Phosphorylation of Rat Thymus Histones, its Control and the Effects thereon of γ-Irradiation

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The phosphate content of rat thymus histones was determined. As expected for a repli-cating tissue, histones 1 and 2B were more phosphorylated and had higher ^{32}P uptakes than did histones from resting liver nuclei; the other histones all showed ^{32}P uptake, but the phosphate content and uptake of histone 2A was about half that for liver histone 2A. When thymus nuclei were incubated in a slightly hypo-osmotic medium, non-histone proteins and phosphorylated histones were released into solution; this was enhanced if ATP was present in the medium. [γ-^{32}P]ATP was incorporated into non-histone proteins, including protein P1, and into the ADP-ribosylated form of histone 1; negligible ^{32}P was incorporated into the other, bound, histones. Histones 1 and 2B added to the incubation medium were extensively, and histones 2A and 4 slightly, phosphorylated. Histones re-leased by increasing the ionic strength of the medium were phosphorylated. Added lyso-zyme and cytochrome *c* were neither bound nor phosphorylated, but added non-histone protein P1 was phosphorylated, causing other histones to be released from the nuclei, especially histones 2A and 3. The released histones were phosphorylated. γ-Irradiation decreased ^{32}P uptake into the non-ADP-ribosylated histones 1 and 4; phosphorylation of histone 1 *in vitro* was unaffected. The importance of non-histone proteins, ATP availa-bility and nuclear protein kinases to the control of histone phosphorylation *in vivo* is discussed.

Phosphorylation of histones is now firmly estab-lished, and quantitative measurements on the extents of phosphorylation and [^{32}P]P_i uptake into the dif-ferent histones are now available for liver in G_0 phase (Ord & Stocken, 1975), but not for thymus, in spite of the extensive studies made on histones from this gland. It is known that [^{32}P]P_i uptake into rat thymus histone 1 is decreased by X-irradiation and that the dose–response curve follows exactly that for ^{32}P up-take into thymus DNA (Ord & Stocken, 1966). Available methods of separation (Candido & Dixon, 1972; Ord & Stocken, 1975) enabled the extents of phosphorylation of thymus histones to be compared with those for liver and the effects of irradiation to be studied in more detail. In thymus, ^{32}P uptakes into histones 4 and 1 were found to be affected equally. Normal restraints on histone phosphorylation were also examined. Dissociation of histone binding to chromatin either by increasing ionic strength or by interaction with non-histone protein promoted phos-phorylation of released histones.

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Materials and Methods

Animals

Male rats of about 120g body wt. were used. When indicated, they were given 100μCi of [^{32}P]P_i (The Radiochemical Centre, Amersham, Bucks., U.K.)/ 100g body wt. intramuscularly 1h before death. Irradiated animals which received 400rd of irradia-tion from a ^{60}Co source were injected immediately after exposure and were killed 1h later.

Isolation, extraction, fractionation and analysis of histones

The histones were prepared from nuclei isolated in 2.2M-sucrose/5mM-MgCl₂/10mM-Tris/HCl, pH7.4, and analysed as described elsewhere (Ord & Stocken, 1975). The identification and purity of histones were established by amino acid analyses and polyacryla-mide-gel electrophoresis (Ord & Stocken, 1975).

ATP content of thymus nuclei from irradiated rats

The pellet of nuclei was suspended in 0.05M-sodium acetate buffer, pH5.1, containing 0.14M-NaCl. Ice-cold 1M-HClO₄ was added to a final concentration of

0.5 M. The sample was kept in ice for 30 min and then centrifuged for 10 min at 5000 g. The nucleotides from the supernatant were absorbed on Darco G 60 (Atlas Powder Co. New York, NY, U.S.A.) and eluted with 50% (v/v) ethanol containing 2.5% (v/v) NH$_3$ at 0°C. The eluate was loaded without concentration at 0°C on a column (0.6 cm diam. × 50 cm long) of Dowex 1 (100–200 mesh). The elution buffer was 1.0 M-acetate buffer, pH 3.6, containing 0.5 M-NaCl. Fractions (2 ml) were collected and assayed by luciferase for ATP content.

