Studies *in vitro* of the Effects of Adenosine 3':5'-Cyclic Monophosphate on the Phosphorylation of Nuclear Proteins in Isolated Rat Heart Nuclei

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Isolated rat heart nuclei were prepared by homogenization and sucrose-density-gradient centrifugation. The protein/DNA ratio of these nuclei was 3.1:1 (w/w), and the histones/non-histone proteins/DNA proportions were 1.4:1.6:1 (by wt.). Non-histone proteins were fractionated into six major groups by elution on a quaternized anion-exchanger (QAE-Sephadex A-50) column with increasing concentrations of NaCl in 5M-urea/0.01M-Tris/HCl buffer (pH 8.3). When isolated nuclei were incubated in a medium containing [γ - 32 P]ATP, a differential distribution of 32 P was observed in the six fractions of non-histone proteins. The fractions eluted from the Sephadex column with 0.35M- and 0.6M-NaCl contained 80% of the total radioactivity incorporated into the non-histone proteins. This incorporation into the 0.35M- and 0.6M-NaCl fractions was increased by 66 and 112% respectively in the presence of cyclic AMP. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of these two particular fractions showed a selective increase in labelling of five protein bands in the presence of cyclic AMP.

In recent years considerable effort has been made to understand the mechanism(s) underlying specific control of gene activation and cell proliferation in eukaryotic cells. Non-histone proteins constitute an integral part of chromatin and are involved in the control of the gene expression. Early increases in the synthesis of non-histone proteins have been reported before induced synthesis of DNA in WI-38 human fibroblasts (Tsuboi & Baserga, 1972), lymphocytes (Levy *et al.*, 1973), synchronized HeLa cells (Stein & Baserga, 1970) and salivary glands after administration of isoproterenol (Kleinsmith & Allfrey, 1969). These proteins are extensively phosphorylated (Ahmad & Ishida, 1971; Shea & Kleinsmith, 1973; Johnson *et al.*, 1974), and the extent of phosphorylation appears to determine their ability to interact with DNA (Wang, 1967; Teng *et al.*, 1971; Kleinsmith, 1973). It has been reported that phosphorylation of non-histone proteins might be associated with the increased rate of RNA synthesis (Kamiyama *et al.*, 1972; Platz *et al.*, 1973; Karn *et al.*, 1974), and suggestions have been made that the former might be one of the mechanisms by which gene expression is controlled at the level of RNA transcription (Stein & Baserga, 1972; Shea & Kleinsmith, 1973; Vidali *et al.*, 1973; Garrard *et al.*, 1974; MacGillivray & Rickwood, 1974).

Most of these studies have been limited to tissues such as liver, kidney and salivary glands, in which cell

division is a normal response to growth stimuli, but little work has been reported in the heart, where growth is due to increase in the size of existing cells. In normal growth or in the experimental hypertrophied heart, the muscle cells increase in size without an increase in their numbers (Richter & Kellner, 1953; Moore & Ruska, 1957; Carney & Brown, 1964; Laks *et al.*, 1969). Under these conditions mitoses are never seen in cardiac muscle cells, although there is an increase in DNA and RNA concentrations in experimental hypertrophy (Beznak *et al.*, 1974). However, cardiac enlargement produced in neonatal animals differs from the adult hypertrophied heart in that it is accompanied by a considerable mitotic activity, and this activity declines rapidly after birth (Peterson & Baserga, 1965; Overy & Priest, 1966; Klinge & Stocker, 1968; Shafiq *et al.*, 1968). Mitotic figures can no longer be detected 3 weeks after birth and thereafter the number of cardiac muscle cells remains the same (Zak, 1973). It is likely therefore that the cardiac hypertrophy in the foetal or neonatal period is due to the presence of a hyperplastic component(s) which is lost during the later period of development of the heart. As a part of investigations of the mechanism of the loss of mitotic activity in adult normal or hypertrophied heart, we have studied the phosphorylation of non-histone proteins in isolated rat heart nuclei. Also, in view of the reported increase in adenylate cyclase activity in perfused rat heart with aortic constriction (Schreiber *et al.*, 1971) and in genetic cardiomyopathy in Syrian hamster (Nair & Umali, 1970), we have investigated the effects of cyclic AMP on phos-

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phorylation of non-histone proteins in rat heart nuclei, and the results of such investigations are reported in the present paper.

