pH 5.5 supernatant. The four fractions were

then dialyzed against either 5 mM potassium phosphate buffer (pH 7.0), or the starting buffer of the DEAE-cellulose chromatography.

For DEAE-cellulose chromatography, Whatman DE-52 cellulose was used, in equilibrium with the starting buffer: 50 mM Tris-acetic acid (pH 7.5) – 1 mM EDTA – 5% sucrose – 0.1 mM dithiothreitol. The columns were eluted stepwise with about 2 column volumes each of this buffer and the same buffer containing 0.1, 0.2 and 0.4 M sodium acetate, respectively.

Protein phosphorylation with (^{32}P)ATP

Cell sap fractions, diluted with 5 mM potassium phosphate buffer (pH 7.0) if necessary, were incubated for different lengths of time at 30°C with (^{32}P)ATP in the presence of 10 mM magnesium acetate. When added, a high concentration of cyclic 3',5'-AMP was used (0.1–0.2 mM) in order to allow for its possible enzymatic degradation. The incubation volume was 0.25 ml and the final (^{32}P)ATP concentration was generally 0.1 mM. The incubation was interrupted by adding 1 ml of 10% trichloroacetic acid and 2 mg of bovine serum albumin in 0.1 ml of water. The precipitated protein was collected by centrifugation, dissolved and reprecipitated four times as described for the histone kinase assays, and its radioactivity was measured. When calculating the extent of the protein phosphorylation it was assumed that the phosphate incorporated had the same specific radioactivity as the (^{32}P)ATP used. All values given are mean values of duplicate determinations and unless otherwise stated refer to material from 1 g of liver (wet weight).

Isolation of protein-bound (^{32}P)SerP and (^{32}P)ThrP

(^{32}P)SerP and (^{32}P)ThrP were isolated from acid hydrolysates of (^{32}P)ATP-incubated protein fractions by chromatography on Dowex 50 and Dowex 1 columns, as previously described (17).

^{32}P-labelling of protein fractions for polyacrylamide gel electrophoresis

100 µl samples containing about 50–220 µg of protein were incubated with 0.125 mM (^{32}P)ATP for 5 min at 30°C in the presence of 10 mM magnesium acetate. The concentration of cyclic 3',5'-AMP when added was 0.17 mM. The total incubation volume was 150 µl. The reaction was interrupted by the addition of 150 µl of 8 M urea containing 2% (v/v) 2-mercaptoethanol and 2% (w/v) sodium dodecyl sulfate. The mixtures were then kept at 45°C for 45 min in order to dissociate the proteins.

Polyacrylamide gel electrophoresis

Electrophoresis in dodecyl sulfate was performed according to Shapiro et al. (22), with the modifications of Duncker & Rueckert (5). The gels, 10% in acrylamide, 0.1% in bis-acrylamide, were prepared in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% dodecyl sulfate. The length of the gels was 10 cm. After pre-electrophoresis for 1 h, 25 µl of the samples and 5 µl of pepsin marker (1 mg/ml) in the same solvent were applied with a Hamilton syringe. Electrophoresis was performed in the anode direction for 7 h with a current of 5 mA/gel. The gels were stained according to Weber & Osborn (25), using a concentration of Coomassie blue of 0.025%. After destaining, the gels were sliced and the radioactivity measured. For radioautography destained gels were halved longitudinally and dehydrated in 45% methanol–7% acetic acid. The gel havles were applied to Whatman 3MM chromatography paper and dried at 70°C.