^{32}P uptake by thymus nuclei in vitro

Nuclei prepared as above were suspended in 5 mM - MgCl$_2$/10 mM - Tris/HCl (pH 7.4)/1 mM - 2 - mercaptoethanol. The concentration of the suspension was checked turbidimetrically so that when nuclei from normal and irradiated rat thymus were to be compared, the incubations were performed with identical concentrations of nuclei. These concentrations (Burton, 1956) normally contained 10–20 mg of DNA/ml of suspension. Nuclear suspension (0.1–0.2 ml) was incubated for 20 min at 37°C in a final volume of 1 ml containing 50 μmol of Tris/HCl, pH 7.4, 5 μmol of MgCl$_2$, 2 μmol of [γ-^{32}P]ATP (Glynn & Chappell, 1964; 4000–30000 c.p.m./nmol of ATP) and, where indicated, 1 mg of purified rat liver histones (obtained as above). The incubation was stopped by plunging the tubes into ice and centrifuging for 15 min at 100000 g at 2°C to separate the soluble

proteins in the supernatant. The nuclear pellet was extracted with 3 × 5 ml of 5% (w/v) HClO$_4$. The extracts were combined, precipitated in 25% (w/v) trichloroacetic acid and recycled in 5 and 25% trichloroacetic acid to give crude histone 1. The histones remaining in the nuclear residue were extracted in 250 mM-HCl, separated into crude histone 2B and the other histones and passed through Bio-Gel P-10 columns (Ord & Stocken, 1975). Crude histone 1 was separated first on columns (15 cm × 1 cm) of DEAE-cellulose to give the non-histone P1, and then on a column (6 cm × 0.5 cm) of BioRex 70 to give the ADP-ribosylated histone 1a and the phosphorylated histone 1b; in small-scale experiments, however, histones 2B and 2A, 3 and 4 were separated electrophoretically (Panyim & Chalkley, 1969). The soluble proteins were also examined electrophoretically (Weber et al., 1973). Sometimes these proteins were precipitated at a final concentration of 25% trichloroacetic acid, washed twice with 2 ml of 25% (w/v) trichloroacetic acid, and their radioactivity was determined by Čerenkov counting after solution in 1 M-NaOH.

After staining with Coomassie Brilliant Blue or Naphthalene Black, the gels were scanned in a Vitatron TLD 100 scanner, the bands cut out and dissolved in 100-vol. H$_2$O$_2$. Radioactivity determinations were by Čerenkov counting (30% efficiency in Beckman CPM 200 liquid-scintillation system). Sufficient counts were recorded to give an accuracy of ±3%.

Table 1. *Phosphate contents and [^{32}P]P$_i$ uptakes by thymus histones from normal and γ-irradiated rats*

[^{32}P]P$_i$ was given 1 h before death. The histones were analysed and isolated as described by Ord & Stocken (1975), where the data for normal and regenerating rat liver are also reported. Irradiated rats received 400 rd total body radiation (45.6 rd/min). Phosphate contents are expressed as mol of phosphate/mol of protein. Where given, the maximum phosphate content and specific radioactivity is that found at the ascending edge of the protein peak eluted from the Bio-Gel. ^{32}P specific radioactivity of the histone phosphate is given as a ratio to that of plasma P$_i$. Results are expressed as means ±S.E.M. with the number of experiments in parentheses; 20 rats were used/group. Histone 1a is the ADP-ribosylated form of histone 1, H1b the form which does not carry ADP-ribose and protein P1 is the non-histone protein also extracted from nuclei in 5% HClO$_4$ (Smith & Stocken, 1975). H = histone.