Experimental

Materials

All chemicals and organic solvents used were of reagent grade. Acrylamide and sodium dodecyl sulphate were purchased from BDH Chemicals, Poole, Dorset, U.K. Ammonium persulphate, *NNN'*-tetramethylethylenamine and bisacrylamide were from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. ATP, cyclic AMP, bovine serum albumin, ovalbumin, trypsin and ribonuclease were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. [γ - 32 P]ATP (over 10 Ci/mmol) was supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Insta-Gel was obtained from Packard Instrument Ltd., Caversham, Berks., U.K., and quaternized anion-exchanger QAE-Sephadex A-50 from Pharmacia (G.B.) Ltd., London W.5, U.K.

Animals

Albino Sprague-Dawley rats weighing 200–300 g and fed *ad libitum* on Purina Laboratory Chow were used for this work. The rats were killed with a blow on the head and the thorax was cut open. The hearts were immediately excised, rinsed twice in ice-cold 0.9% (w/v) NaCl and trimmed of all visible connective tissue. They were either used for the isolation of nuclei on the same day, or frozen in solid CO₂ and stored at –25°C until use. Hearts from 10–15 rats were used for each experiment.

Isolation of nuclei

All procedures, unless otherwise stated, were carried out at 4°C. The procedure for isolation of nuclei was mainly as described by Widnell & Tata (1964). The heart tissue was minced finely with a pair of curved scissors in 3 vol. of a medium composed of 0.25 M-sucrose, 3 mM-CaCl₂ and 0.01 M-Tris/HCl (pH 7.4). The minced tissue was then homogenized for 1.5 min in an Ultra-Turrax TP 18/2 tissue disintegrator (Janke and Kunkel K.G., Staufen i. Br., Germany) run at 80 V (mains, 220 V). The homogenate (35 ml) was filtered through two layers of nylon bolting cloth (mesh 110) and then underlayered by 10 ml of 0.32 M-sucrose/3 mM-CaCl₂/0.01 M-Tris/HCl (pH 7.4) and centrifuged at 700 g for 5 min at 4°C in a refrigerated centrifuge. The pellet was suspended in 25 ml of 0.25 M-sucrose/3 mM-CaCl₂/0.01 M-Tris/HCl (pH 7.4) and the above step repeated once. The pellet was then homogenized in 60 ml of 2.2 M-sucrose/3 mM-CaCl₂/0.01 M-Tris/HCl (pH 7.4), underlayered by 15 ml of the same medium

and centrifuged at 40 000 g for 1 h (SW3 × 23 ml rotor) in an MSE 65 ultracentrifuge. The pelleted nuclei were suspended in 20 ml of 0.25 M-sucrose/3 mM-CaCl₂/0.01 M-Tris/HCl (pH 7.4) and centrifuged at 1000 g for 10 min. This was repeated twice. The nuclear preparations when examined under a phase-contrast microscope were mainly intact and mostly free of myofibrils, erythrocytes, intact muscle cells or any cytoplasmic contamination.

Phosphorylating medium

The medium used for phosphorylation of isolated nuclei was the same as described by Rickwood *et al.* (1973) and contained the following: 0.25 M-sucrose, 0.1 M-Tris/HCl (pH 8.3), 0.01 M-MgCl₂, 0.025 M-NaCl, 0.01 mM-ATP, 1 mM- β -mercaptoethanol and 100 μ Ci of [γ - 32 P]ATP/ml. This medium will be referred to as the 'phosphorylating medium' in the text. The ratio of the weight of nuclei to [γ - 32 P]ATP in the phosphorylating medium was always kept constant. For each 100 mg (wet wt.) of the isolated nuclei, 1 ml of the phosphorylating medium was used for incubation.

Phosphorylation of isolated nuclei

A known amount of nuclei was suspended in the phosphorylating medium and incubated for 6 min at 37°C. The reaction was stopped by the addition of 10 ml of ice-cold phosphorylating medium, from which [γ - 32 P]ATP was omitted but containing 2.5 mM-ATP. The nuclei were pelleted by centrifugation at 1000 g for 10 min. The supernatant was poured off and the nuclei were washed twice in the non-radioactive phosphorylating medium before extraction of the nuclear proteins.