Estimation of molecular weight of phosphorylated components

The molecular weights of the major ^{32}P-labelled components of Fraction 3 were estimated by comparing their migration rates on polyacrylamide gel electrophoresis with those of bovine serum albumin and α-chymotrypsinogen (Sigma), hog pepsin (Sigma) and rabbit muscle phosphorylase *b* (Boehringer). The reference substances were dissolved, each to a concentration of 2 mg/ml, in a solution of 4 M urea containing 1% 2-mercaptoethanol and 1% dodecyl sulfate. 550 µg of the protein from fraction 3 which was eluted with 0.1 M sodium acetate on DEAE-cellulose chromatography was phosphorylated as described above. The ^{32}P-labelled inactivated material was mixed with an equal volume of reference protein solution before electrophoresis. In order to obtain ^{32}P-labelled phosphorylase *a* as an internal marker, the phosphorylation was also performed in the presence of 25 µg of rabbit muscle phosphorylase *b* kinase purified according to DeLange et al. (2) with the modifications described by Soderling et al. (23).

RESULTS

Protein phosphorylation with (^{32}P)ATP and enzyme content of different fractions

Cell sap from two fasted rats and from two fed rats was incubated at 20°C for 60 min in order to dephosphorylate phosphorylase (24), glycogen synthetase (24, 9), and probably also other phosphoproteins by the action of phosphoprotein phosphatases. Fractions 1–4 were then prepared as described under Experimental. The fractions were tested for phosphate-incorporating activity and for phosphorylase, phosphorylase kinase and glycogen synthetase activities. The results are given in Table I.

Most of the phosphorylation was obtained in Fraction 1. In this fraction there was a moderate increase of the phosphorylation in the presence of cyclic 3',5'-AMP; no marked differences between fed and fasted rats were found. There was hardly any incorporation at all in Fractions 2 and 4, but Fraction 3 was phosphorylated to a con-

Table I. *Protein phosphorylation with (^{32}P)ATP and enzyme content of different fractions*

Cell sap incubated at 20°C for 60 min and fractionated by pH 5.5 precipitation and ammonium sulfate as described in the text. Four fractions were obtained which were incubated at 30°C with 1 mM (^{32}P)ATP for 20 min in the presence of 0.1 mM cyclic 3′,5′-AMP or in its absence. Values within brackets were obtained on incubation with 0.1 mM (^{32}P)ATP for 5 min. Samples were diluted to give a maximal phosphorylation of 0.2 nmol with 0.1 mM (^{32}P)ATP and 0.5 nmol with 1 mM (^{32}P)ATP. The values given for glycogen synthetase (measured in the presence of glucose-6 phosphate), were obtained from a separate experiment. All values refer to 1 g of tissue (wet weight)

Fraction	Protein (mg)	Phosphate incorporation (nmole)		Phosphorylase (units)		Glycogen synthetase (units)	Phosphorylase kinase (units)
		−cAMP	+cAMP	+salt	−salt		
Fed rats							
1	14	19.8 (4.9)	25.2 (6.7)	0.2	0.2	0.03	1.8
2	1	0.3	0.3	0.0	0.0	0.00	0.0
3	15	3.1 (0.5)	6.6 (4.0)	1.2	0.9	0.00	0.0
4	12	0.3	0.2	0.1	0.1	0.00	0.0
Fasted rats							
1	15	24.6	28.4	0.9	0.5	0.37	0.4
2	1	0.2	0.1	0.0	0.0	0.00	0.0
3	19	2.4	5.3	5.8	4.2	0.00	0.0
4	23	0.3	0.2	0.2	0.2	0.00	0.0

siderable extent. The presence of cyclic 3′,5′-AMP stimulated the phosphorylation of Fraction 3 to a high degree, especially at a (^{32}P)ATP concentration of 0.1 mM. In general the protein phosphorylation of Fraction 3 from fed rats was slightly higher than that from fasted rats, but this difference was not further examined.

Since the phosphorylation of Fraction 3 was proportionately more stimulated by cyclic 3′,5′-AMP than Fraction 1, it was of interest to relate the phosphorylation obtained to the enzymes known to be phosphorylated under the influence of cyclic 3′,5′-AMP, i.e. phosphorylase, phosphorylase kinase, and glycogen synthetase. The two latter enzyme activities were only found in Fraction 1. Phosphorylase activity was obtained mainly in Fraction 3, and to a much higher degree in fractions from fasted rats than in those from fed rats. Since the phosphorylation was about the same in the two cases, it could be concluded that most of the cyclic 3′,5′-AMP-dependent protein phosphorylation of Fraction 3 from fed rats was not due to phosphorylation of phosphorylase. Fed rats were therefore used for all the subsequent experiments.