	Resting liver		Normal rat thymus		Rat thymus after 400 rd	
Protein	Phosphate content	^{32}P specific radioactivity	h phate content	^{32}P specific radioactivity	Phosphate content	^{32}P specific radioactivity
H1a	0.75	—	1.2 ±0.5 (5)	0.20±0.02 (3)	1.1 ±0.2 (3)	0.23±0.06 (3)
H1b	0.12±0.07 (3)	0.06	0.31±0.06 (5)	0.59±0.09 (3)	0.23±0.07 (3)	0.22±0.05 (3)
Protein P1	2.3 ±0.64 (3)	0.22	1.9 ±0.8 (5)	0.08±0.01 (3)	1.8 ±0.6 (3)	0.13±0.08 (3)
H2B (max.)	0.07±0.07 (5)	0	0.23±0.09 (4) 1.7	0.18±0.03 (3)	0.18±0.02 (3) 1.6	0.20±0.07 (3)
H2A (max.)	0.37±0.05 (5)	0.67±0.1 (5)	0.16±0.04 (5) 0.5	0.32±0.09 (3)	0.19±0.09 (3) 0.4	0.39±0.08 (3)
H3	0.24±0.06 (5)	0	0.18±0.03 (4)	0.20±0.07 (3)	0.08±0.01 (3)	0.18±0.02 (3)
H4 (max.)	0.08±0.05 (5)	0.10	0.05±0.02 (4)	0.36±0.03 (3)	0.04±0.02 (3)	0.15±0.04 (3)
H3 complex	19.5	—	16±3 (5)	0.03±0.02 (3)	25±4 (3)	0.04±0.02 (3)

Results

Phosphorylated state of normal rat thymus histones

The phosphorylated states of histones from thymus gland of young rats are predictable for a tissue with a mitotic index of 1–5%. Histones 1b and 2B, which are almost non-phosphorylated in liver in G_0 phase and show little phosphate turnover, contain more
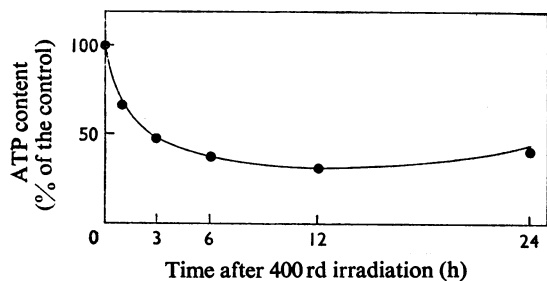
Fig. 1. *Effect of 400 rd total body irradiation on the ATP content of rat thymus nuclei*

Nuclei and extracts therefrom were prepared as described in the text. The ATP content was estimated by luciferase. Each point is derived from four groups of six animals. The s.e. was ±7%.

Table 2. *Effect of added ATP or protein on protein release from thymus nuclei in vitro*

Nuclei were prepared and incubated in the presence or absence of ATP (final concn. 2mM-ATP) for 20min at 37°C as described in the text. Nuclei equivalent to 1–1.2mg of DNA were present/ml of final suspension. The soluble proteins were those present in the supernatant after centrifuging the suspension for 15min at 100000g. Protein concentration [measured as described previously (Ord & Stocken, 1975)] is expressed as mg/ml; the results are the means of duplicate experiments and differed by less than ±1%.

