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Differential effects of polyamines on the phosphorylation of chromatin-associated proteins

Khalil AHMED,* Alan T. DAVIS and Said A. GOUELI
Toxicology Research Laboratory, Department of Laboratory Medicine and Pathology, University of Minnesota, Veterans Administration Medical Center, Minneapolis, MN 55417, U.S.A.

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Studies are presented on the nature of chromatin-associated phosphoproteins whose phosphorylation is influenced by polyamines. After labelling with ^{32}P, chromatin-associated proteins were separated into four fractions. Fraction I comprised neutral and basic non-histone phosphoproteins, including high-mobility-group non-histones; fraction II consisted mostly of histones; fraction III consisted of a class of (salt-soluble) acidic non-histone phosphoproteins; and fraction IV consisted of residual (salt-insoluble) acidic non-histone phosphoproteins. The average relative distribution of protein in the four fractions (I–IV) was about 1:4:2:1 for both liver and prostate. However, tissue-dependent differences were observed in the incorporation of ^{32}P in various protein fractions. In the presence of polyamines (e.g. 1mM-spermine or 2mM-spermidine) maximal stimulation of phosphorylation was observed in non-histone proteins of fraction I (160–180%), followed by that in non-histone proteins of fraction III (80–110%). The phosphorylation of residual non-histone proteins in fraction IV, and the small extent of phosphorylation of histones in fraction II, remained unaltered in the presence of polyamines. Thus polyamines do not stimulate the phosphorylation of all non-histone proteins; their stimulative effect is most prominent in the phosphorylation of neutral and basic non-histone proteins and a class of salt-soluble acidic non-histone proteins. In accord with our hypothesis, these differential effects of polyamines on phosphorylation of endogenous non-histone proteins may relate to the conformation of these substrates rather than to endogenous kinases.

The phosphorylation of chromosomal proteins (and especially non-histone proteins), which have been implicated as regulatory agents in the control of transcription (for reviews see, e.g., Allfrey, 1974; Olson & Busch, 1974; Stein *et al.*, 1974; Ahmed, 1975; Gilmour & Paul, 1975; Ahmed & Wilson, 1978) is mediated by the endogenous nuclear protein kinase reactions (Ahmed & Ishida, 1971; Teng *et al.*, 1971; Ishida & Ahmed, 1974; Kish & Kleinsmith, 1974). In the prostate, these reactions may be germane to androgen action on this target organ, as they are profoundly and differentially influenced by the androgenic status of the animal (Ahmed, 1975; Ahmed & Wilson, 1978; Ahmed *et al.*, 1979, 1981*a*; Goueli *et al.*, 1980).

The activity of nuclear protein kinases (regardless of the tissue from which they are derived) is influenced *in vitro* by a number of factors (Kaplowitz *et al.*, 1971; Ahmed *et al.*, 1979) including the aliphatic polyamines (spermine, spermidine) (Imai *et al.*, 1975; Ahmed *et al.*, 1978). The precise function of these naturally occurring amines remains unclear; however, they appear to be involved in growth-related activities of cells (for reviews see, e.g., Williams-Ashman *et al.*, 1969, 1976; Tabor & Tabor, 1976; Scalabrino & Ferioli, 1981). In the prostate, their synthesis is strictly controlled by androgenic hormones, as are the development and function of this organ (Williams-Ashman *et al.*, 1969, 1976; Pegg *et al.*, 1970). In view of this, and the possible relevance of prostatic nuclear phosphoproteins to the mechanism of androgen action, we decided to examine the effects of polyamines on various prostatic chromatin-associated protein kinases.

In a previous paper (Ahmed *et al.*, 1978) we reported that polyamines stimulated chromatin- and

Abbreviation used: SDS, sodium dodecyl sulphate.

* To whom reprint requests should be addressed, at: Veterans Administration Medical Center, 4801 East 54th Street, Minneapolis, MN 55417, U.S.A.

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non-histone-protein-associated protein kinase reactions that utilized exogenous acidic-proteins (such as dephosphophosvitin) or endogenous non-histone proteins of chromatin, but not exogenous or endogenous histones, as substrates. We also proposed that these effects of polyamines might be mediated, at least in part, by their influencing the conformation of the phosphoprotein acceptor. We now report that among the various chromatin-associated non-histone proteins, the phosphorylation of a neutral and basic non-histone-protein fraction is stimulated the most by polyamines, whereas only some of the acidic non-histone proteins respond similarly. A preliminary account of this work has been given (Ahmed *et al.*, 1981*b*).