Extraction and dissociation of chromatin

The nuclei were homogenized twice for 15 s in 10 vol. of 0.14 M-NaCl/0.01 M-EDTA/0.1 M-Tris/HCl (pH 7.4) with the aid of a Potter homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 1000 g for 10 min. The supernatants from the two extractions were pooled and taken as 'saline-soluble proteins' fraction. The pelleted chromatin was homogenized in 20 vol. of 7 M-urea/3 M-NaCl and the homogenate stirred at 4°C for 30 min. The dissociated chromatin was then centrifuged for 24 h at 190 000 g to sediment DNA. The supernatant, which contained histones and non-histone proteins, was subjected to ion-exchange chromatography as described in the following section.

Fractionation of chromatin proteins

The method of Gilmour & Paul (1970) as modified by Chaudhuri (1973) was used to fractionate the

chromatin proteins on a QAE-Sephadex A-50 column. The supernatant from the previous step, usually 10ml, was extensively dialysed against 3×200 ml of 5M-urea/0.01M-Tris/HCl (pH8.3)/1mM- β -mercaptoethanol for 24h. The dialysed protein sample (10–12ml) was applied to a column (20cm $\times$ 1.2cm) of QAE-Sephadex A-50, which was equilibrated at room temperature with 5M-urea/0.01M-Tris/HCl (pH8.3)/1mM- β -mercaptoethanol. After the sample had passed into the Sephadex column, the flow was stopped. After 10min the flow was resumed and the column washed with 75 ml of the equilibration buffer. The unretained protein fractions consisted of histones. The column was then washed with either 3M-NaCl/5M-urea/0.01M-Tris/HCl (pH8.3) to elute the non-histone proteins in one step or with 75 ml each of 0.05M-, 0.15M-, 0.35M-, 0.6M-, 1.0M- and 3.0M-NaCl in 5M-urea/0.01M-Tris/HCl (pH8.3) to elute the non-histone proteins in six fractions. These fractions are referred in the text as I–VI according to their order of elution from the column (see Table 3).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

The method of Laemmli (1970) was followed to prepare the gels for electrophoresis. The fractions from the QAE-Sephadex A-50 column were processed by the method of MacGillivray *et al.* (1972). After extensive dialysis against 0.1% (w/v) sodium dodecyl sulphate for 48h, the samples were freeze-dried and then dissolved in 8M-urea. They were further dialysed overnight against 8M-urea/1% (w/v) sodium dodecyl sulphate/1mM- β -mercaptoethanol/0.01M-Tris/HCl (pH7.0). The samples were then dialysed at 37°C for 2h against a solution with the same composition as the above buffer except that it contained 0.1% (w/v) sodium dodecyl sulphate. Portions (0.2ml) of these samples (100–150 μ g of protein) mixed with 5 μ l of Bromophenol Blue were applied to the gels and the electrophoresis was carried out at room temperature for 6h at 2mA per gel. The composition of the buffer used was: 3g of Tris, 14.4g of glycine and 1g of sodium dodecyl sulphate made up to 1 litre with water. The gels were stained overnight in Coomassie Brilliant Blue (93mg) dissolved in methanol/water (25:62, v/v) containing sulphosalicylic acid (3g) and trichloroacetic acid (10g). The gels were destained by elution in acetic acid/methanol/water (7:30:63, by vol). After the background colour had considerably decreased the gels were kept in 10% (w/v) acetic acid and then scanned at 620nm with a Chromoscan apparatus (Joyce-Loebl and Co. Ltd., Gateshead, Tyneside, U.K.). For 32 P profiles the gels were sliced into segments of 1 mm thickness with a gel slicer. The proteins used as markers were the following: ribonuclease (mol.wt. 13700), trypsin (mol.wt. 23300),

ovalbumin (mol.wt. 45000) and bovine serum albumin (mol.wt. 68000).

Analytical

DNA was estimated by reading u.v. absorbance at 260nm (21 absorbance units equivalent to 1mg of DNA/ml) and protein by the method of Schaffner & Weissman (1973). Suitable portions of protein samples were counted for radioactivity in 10ml of Insta-Gel (95%, v/v) scintillation fluid in an Inter-technique liquid-scintillation counter.