The incorporation of (^{32}P)phosphate into Fractions 1 and 3 was about the same regardless of whether or not the original cell sap had been incubated at 20°C.

Rate and extent of protein phosphorylation of Fractions 1 and 3 at different (^{32}P)ATP concentrations. Effect of cyclic 3′,5′-AMP

Samples of Fractions 1 and 3 prepared from non-incubated cell sap from fed rats were incubated at 30°C for different lengths of time with 0.1, 1 and 5 mM (^{32}P)ATP in the presence of 0.2 mM cyclic 3′,5′-AMP. In parallel, incubations were performed without the cyclic nucleotide. In addition, the amounts of Fractions 1 and 3 were varied in the series with the two lower (^{32}P)ATP concentrations.

The results are shown in Figs. 1–4. It can be seen that the magnitude of the maximal phosphorylation of both Fraction 1 and Fraction 3 was highly dependent on the (^{32}P)ATP concentration. At 0.1 mM (^{32}P)ATP the maximal value was obtained within 5–10 min. On the other hand at 5 mM (^{32}P)ATP the reaction was still not complete in 60 min. Cyclic 3′,5′-AMP had only minor effects on the phosphorylation of Fraction 1 (Fig. 1), but greatly stimulated the reaction with Fraction 3 (Fig. 2), especially on incubation for a fairly short time. 0.1 mM (^{32}P)ATP and an incubation time of 5 min at 30°C were found suitable for assays of cyclic AMP-dependent endogenous phosphorylation.

When the amounts of Fractions 1 and 3 were varied on incubation with 0.1 mM (^{32}P)ATP for 5

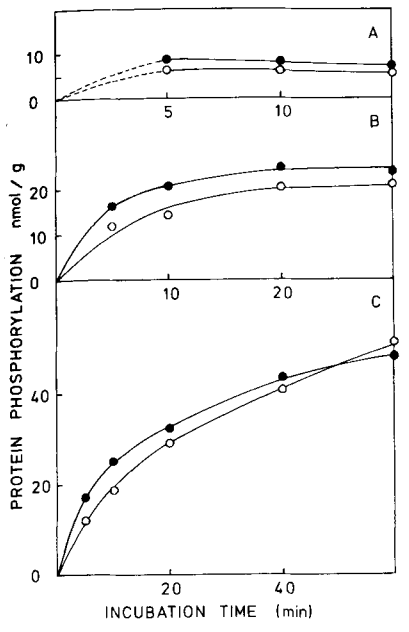

Fig. 1. Time course of phosphorylation of Fraction 1 at different (^{32}P)ATP concentrations in the presence (●—●) and absence (○—○) of 0.2 mM cyclic 3′,5′-AMP. Material from 1/12 g of liver incubated with (A) 0.1 mM, (B) 1 mM, and (C) 5 mM (^{32}P)ATP.

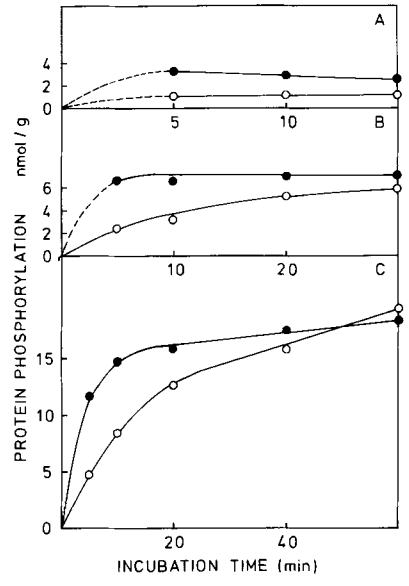

Fig. 2. Time course of phosphorylation of Fraction 3 at different (^{32}P)ATP concentrations in the presence (●—●) and absence (○—○) of 0.2 mM 3′,5′-AMP. Material from 1/15 g of liver incubated with (A) 0.1 mM, (B) 1 mM, and (C) 5 mM (^{32}P)ATP.