Materials and methods

Animals

Male Sprague–Dawley rats weighing 295–325g (from Harlan/Sprague–Dawley Co., Madison, WI, U.S.A.) were used as the source of ventral prostate and liver as before (Ahmed *et al.*, 1978).

Chemicals

Polyamines (spermine and spermidine hydrochlorides) and other aliphatic amines were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. They were adjusted to pH 7.4 before use by adding an appropriate amount of Tris base. All other reagents used were of the highest purity available.

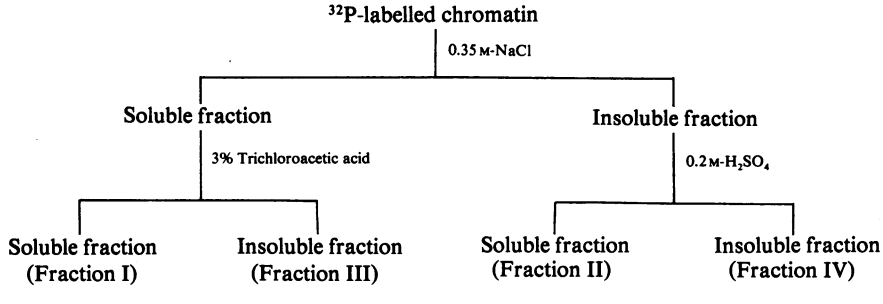
Preparation of ^{32}P-labelled chromatin

The details of preparation and properties of purified rat ventral-prostate nuclei and water-soluble chromatin material derived therefrom have been given (Ahmed, 1971; Ahmed & Wilson, 1975; Ahmed *et al.*, 1978). Chromatin (equivalent to 2.0mg of protein) was labelled with ^{32}P by incubation for 30min at 37°C in a reaction medium consisting of 30mM-Tris/HCl (pH 7.45 at 37°C), 5mM-$MgCl_2$, 1mM-dithiothreitol and 5μM-[γ-^{32}P]-ATP (sp. radioactivity 2.5×10^6d.p.m./nmol of ATP) in a final volume of 5ml. Under these experimental conditions, ^{32}P incorporation into chromatin proteins attained a steady state. It was previously established that polyamines stimulated by 48% the extent of ^{32}P incorporation in total chromatin-associated proteins (Ahmed *et al.*, 1978).

Fractionation of ^{32}P-labelled chromatin

^{32}P-labelled chromatin prepared as above was fractionated into four groups of proteins by the protocol shown in Scheme 1. At the end of the reaction sufficient 2M-NaCl was added, with rapid mixing, to give a final NaCl concentration of 0.35M. The mixture was stirred on ice for 60min and was then centrifuged at 10000*g* for 10min. The supernatant was collected, and the pellet was re-extracted with 0.35M-NaCl in a similar manner. The pellet was kept and the supernatant fractions were pooled. The latter was made up to 3% (w/v) with respect to trichloroacetic acid, and centrifuged at 20000*g* for 20min. The residue was designated 'fraction III' and consists of 'acidic' non-histone proteins, which are loosely bound to chromatin. The supernatant fraction from this step was further treated as described by Goodwin *et al.* (1975), to yield the final pellet, which was dried under vacuum and stored at −20°C. This fraction (designated 'fraction I') represents the 'neutral and basic' non-histone proteins (Ahmed *et al.*, 1980), including the 'high-mobility-group' non-histone (HMG) proteins (Goodwin *et al.*, 1975).

The residue obtained after the initial 0.35M-NaCl extraction was treated with 0.2M-H_2SO_4 for 60min on ice. After centrifugation at 10000*g* for 10min, the pellet was re-extracted similarly for 30min. The two supernatant fractions were pooled and treated as described by Bonner *et al.* (1968) to yield 'fraction II', which consists of histones. The pellet remaining after the 0.2M-H_2SO_4 extraction step comprises



Scheme 1. *Procedure for the fractionation of ^{32}P-labelled chromatin proteins*
For details, please see the text.

residual (salt-insoluble) acidic non-histone proteins, and is designated 'fraction IV'. Fractions III and IV isolated above were further processed by the procedure of Levy *et al.* (1972) to remove DNA and RNA. The supernatants were dialysed against 0.1% 2-mercaptoethanol for a period of 8h with two changes (4 litres each time). The non-diffusible material was freeze-dried and stored at −20°C.

Polyacrylamide-gel electrophoresis of various protein fractions

The proteins in fraction I (neutral and basic non-histones) and fraction II (histones) were analysed by the procedure of Panyim & Chalkley (1969). ^{32}P-labelled protein samples (generally 45–75μg of protein) were dissolved in 0.9M-acetic acid/6M-urea/15% (w/v) sucrose. After electrophoresis, the gels were stained directly with Coomassie Brilliant Blue G-250 (Blakesley & Boezi, 1977) or were fixed in 20% (w/v) trichloroacetic acid.