Results

Nuclear proteins and DNA isolated from rat heart nuclei

The amount of DNA and nuclear proteins are presented in Table 1. The protein/DNA ratio was 3.1:1 (w/w) and the proportions of histones/non-histone/proteins/DNA were 1.4:1.6:1 (by wt.). The proteins soluble in 0.14M-NaCl constituted only 5.5% of the total nuclear proteins. The amount of protein in the DNA pellet was always less than 10% of the total protein in the nuclei (results not shown), indicating that the dissociation of chromatin by 7M-urea/3M-NaCl into DNA and proteins was almost complete. Although the protein recoveries from the QAE-Sephadex column ranged from 82 to 86%, the resolution of the different protein fractions was very high. When histone and non-histone protein fractions were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis very little cross-contamination between these fractions was observed (Plate 1). The non-histone proteins fraction contained traces of histones and separated into about 30 individual bands. The electrophoretic pattern of saline-soluble proteins was similar to that of the non-histone proteins (Plate 1).

Effects of cyclic AMP on phosphorylation of nuclear proteins

The results from experiments carried out to investigate the effects of cyclic AMP on incorporation of 32 P into nuclear proteins are given in Table 2. In the presence of 5 μ M-cyclic AMP (5 μ M was the optimum concentration in the present experimental conditions), 32 P incorporation into the saline-soluble proteins fraction was increased by 37% over that of the control. Under the same conditions the incorporation of 32 P into histone and non-histone protein fractions was increased by 21.8 and 80% respectively.

Distribution of 32 P into different fractions of non-histone proteins

Since non-histone proteins are extremely heterogeneous in nature, it was decided to investigate the distribution of 32 P into different fractions of this

Table 1. *Nuclear proteins and DNA isolated from rat heart nuclei*

Isolated nuclei (120mg wet wt.) were analysed for their contents of proteins and DNA. Nuclear proteins soluble in 0.14M-NaCl/0.01M-EDTA were extracted as described in the text. The dissociated chromatin was centrifuged at 190000g for 24h (to sediment DNA) and the supernatant fractionated on QAE-Sephadex A-50 column. After the histones had passed unretained through the column, the non-histone proteins were eluted in one step with 5M-urea containing 3M-NaCl, 0.01M-Tris/HCl (pH8.3) and 1mM- β -mercaptoethanol. DNA and protein were estimated as described in the text.

Exp. no.	Protein (mg/100mg of isolated nuclei)			DNA (mg)
	Saline-soluble	Histones	Non-histone proteins	
I	0.156	1.666	1.776	1.081
II	0.225	1.477	1.641	1.137
III	0.213	1.597	1.911	1.187
Average	0.198	1.580	1.776	1.135

group of proteins. As shown in Table 3, when isolated nuclei were incubated with [γ - 32 P]ATP and the non-histone proteins fractionated on a QAE-Sephadex A-50 column a differential distribution of 32 P into non-histone protein fractions was observed: 80% of the total radioactivity incorporated into non-histone proteins was associated with fractions III and IV, eluted with 5M-urea containing 0.35M- and 0.6M-NaCl respectively. The largest amount of radioactivity appeared in fraction IV, eluted with 0.6M-NaCl, whereas the lowest incorporation was observed in fraction VI, eluted with 3M-NaCl. The 32 P incorporation into the remaining fractions (I, II and V), eluted with 0.05M-, 0.15M- and 1.0M-NaCl respectively, was 3.3, 10.5 and 4.7% respectively.

Effects of cyclic AMP on phosphorylation of non-histone proteins

The effects of cyclic AMP on the distribution of 32 P into different fractions of non-histone proteins is shown in Table 4. When isolated nuclei were phosphorylated in the presence of cyclic AMP, the largest stimulation of 32 P incorporation into non-histone proteins (112%) was observed in fraction IV eluted from the QAE-Sephadex A-50 column. An increase of 66% was also observed in the specific radioactivity of fraction III. No significant increase in the specific radioactivity was observed in the remaining fractions in the presence of cyclic AMP.

Table 2. *Effects of cyclic AMP on phosphorylation of nuclear proteins in isolated rat heart nuclei*

Isolated nuclei (90mg wet wt.) were incubated for 6min at 37°C in 0.9ml of the phosphorylating medium containing 90 μ Ci of [γ - 32 P]ATP in the presence and absence of 5 μ M-cyclic AMP; 1mM-theophylline was added to both control and test samples. Extraction of saline-soluble proteins and fractionation of histone and non-histone proteins were carried out as described in the text. After the histones had passed unretained through the QAE-Sephadex A-50 column, the non-histone proteins were eluted with 3M-NaCl in 5M-urea/0.01M-Tris/HCl (pH8.3)/1mM- β -mercaptoethanol. The radioactivity in different protein fractions was determined as described in the text. The results are expressed as the means $\pm$ S.E.M. of three experiments, and the significance of the difference was assessed by Student's *t* test. Significance level: **P*<0.01.