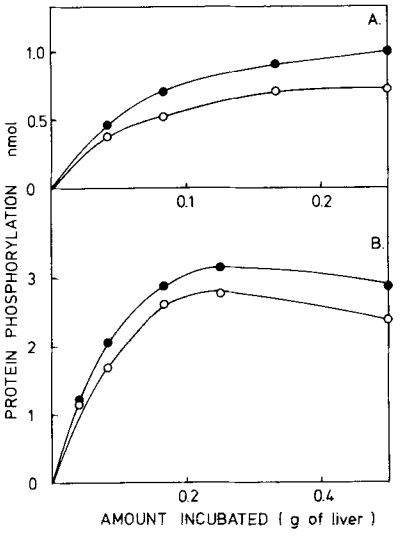

Fig. 3. Phosphorylation of different amounts of Fraction 1 on incubation with (A) 0.1 mM (^{32}P)ATP for 5 min and (B) 1 mM (^{32}P)ATP for 20 min in the presence (●—●) and absence (○—○) of 0.2 mM cyclic 3′,5′-AMP.

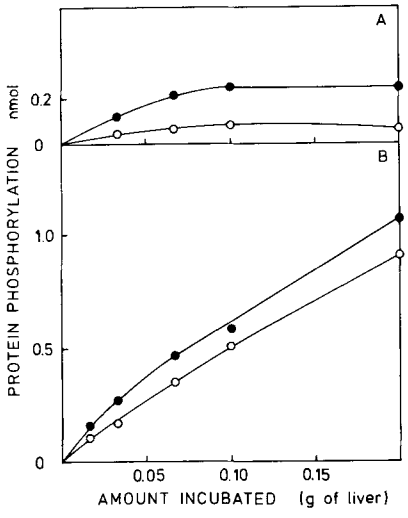

Fig. 4. Phosphorylation of different amounts of Fraction 3 on incubation with (A) 0.1 mM (^{32}P)ATP for 5 min and (B) 1 mM (^{32}P)ATP for 20 min in the presence (●—●) and absence (○—○) of 0.2 mM cyclic 3′,5′-AMP.

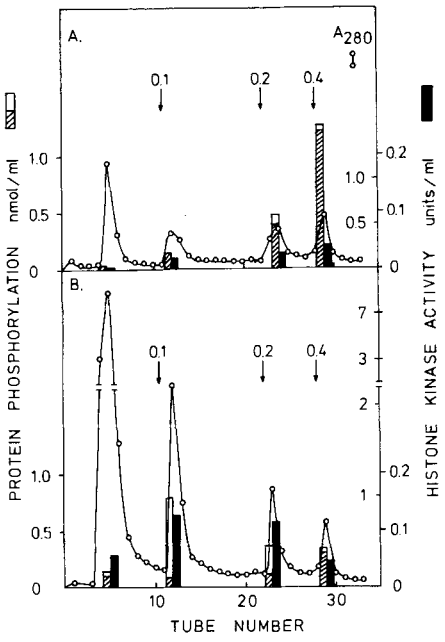

Fig. 5. DEAE-cellulose chromatogram of (A) Fraction 1 from 6.6 g of liver and (B) Fraction 3 from 6.8 g of liver. 1.7×6.3 cm columns eluted with 50 mM Tris-acetic acid buffer (pH 7.5) containing 1 mM EDTA, 5% sucrose and 0.1 mM dithiothreitol. At the arrows, the buffer was changed to buffers also containing 0.1, 0.2 and 0.4 M sodium acetate, respectively. 10 ml fractions were eluted every 10 min and analyzed for absorbance at 280 nm (O—O). Protein phosphorylation and histone kinase activity were estimated in the fractions with the highest protein concentration after each change of elution buffer. Open bars denote phosphorylation in the presence of cyclic 3′,5′-AMP; cross-hatched in the absence of cyclic 3′,5′-AMP.

min or with 1 mM (^{32}P)ATP for 20 min the results shown in Figs. 3–4 were obtained. From the curves the range for a nearly linear relationship between phosphate incorporation and amount of sample incubated could be estimated and used for the quantitative phosphorylation tests.