The proteins in fractions III and IV (i.e. fractions containing acidic non-histones) were analysed by SDS/urea/polyacrylamide-gel electrophoresis by the procedure of MacGillivray *et al.* (1972). ^{32}P-labelled protein samples as above (30–100μg) were dissolved in 8M-urea/0.1% (w/v) SDS/1% 2-mercaptoethanol/0.01M-sodium phosphate buffer, pH7.0. After electrophoresis the gels were fixed in 20% (w/v) trichloroacetic acid for measurement of radioactivity. Duplicate gels were washed successively with 33% (v/v) methanol/10% (w/v) trichloroacetic acid and 10% (w/v) trichloroacetic acid, each for 2–4h, and then were stained with Coomassie Brilliant Blue G-250 (Blakesley & Boezi, 1977).

Measurement of radioactivity

Gels fixed in 20% (w/v) trichloroacetic acid were sliced into 1mm-thick pieces with a Mickle Gel Slicer. Individual slices were placed in vials containing 5ml of Omnifluor scintillation fluid (New England Nuclear, Boston, MA, U.S.A.) and their radioactivity was determined with a Packard Tri-Carb Spectrometer.

Other procedures

All pH values refer to final medium or reaction pH at the given temperature, and were measured with pH-meter 26 (Radiometer, Copenhagen, Denmark) with a glass and Ag/AgCl combination electrode (A. H. Thomas Co., Philadelphia, PA, U.S.A.) standardized at the appropriate temperature. Protein was assayed by the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Distribution of protein and ^{32}P radioactivity in various protein fractions extracted from ^{32}P-labelled chromatin

Chromatin-associated proteins were separated into the four fractions (I–IV) described above by a combination of procedures as detailed in the Materials and methods section (Scheme 1); in essence, these fractions represent groups of proteins based on their physical-chemical characteristics. A summary of the amounts of protein and radioactivity in various fractions from prostatic and liver ^{32}P-labelled chromatin is given in Table 1. The initial extraction with 0.35M-NaCl separated the chromatin proteins into loosely and tightly bound proteins. The former was then fractionated into two groups based on solubility in 3% trichloroacetic acid; those that were soluble (fraction I) represent the neutral and basic non-histones (including the HMG proteins), whereas the insoluble proteins are a class of acidic non-histone proteins (fraction III). These acidic non-histones, because of their easy extraction in 0.35M-NaCl, are believed to be loosely bound to DNA in chromatin. The fraction that was insoluble in 0.35M-NaCl, i.e. bound tightly to chromatin, was separated into two groups based on its solubility in 0.2M-H_2SO_4. This procedure yielded

Table 1. *Recovery of protein and ^{32}P radioactivity in various protein fractions extracted from ^{32}P-labelled chromatin of rat ventral prostate and liver*

Chromatin (equivalent to 2.0mg of protein) was labelled to a steady state with ^{32}P from [γ-^{32}P]ATP, and separated into four fractions as described in the Materials and methods section.

	Ventral-prostate chromatin			Liver chromatin		
	Amount of protein recovered			Amount of protein recovered		
Protein fraction	(mg)	(%)	^{32}P (pmol/mg of protein)	(mg)	(%)	^{32}P (pmol/mg of protein)
I	154	11	822	183	12	493
II	723	49	32	760	48	40
III	400	27	320	385	24	339
IV	190	13	207	261	16	519

a fraction (II) predominantly rich in the basic proteins, histones, and a residual fraction (IV) representing another class of acidic non-histone proteins which differ from those in fraction III in their apparent affinity for DNA in chromatin.

In accord with other studies, nearly half (49%) of the protein was present in fraction II, i.e. as histones. The acidic non-histones in fractions III and IV comprised 28% and 12% of the protein respectively. The smallest fraction belonged to the neutral and basic non-histone group (fraction I), which constituted 11% of the total protein. These recovery values are based on experiments with ^{32}P-labelled chromatin from the ventral prostate. Essentially similar results were obtained with chromatin from liver. However, the distribution of radioactivity in the above fractions did not correlate with the amounts of protein present. On the basis of results with prostate chromatin, the highest specific radioactivity was observed in the neutral and basic non-histones (fraction I), followed by somewhat lower values for proteins of fractions III and IV. The histones (fraction II) as demonstrated previously (Ahmed *et al.*, 1978; Wilson & Ahmed, 1977), contained the lowest amount of radioactivity. The results with liver chromatin were qualitatively similar to those with prostate chromatin, although some quantitative differences in the two were apparent (Table 1). In particular, it appears that the amount of radioactivity per mg of non-histone proteins was lower in fraction I but higher in fraction IV in liver, as compared with corresponding fractions of prostatic chromatin. These differences may relate to tissue-specific amounts of either substrates or enzymes involved in their phosphorylation.