Protein fraction	Radioactivity incorporated (c.p.m./mg of protein)		Increase over control (%)
	Control	+5 μ M-Cyclic AMP	
Saline-soluble	19363 $\pm$ 1311	26542 $\pm$ 1034	37.1*
Histones	2019 $\pm$ 59	2459 $\pm$ 68	21.8*
Non-histones	90644 $\pm$ 6068	163184 $\pm$ 9104	80.0*

Polyacrylamide-gel electrophoresis of non-histone protein fractions

In an attempt to investigate the nature of the non-histone proteins in fractions III and IV, which were preferentially phosphorylated in comparison with the other non-histone protein fractions, an electrophoretic analysis of the different non-histone protein fractions was carried out. Densitometer tracings of non-histone proteins separated on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis are given in Fig. 1. There was a strong similarity between the tracings of non-histone proteins in fractions I and II, III and IV, V and VI. The fractions I and II contained, in addition to higher-molecular-weight proteins, a large proportion of proteins of mol.wt. ranging from 13000 to 45000. Fewer low-molecular-weight proteins were present in fractions III and IV, which were richer in proteins of high molecular weights. In contrast, fractions V and VI consisted mainly of two high-molecular-weight proteins.

Distribution of 32 P in non-histone proteins separated on polyacrylamide-gel electrophoresis

Since the protein fractions III and IV contained non-histone proteins that were preferentially phosphorylated in isolated nuclei in the presence of cyclic AMP, the distribution of 32 P in different bands of the

EXPLANATION OF PLATE I

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the nuclear proteins isolated from rat heart nuclei
Saline-soluble, histone and non-histone proteins were isolated from rat heart nuclei as described in the text. Electrophoresis of the proteins in sodium dodecyl sulphate/polyacrylamide-gels were carried out as described in the text. Fractions I–VI (see Table 3) of non-histone proteins are the ones eluted from QAE-Sephadex A-50 column with increasing concentrations of NaCl in 5 M-urea/0.01 M-Tris/HCl (pH 8.3)/1 mM- β -mercaptoethanol.

Table 3. *Phosphorylation of proteins in isolated rat heart nuclei in the presence of [γ - 32 P]ATP*

Isolated nuclei (85 mg wet wt.) were incubated for 6 min at 37°C in 0.85 ml of the phosphorylating medium containing 85 μ Ci of [γ - 32 P]ATP. Saline-soluble proteins were extracted and DNA was sedimented as described in the text. After the histones had passed through the QAE-Sephadex A-50 column, the non-histone proteins were eluted with 75 ml of 5 M-urea/0.01 M-Tris/HCl (pH 8.3)/1 mM- β -mercaptoethanol containing increasing amounts of NaCl as shown in the Table. The results are expressed as the means $\pm$ S.E.M. of three experiments.

Protein fraction	NaCl concn. in the elution buffer (M)	Total protein eluted from the column (mg)	Radioactivity incorporated (c.p.m./mg of protein)	Radioactivity in non-histone proteins (% of total)
Histones	0	1.329 $\pm$ 0.116	2108 $\pm$ 109	—
Non-histones				
I	0.05	0.229 $\pm$ 0.010	3266 $\pm$ 165	3.3
II	0.15	0.265 $\pm$ 0.003	10241 $\pm$ 903	10.5
III	0.35	0.242 $\pm$ 0.006	31924 $\pm$ 1203	32.7
IV	0.6	0.201 $\pm$ 0.007	46672 $\pm$ 2453	47.8
V	1.0	0.175 $\pm$ 0.006	4639 $\pm$ 177	4.7
VI	3.0	0.107 $\pm$ 0.004	973 $\pm$ 81	1.0
Saline-soluble proteins		0.221 $\pm$ 0.012	23871 $\pm$ 109	—

Table 4. *Effects of cyclic AMP on phosphorylation of nuclear proteins in isolated rat heart nuclei*

Isolated nuclei (100 mg wet wt.) were incubated for 6 min at 37°C in 1.0 ml of the phosphorylating medium containing 100 μ Ci of [γ - 32 P]ATP in the presence and absence of 5 μ M-cyclic AMP; 1 mM-theophylline was present in both the control and test samples. Extraction and fractionation of non-histone proteins were as described in the text. The results are expressed as the means $\pm$ S.E.M. of three experiments, and the significance of the difference was assessed by Student's *t* test. Significance level: *not significant; $\dagger P < 0.01$.