Isolation of (^{32}P)SerP and (^{32}P)ThrP from (^{32}P)ATP-incubated Fractions 1 and 3

From an acid hydrolysate of (^{32}P)ATP-incubated fraction 1, (^{32}P)SerP and (^{32}P)ThrP were isolated by chromatography on Dowex 50×8 and Dowex 1×8. The ratio of (^{32}P)SerP to (^{32}P)ThrP obtained after correction for losses during hydrolysis was about 20. From Fraction 3 (^{32}P)SerP was isolated, but not significant amounts of (^{32}P)ThrP.

DEAE-cellulose chromatography of Fractions 1 and 3

Fractions 1 and 3 from cell sap incubated at 20°C for 60 min were chromatographed on DEAE-cellulose eluted stepwise as described in Fig. 5. The chromatographic fractions with the highest protein concentration appearing after each change of elution buffer were tested for phosphate-incorporating activity by incubation with 0.1 mM (^{32}P)ATP for 5 min, in the presence or absence of cyclic 3′,5′-AMP. In addition, the fractions were tested for protein kinase activity.

As seen in Fig. 5A, most of the phosphate-incorporating activity of Fraction 1 was eluted with the buffer containing 0.4 M sodium acetate (Fraction 1:0.4). Some phosphate-incorporating material also appeared with the buffer containing 0.2 M sodium acetate. The dependence of these fractions on cyclic 3′,5′-AMP was very low.

As shown in Fig. 5B, on chromatography of Fraction 3 most phosphate-incorporating material was eluted with the buffers containing 0.1 and 0.2 M sodium acetate (Fractions 3 : 0.1 and 3 : 0.2, respectively). The phosphorylation of this protein was markedly stimulated by cyclic 3′,5′-AMP, in contrast to the low protein-phosphorylation of the protein eluted in the last peak (Fraction 3 : 0.4).

Histone kinase activity was found mainly in the chromatographic fractions of Fraction 3.

The DEAE-cellulose chromatograms of material from fed rats were very similar to those of material from rats that had been fasting for 16–20 h. Incubation of the cell sap at 20°C did not influence the appearance of the chromatogram appreciably.

Resolution of phosphorylated protein on polyacrylamide gel electrophoresis

In order to estimate the number and relative amounts of phosphorylated proteins, polyacrylamide gel electrophoresis in dodecyl sulfate was performed on ^{32}P-labelled fractions derived from incubated cell sap from fed rats. Fraction 1 and its DEAE-cellulose subfractions displayed a very heterogeneous pattern, as exemplified by Fraction 1 : 0.4 (Fig. 6A). The pattern of Fraction 3, on the other hand, was less complex, with two dominating ^{32}P-labelled components (Fig. 6C). After DEAE-cellulose chromatography of Fraction 3, these components were recovered in Fraction