Effects of various amines on ^{32}P incorporation in chromatin proteins

The effects of spermine, spermidine and other amines on the phosphorylation of proteins in various prostatic chromatin fractions (as above) were investigated (Figs. 1–4). Univalent salts (at about 150mM) can also stimulate nuclear-associated protein kinase reactions (Ahmed, 1975; Ahmed & Wilson, 1975; Ahmed *et al.*, 1978). To provide an equivalent increment in total ionic strength to that given by 1mM-spermine at pH 7.4 and 37°C (Hirschman *et al.*, 1967), controls containing 8mM-NaCl were also included for comparison. Mg^{2+} does not replace polyamines in those reactions, and indeed an optimal concentration of Mg^{2+} (about 5mM) is essential for the stimulative effects of polyamines (Ahmed *et al.*, 1978). Other bivalent cations, such as Ca^{2+}, Mn^{2+}, Fe^{2+} and Co^{2+}, were inhibitory at this concentration. The results in Fig. 1(*a*) demonstrate that ^{32}P incorporation in proteins of fraction I (i.e. neutral and basic non-histones) was the same in the presence of absence of 8mM-NaCl.

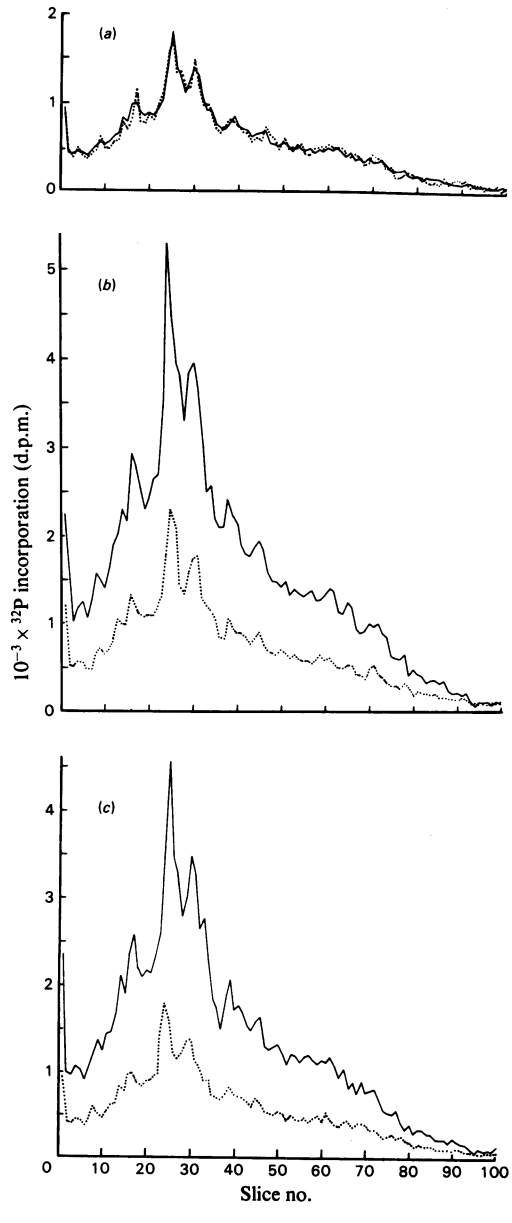


Fig. 1. *Effects of polyamines on ^{32}P incorporation in proteins of fraction I isolated from ^{32}P-labelled chromatin from rat ventral prostate*

Chromatin (equivalent to 2.0mg of protein) was labelled with ^{32}P, and fraction I was isolated as described in the Materials and methods section. Fraction I is as defined in Scheme 1. (*a*) ——, control (no additions); ····, 8mM-NaCl; (*b*) ——, 1mM-spermine; ····, 4mM-hexane-1,6-diamine; (*c*) ——, 2mM-spermidine; ····, 0.5mM-methylglyoxal bis(guanylhydrazone). Gel electrophoresis was from left (+) to right (−). Each slice is 1mm in thickness. All other details were as described in the Materials and methods section.