Non-histone-proteins fraction	Radioactivity incorporated (c.p.m./mg of protein)		Increase over control (%)
	Control	+5 μ M-Cyclic AMP	
I	4688 $\pm$ 426	5243 $\pm$ 619	11.8*
II	8029 $\pm$ 445	8466 $\pm$ 342	5.4*
III	39721 $\pm$ 869	65936 $\pm$ 1165	66.0 $\dagger$
IV	44129 $\pm$ 1321	93553 $\pm$ 1187	112.0 $\dagger$
V	4152 $\pm$ 418	4665 $\pm$ 506	12.4*
VI	1947 $\pm$ 204	2026 $\pm$ 263	4.1*

proteins on polyacrylamide-gel electrophoresis was investigated. Figs. 2 and 3 show 32 P profiles of fractions III and IV respectively in the presence and absence of cyclic AMP. The profiles of these two fractions were strikingly similar. In the high-molecular-weight region of the profiles, three proteins of approx. mol.wts. 60000, 88000 and 100000 showed average increases of 80, 55 and 140% respectively in 32 P incorporation in the presence of cyclic AMP; another protein of about 120000 mol.wt. also showed increased incorporation, but to a much

lesser extent. In the low-molecular-weight region of the profile only one protein showed an appreciable increase (70% over control): this was of approx. mol.wt. 20000. In addition, there were three other proteins of mol.wts. ranging from 30000 to 45000 in which 32 P incorporation was only slightly increased under these conditions.

Discussion

The exact molecular changes in the heart leading to its hypertrophy are not known. Investigations in the past have been hampered partly by the fact that heart muscle, as compared with liver, kidney and glands, is more resistant to subcellular fractionation. In the present work, by careful and controlled homogenization of rat heart, we have isolated intact nuclei with infrequent cytoplasmic contaminants. Several methods (Wang, 1967; Shirley & Huang, 1969; Suria & Liew, 1974; Racha & Fredericq, 1975) have been used in different laboratories to isolate nuclear proteins from DNA and to separate non-histone proteins from histones. We found the method of Gilmour & Paul (1970) as modified by Chaudhuri (1973) most satisfactory for this purpose. The advantage of this isolation procedure is that it provides a very good separation of non-histone proteins from histones. In addition, the non-histone proteins can be fractionated into six major groups depending on their solubility at different ionic strengths. By the use of this method the protein/DNA ratio in isolated nuclei was 3.1:1 and the histone/non-histone protein/DNA proportions were 1.4:1.6:1. These ratios vary from tissue to tissue and depend on the method used for preparation of nuclei and isolation of proteins (MacGillivray *et al.*, 1972;

Fig. 1. Polyacrylamide-gel electrophoresis of different non-histone-protein fractions isolated from rat heart nuclei. Non-histone proteins were separated into six fractions using QAE-Sephadex A-50. The six fractions (see Table 3) were eluted from the Sephadex column with increasing concentrations of NaCl in 5M-urea/0.01M-Tris/HCl (pH8.3)/1mM- β -mercaptoethanol. Electrophoresis of non-histone proteins in sodium dodecyl sulphate/polyacrylamide gels was carried out as described in the text. After the background had destained considerably, the gels were scanned at 620nm with a Joyce-Loebl Chromoscan instrument.

Fig. 2. Effects of cyclic AMP on incorporation of ^{32}P into non-histone-protein fraction III

Isolated nuclei were incubated in [γ - ^{32}P]ATP containing phosphorylating medium in the presence (●) and absence (○) of 5 μM -cyclic AMP and nuclear proteins were fractionated as described in the text. Non-histone proteins (fraction III; 150 μg) eluted from the QAE-Sephadex A-50 column with 0.35M-NaCl/5M-urea/0.01M-Tris/HCl (pH8.3)/1mM- β -mercaptoethanol, was subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The gels were frozen on solid CO_2 and cut into 1 mm slices and the radioactivity in each slice was determined by using 10ml of 95% (v/v) Insta-Gel.