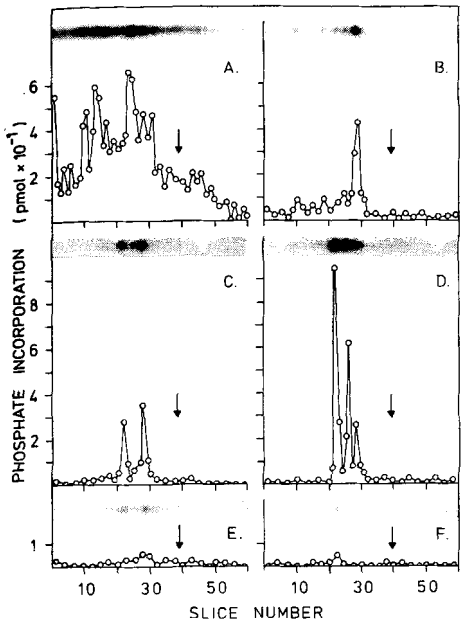

Fig. 6. Polyacrylamide gel electrophoresis in dodecyl sulfate of phosphorylated protein fractions. 100 μl of Fractions 1, 3, 3:0.1 and 3:0.2 (containing 0.22, 0.22, 0.10, and 0.05 mg of protein, respectively), were phosphorylated in the presence of cyclic 3′,5′-AMP (A-D). In addition, Fractions 3 and 3:0.1 were phosphorylated in the absence of the cyclic nucleotide (E and F). Electrophoresis was performed in duplicate. One of each gel pair was halved longitudinally and the radioactivity of each slice determined. The phosphate incorporation values given are calculated to correspond to a total amount of 1 mg of protein for each gel. The other gel was used for radioautography, the result of which is shown in the figure. The arrows indicate the relative position of pepsin. (A) Fraction 1:0.4, (B) Fraction 3:0.2, (C and E) Fraction 3, and (D and F) Fraction 3:0.1

3:0.1 with a third component interposed between them (Fig. 6D). Fraction 3:0.2 mainly contained the component with the greatest mobility (Fig. 6B). Fraction 3:0.4—not shown—resulted in several diffuse bands. When cyclic 3′,5′-AMP was omitted, the phosphate incorporation into all the main phosphorylated components described above for Fractions 3 and 3:0.1 was almost abolished (Fig. 6E and F).

The molecular weights of the ^{32}P-labelled components from the material of Fraction 3:0.1 were determined by polyacrylamide gel electrophoresis in dodecyl sulfate with reference protein substances of known molecular weights. Figs. 7–8 illustrate a typical experiment. The estimated molecular weights for the three components corresponded to about 62000, 55000 and 47000, respectively.

When Fraction 3:0.1 was incubated with ^{32}P(ATP) in the presence of rabbit muscle phosphorylase *b* kinase, an additional ^{32}P-labelled component with an estimated molecular weight of 93000 appeared (Fig. 7B). It had the same relative mobility as the subunit of rabbit muscle phosphorylase *b* used as a reference. It is concluded that this additional phosphorylated peak derived from endogenous phosphorylase *b* in the fraction being phosphorylated by the added phosphorylase *b* kinase.

DISCUSSION

In this investigation it was obvious that cyclic 3′,5′-AMP greatly stimulated the phosphorylation of some proteins, while it had no or only

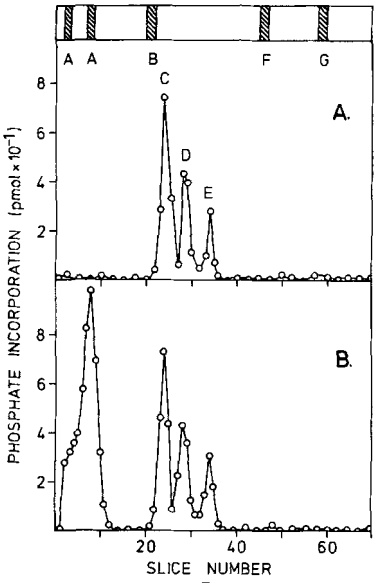

Fig. 7. Polyacrylamide gel electrophoresis of Fraction 3:0.1 and marker proteins on 10% polyacrylamide gels in dodecyl sulfate. Material corresponding to 0.22 mg of proteins was phosphorylated in the absence (Fig. 7A) and presence (Fig. 7B) of added phosphorylase *b* kinase. Phosphorylation and electrophoresis were performed as described under Experimental. The values given for the phosphate incorporation are calculated to correspond to a total of 1 mg of protein. The bands are designated as follows: (A) phosphorylase *b* (giving two bands), (B) bovine serum albumin, (C–E) Fraction 3:0.1 components, (F) pepsin, and (G) α-chymotrypsinogen.