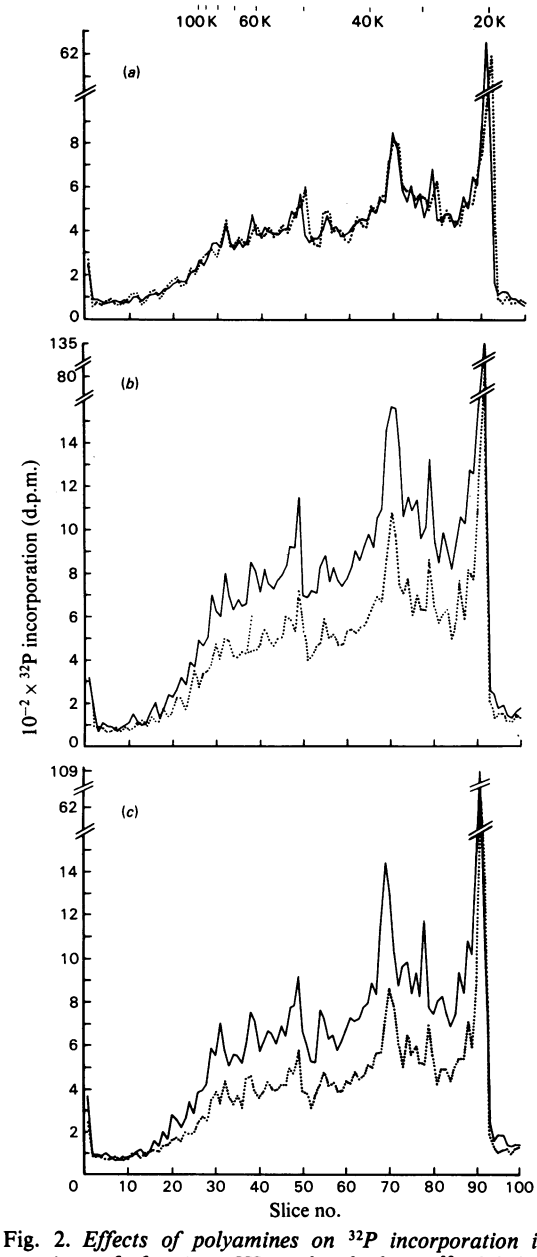


Fig. 2. *Effects of polyamines on ^{32}P incorporation in proteins of fraction III isolated from ^{32}P-labelled chromatin from rat ventral prostate*

All experimental details were the same as for Fig. 1. Fraction III is as defined in Scheme 1. Gel electrophoresis was from left (−) to right (+). The legend to each of the three panels is the same as for the respective panels in Fig. 1. Molecular weights are indicated on the top ('100K' = 100000 etc.)

Several peaks of radioactivity were observed and were present in slowly migrating proteins in the acid/urea/acrylamide gels (e.g. in slice nos. 10–40). In the presence of 1mM-spermine (Fig. 1*b*) or 2mM-spermidine (Fig. 1*c*) a severalfold increase in phosphorylation of several proteins was observed. This effect was most pronounced in proteins with somewhat slower mobility (i.e. slice nos. 10–40) than in those with higher mobility (slice nos. 40–90). By comparison, hexane-1,6-diamine, which is not a naturally occurring polyamine, had no effect on these phosphoproteins. Even the more highly ionized polyamine analogue methylglyoxal bis-(guanylhydrazone) {1,1′-[(methylethanediylidine)-dinitrilo]diguanidine}, which is also a specific inhibitor of putrescine-activated *S*-adenosylmethionine decarboxylase (Williams-Ashman & Schenone, 1972), did not stimulate protein phosphorylation. Two-dimensional gel electrophoresis (acid/urea disc gel in the first and SDS/urea slab gel in the second dimension) followed by autoradiography revealed that highly phosphorylated proteins ranged in molecular weight from 15000 to 100000, the prominent bands being present in the 100000, 80000, 65000, 60000, 40000, 35000, 30000, 25000, 20000 and 17000 regions (results not shown). Previously it was shown that proteins HMG1 and HMG2 were not phosphorylated in chromatin (Ahmed *et al.*, 1980), whereas HMG 14 and HMG 17 were (Saffer & Glazer, 1980). These were not specifically identified in the above fraction by us.

Fraction III (loosely bound acidic non-histone proteins) also contained numerous radioactively labelled proteins with different mobilities on the gel, although less radioactivity was present in the high-molecular-weight ('100K') proteins (Figs. 2*a*–2*c*). Spermine and spermidine markedly stimulated ^{32}P incorporation in most of these proteins, although the total extent was somewhat smaller than that in fraction I. Again, no effect of 8mM-NaCl or 4mM-hexane-1,6-diamine or 0.5mM-methylglyoxal bis(guanylhydrazone) was observed.