Gonzalez-Mujica & Mathias, 1973; Suria & Liew, 1974).

Several protein kinases have been detected in the acidic-protein fraction of rat liver nuclei (Kleinsmith, 1975). It was consistently observed in the present work that when isolated nuclei are incubated with [γ - ^{32}P]ATP the non-histone proteins are phosphorylated to a greater extent than histones. This is in general agreement with the pattern of phosphorylation of nuclear proteins in liver (Kleinsmith *et al.*, 1966; Chae *et al.*, 1972) and rat ventral prostate (Ahmad, 1971). A differential distribution of ^{32}P into fractions III and IV (Table 3) suggests that these fractions are probably

Fig. 3. Effects of cyclic AMP on incorporation of ^{32}P into non-histone protein fraction IV

Isolated nuclei were incubated in [$\gamma\text{-}^{32}\text{P}$]ATP containing phosphorylating medium in the presence (●) and absence (○) of $5\mu\text{M}$ -cyclic AMP. The other experimental details were the same as in Fig. 2, except that the non-histone proteins analysed in this experiment were those eluted from the Sephadex column with 0.6M -NaCl in 5M -urea/ 0.01M -Tris/HCl (pH 8.3)/ 1mM - β -mercaptoethanol.

rich in substrates for the protein kinases. One-dimensional gel-electrophoresis, however, showed no significant qualitative difference between these fractions and fractions I, II, V and VI, which were eluted from QAE-Sephadex A-50 with buffers of different ionic strengths.

There has been a controversy about the role of cyclic AMP in regulation of cellular growth. It has been reported that the concentration of cyclic AMP was increased in normal liver cells induced to synthesize DNA and divide (MacManus *et al.*, 1972) and that dibutyryl cyclic AMP caused an increase in nuclear DNA synthesis in liver (Short *et al.*, 1975). In contrast with these observations, studies from several laboratories strongly suggest that cyclic AMP has a regulatory effect in limiting the rate of proliferation of cells in culture. The concentration of cyclic AMP increased as untransformed cells approached confluency and stopped growing (Otten *et al.*, 1972; Anderson & Pastan, 1973). It has also

been reported that the addition of cyclic AMP, analogues of cyclic AMP or agents that raise the intracellular concentrations of cyclic AMP decreased or stopped the growth rate of several cell lines (Ryan & Heidrick, 1968; Sheppard, 1971; Johnson & Pastan, 1972; Blat *et al.*, 1973; Carchman *et al.*, 1974).

The proposed role of cyclic AMP in growth control of cells is further complicated by reports that non-histone proteins are extensively phosphorylated (Kleinsmith & Allfrey, 1969; Ahmad & Ishida, 1971; Shea & Kleinsmith, 1973; Johnson *et al.*, 1974) and this phosphorylation can be related to increased RNA synthesis during the cell cycle (Kamiyama *et al.*, 1972; Platz *et al.*, 1973; Karn *et al.*, 1974). Thus any increase in the concentration of cyclic AMP would result, through activation of protein kinases, in increased phosphorylation of non-histone proteins and thus increased synthesis of RNA. In view of this it seems difficult to reconcile the reported increased concentration of cyclic AMP with the inhibition of growth in different cell lines. In the presence of cyclic AMP a selective stimulation of phosphorylation of some acidic proteins was observed in rat liver nuclei (Johnson & Allfrey, 1972) and bovine anterior pituitary gland (Jolicœur & Labrie, 1974). In the present investigations cyclic AMP caused a general increase in phosphorylation of nearly all non-histone-protein fractions, but a preferential increase in ^{32}P incorporation was observed in fractions III and IV, which were eluted with 0.35M - and 0.6M -NaCl from the Sephadex column. On further analysis of ^{32}P distribution in non-histone proteins in these fractions, a selective increase in incorporation of the label was consistently observed in five proteins of mol.wts. 20000, 60000, 88000, 100000 and 120000. Since about 100 non-histone proteins from rat liver nuclei have been detected by two-dimensional gel electrophoresis (Yeoman *et al.*, 1973), it is possible that these bands might each consist of several protein components. Further isolation and characterization of these proteins should help to clarify the significance of this cyclic AMP-stimulated phosphorylation of non-histone proteins in the heart.

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