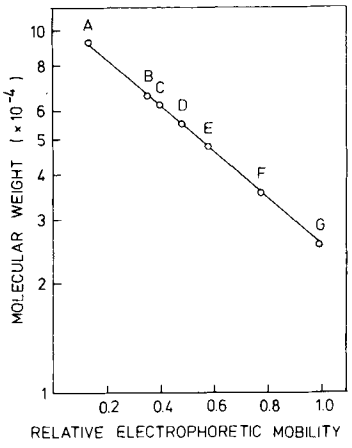

Fig. 8. The electrophoretic mobilities of phosphorylated Fraction 3:0.1 and marker proteins plotted against the logarithm of their molecular weights (22). (A) phosphorylase *b* (molecular weight 92500 (20), (B) bovine serum albumin (66000), (C–E) Fraction 3:0.1 protein components corresponding to molecular weights of 62000, 55000, and 47000, respectively, (F) pepsin (35000), and (G) α-chymotrypsinogen (25700). The electrophoretic mobilities are given relative to the mobility of α-chymotrypsinogen.

a very small effect on the phosphorylation of others. This supports the view that cyclic 3′,5′-AMP-dependent kinases have a substrate specificity which is different from that of cyclic 3′,5′-AMP-independent kinases, which means that the phosphoproteins formed in kinase-catalyzed reactions might be divided into the two corresponding groups.

The quantitative data for the protein phosphorylations must be interpreted with caution. This is because the reactions are dependent on the presence of both protein kinase activity and phosphorylatable protein in the same fractions. In addition, the possible presence of ATPase (EC 3.6.1.3), adenylate kinase (EC 2.7.4.3) and phosphoprotein phosphatase (EC 3.1.3.16) in some fractions might influence the protein phosphorylation, as described for unfractioned cell sap (17).

On polyacrylamide gel electrophoresis under dissociating conditions several proteins were shown to be labelled upon incubation with (^{32}P)ATP. While material from Fraction 1 displayed a complex pattern, Fraction 3 contained only two major phosphorylatable proteins. On DEAE-cellulose chromatography an additional component appeared, eluted with 0.1 M sodium acetate. It is possible that the protein corresponding to this component was not phosphorylated in Fraction 3 due to the presence of an inhibitor or a specific protein phosphatase. It is also possible that the chromatography and the resulting higher ionic strength might have dissociated a protein complex, unmasking an additional phosphorylatable protein. In addition, the possibility that the component might have appeared as a result of proteolytic cleavage of the phosphorylated protein with the highest molecular weight cannot be ruled out. As omission of cyclic 3′,5′-AMP resulted in a very low incorporation of phosphate into all three components, it is concluded that the corresponding proteins were all phosphorylated by cyclic 3′,5′-AMP-dependent protein kinase.

In view of the results presented in Table I and Figs. 6 and 7 it is unlikely that these proteins were active phosphorylase, phosphorylase kinase or glycogen synthetase. As the estimated molecular weights of the ^{32}P-labelled components were fairly high (47000, 55000 and 62000, respectively), it is improbable that they were derived from histones, considering the generally lower molecular weights of such proteins (3). A phosphorylation of ribosomal proteins has been described by several authors (10). At present the possibility cannot be excluded that any of the main phosphorylated components were of ribosomal origin, even if most ribosomal proteins give lower molecular weights on polyacrylamide gel electrophoresis in detergent (4). However, due to the known metabolic effects of cyclic 3′,5′-AMP it seems probable that they originated from enzymes which are regulatively phosphorylated–dephosphorylated during their action. As is shown in another paper (18) the component with the highest molecular weight derives from pyruvate kinase (EC 2.7.1.40).

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