In contrast with the above results, spermine or spermidine had no or minimal effect on the phosphorylation of acidic non-histone proteins in fraction IV (Figs. 3*a*–3*c*). Although a significant amount of radioactivity was present in several proteins with molecular weights of 70000–80000, and lower than 20000, most of the label was incorporated in proteins with molecular weights of 20000–40000 and about 10000. Likewise, no stimulation of the phosphorylation of histones in fraction II (between slice nos. 50 and 60 on the gel) was observed in the presence of any of the amines tested (Figs. 4*a*–4*c*).

Effect of various amines on phosphorylation of liver chromatin proteins

The above observations of specific polyamine effects on the phosphorylation of select groups of chromosomal proteins were not limited to prostatic chromatin. As shown in Fig. 5, qualitatively similar

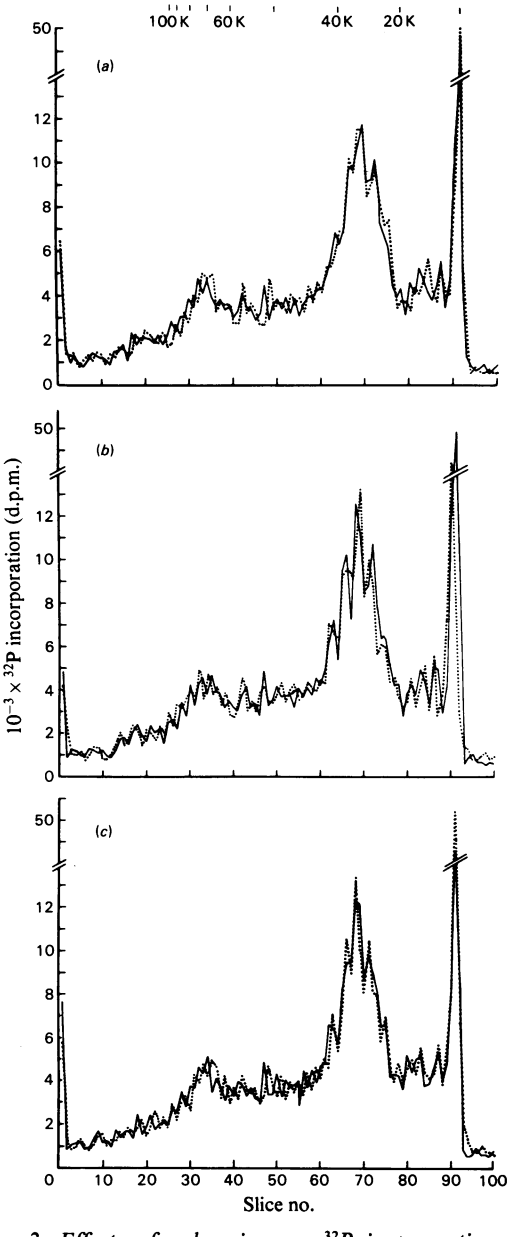


Fig. 3. *Effects of polyamines on ^{32}P incorporation in proteins of fraction IV isolated from ^{32}P-labelled chromatin from rat ventral prostate*

All experimental details were the same as for Fig. 1. Fraction IV is as defined in Scheme 1. Gel electrophoresis was from left (−) to right (+). The legend to each of the three panels is the same as for the respective panels in Fig. 1. Molecular weights are indicated on the top ('100K' = 100000 etc.)

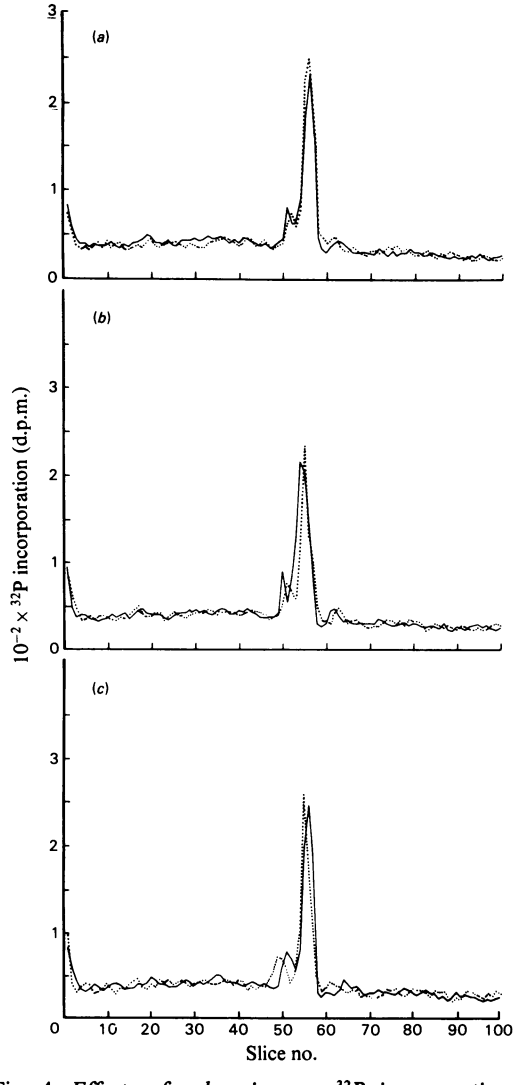


Fig. 4. *Effects of polyamines on ^{32}P incorporation in proteins of fraction II isolated from ^{32}P-labelled chromatin from rat ventral prostate*

All experimental details were the same as for Fig. 1. Fraction II is as defined in Fig. 1. Gel electrophoresis was from left (+) to right (−). The legend to each of the three panels is the same as for the respective panels in Fig. 1.

results were observed with liver chromatin, although some quantitative differences in the degree of effects of polyamines were noted. In particular, 1mM-spermine gave a small but reproducible stimulation (20%) of phosphorylation of some proteins in fraction IV of liver chromatin; this was not observed with prostatic chromatin. Further summary of comparison of these results is as follows: 1mM-spermine stimulated the phosphorylation of fractions I and III of prostatic chromatin by 180% and 110% respectively, whereas 2mM-spermidine stimulated it by 160% and 80% respectively. In the same

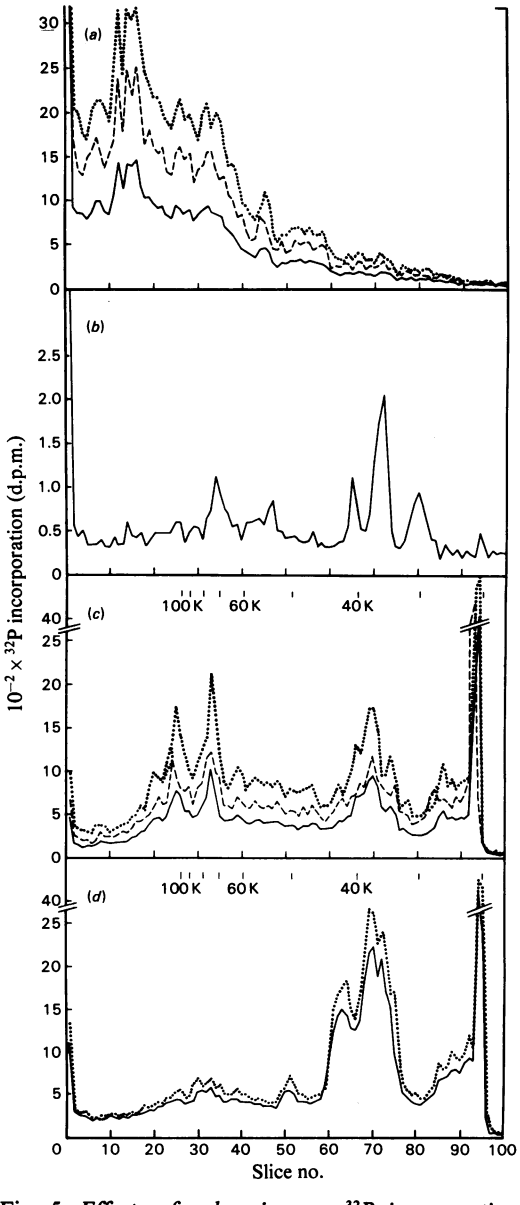


Fig. 5. *Effects of polyamines on ^{32}P incorporation in proteins of various fractions isolated from ^{32}P-labelled chromatin from rat liver*

All experimental details were the same as in Fig. 1. Various protein fractions are as defined in Scheme 1. (*a*) ^{32}P incorporated in proteins of fraction I. ——, Controls (no additions), or 4mM-hexane-1,6-diamine, or 0.5mM-methylglyoxal bis(guanylhydrazone); ····, 1mM-spermine; – – – –, 2mM-spermidine. Gel electrophoresis was from left (+) to right (–). (*b*) ^{32}P incorporated in proteins of fraction II. ——, controls (no additions), or 1mM-spermine, or 2mM-spermidine, or 4mM-hexane-1,6-diamine. or 0.5mM-methylglyoxal bis(guanylhydrazone). Gel electrophoresis was from left (+) to right (–). (*c*) ^{32}P incorporated in proteins of fraction III. The legend for each graph is the same as for Fig. 5(*a*). Gel electrophoresis was from left (–) to right (+). Molecular weights are indicated on top ('100K' = 100000 etc.). (*d*) ^{32}P incorporated in proteins of fraction IV. The legend for the two lines is the same as for Fig. 5(*a*), except that the solid line also represents 2mM-spermidine. Other details are the same as for Fig. 5(*c*).

cases for liver chromatin, the stimulation was 140% and 100%, respectively, in the presence of 1mM-spermine, and 80% and 50%, respectively, in the presence of 2mM-spermidine. Thus, in general, the stimulative effect on phosphorylation of liver chromatin proteins (except that in fraction IV, in the presence of spermine) was somewhat smaller than that for prostatic chromatin proteins. No effect was seen in fraction II in either case. Likewise, no stimulation in any fraction was observed in the presence of 8mM-NaCl, or 4mM-hexane-1,6-diamine, or 0.5mM-methylglyoxal bis(guanylhydrazone).

Discussion

It has previously been shown that the total non-histone protein fraction can serve as substrates for polyamine-stimulated nuclear protein kinase reactions (Imai *et al.*, 1975; Ahmed *et al.*, 1978; Atmar *et al.*, 1978; Farron-Furstenthal & Lightholder, 1978). However, their detailed analysis was not undertaken. In accord with others (Teng *et al.*, 1971; and several reviews cited above), we have observed that many prostatic, as well as liver, chromatin-associated proteins are capable of incorporating ^{32}P from [γ-^{32}P]ATP, mediated via intrinsic protein kinase reactions. The phosphorylation of various protein fractions (and possibly individual proteins within each fraction) appears to be differentially stimulated in the presence of polyamines. Thus phosphorylation of neutral and basic non-histone proteins (Ahmed *et al.*, 1980) was the most responsive to the stimulative effect of polyamines. Phosphorylation of acidic non-histone proteins in fraction III (i.e. salt-extractable, acid-insoluble proteins) was also significantly enhanced in their presence. On the other hand, phosphorylation of the even more highly acidic residual non-histone proteins (fraction IV) did not respond to the stimulative effect of polyamines. The marked differences in the action of polyamines on the phosphorylation of various proteins in chromatin further support our previous conclusion that these effects may relate more to alterations of substrate conformation owing to the charge and geometry of polyamine molecules than to their direct activation of these kinase reactions (Ahmed *et al.*, 1978). In this regard, it is

conceivable that the extent of existing phosphorylation of a protein substrate may be one of the factors which influence its conformation in response to polyamines. This possibility needs further investigation.

Other plausible explanations for a lack of stimulation of phosphorylation of proteins in fraction IV may be considered. It may be argued that proteins in this fraction possess a large amount of endogenous covalently bound polyamines (e.g. Williams-Ashman & Canellakis, 1980), rendering them unresponsive to added amines in the reaction. This seems unlikely, in view of the observations that both liver and prostatic proteins behaved similarly, even though intracellular content of polyamines in the two tissues is markedly different, and the observation (Ahmed *et al.*, 1978) that the percentage stimulation by polyamines of phosphorylation of the prostatic chromatin proteins from castrated animals (i.e. with decreased intracellular polyamine content) was not markedly different from that of normal controls. However, in the absence of clear information on the subcellular distribution of polyamines, a definitive answer to this question will only be obtained by direct analysis of the protein fractions for covalently bound polyamines. It is also possible that lack of polyamine effect on proteins of fraction IV is due to their tight binding to DNA. However, it is noteworthy that polyamines *in vitro* freely reversed the inhibitory effects of added polynucleotides in the non-histone-protein-associated protein kinase reactions (Ahmed *et al.*, 1978). Further, protein kinase reactions associated with non-histone proteins isolated from chromatin and essentially devoid of DNA or RNA are also markedly stimulated by polyamines (Ahmed *et al.*, 1978, 1979).

The stimulation of phosphorylation of nuclear proteins in the presence of polyamines appears to be a general phenomenon with no tissue-specificity. Any possible physiologic significance of these observations remains highly speculative. Since, at least *in vitro*, the phosphorylation of chromatin-associated non-histone proteins is stimulated more by polyamines than by salts such as NaCl, it is conceivable that these naturally occurring amines are preferred in promoting the phosphorylation of specific nuclear proteins which have been implicated in the control of transcription (references cited above).

The particularly high extent of phosphorylation and its stimulation by polyamines in fraction I of prostatic chromatin (as compared with liver) is intriguing in the context of androgen action, since phosphorylation of chromatin apparently enhances its acceptor activity for 5α-dihydrotestosterone–receptor complex (Klyzsejko-Stefanowicz *et al.*, 1976). Presumably, these proteins are among the constituents of non-histone proteins which may serve such acceptor function (Tymoczko & Liao, 1971; Mainwaring *et al.*, 1976; Nyberg & Wang, 1976).

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