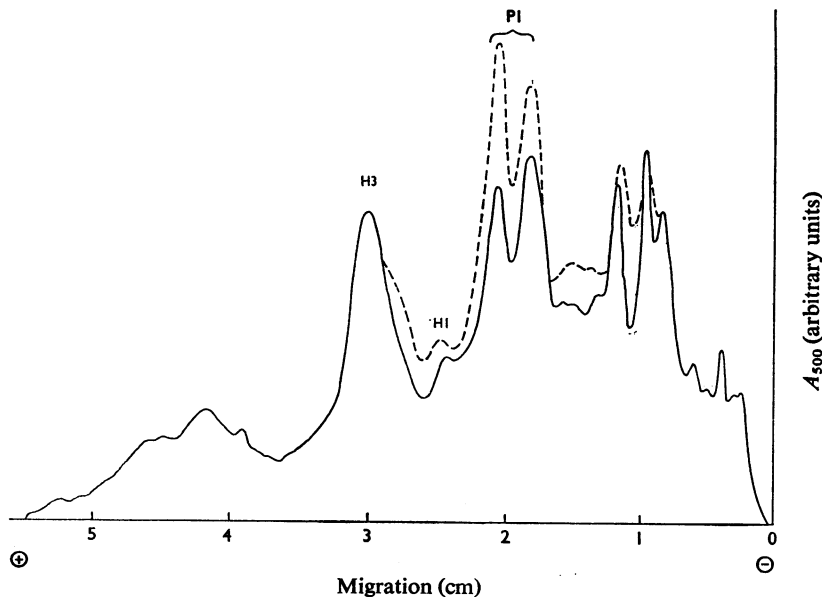| Expt. 1	ATP	Protein added	Protein release
Control, stop 0 min	–	0	0.23
Control, stop 20 min	–	0	0.24
Control, stop 0 min	+	0	0.22
Control, stop 20 min	+	0	0.27
Expt. 2			
Control	–	0	0.27
+protein HMG 1	–	0.25	0.29
Control	+	0	0.35
+protein HMG 1	+	0.25	0.48
Expt. 3			
Control	+	0	0.36
+H2A	+	1.0	0.40
+H2B	+	1.0	0.41
+H4	+	1.0	0.27

Fig. 2. *Effects of ATP on the release of soluble proteins from rat thymus nuclei*

Nuclei equivalent to 2.4mg of DNA/ml were prepared and incubated for 20min at 37°C in the presence or absence of 2mM-ATP as described in the text. Samples (20μl; 27 and 24μg respectively) from the 100000g supernatant were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The gels were stained with Coomassie Blue and scanned in a Vitatron scanner. ——, Soluble protein from nuclei incubated without ATP. ----, Soluble protein from nuclei incubated in the presence of 2mM-ATP. Non-histone protein P1 runs as a doublet at about 2cm, H1 at 2.5cm and H3 at 3cm. The other histones run between 3.5 and 5cm.

phosphate in thymus, with detectable ^{32}P uptake, characteristic of replicating and dividing cells (Lake et al., 1972; Gurley et al., 1973; Bradbury et al., 1973) (Table 1). The specific radioactivities of histones 3 and 4 are also increased. Histone 3 is believed to be phosphorylated only in mitosis (Gurley et al., 1973). Conversely, histone 2A, which shows a high ^{32}P turnover in liver, is less phosphorylated in thymus. This phosphorylation is especially evident in cells with high protein synthesis, such as liver and HeLa cells (see Thrower et al., 1975). Little difference was noted between liver and thymus histone 1a and non-histone protein P1, except that from thymus the ADP-ribosylated histone 1a was only 10% of the total histone 1, in contrast with liver, where it comprises 20–30%. Histone 3 associated with ribonucleotide (histone 3 complex) was about 1% of the total histones from thymus, as in liver; in preliminary experiments it showed a higher [^{3}H]lysine uptake compared with free histone 3 (cf. Ord & Stocken, 1975).

Effects of γ-irradiation on the phosphorylation of thymus histones in vivo

Total body irradiation with 400rd decreased ^{32}P uptake into thymus DNA in these rats to 43% of the value in normal animals. A similar decrease is found for ^{32}P uptake into histones 1b and 4 (Table 1). Histone 3, which had a lower ^{32}P specific radioactivity than histones 1b and 4, showed no effect of irradiation on ^{32}P uptake, but the phosphate content was decreased to the same extent as that for histone 4. No decreases in phosphorylation of the other proteins were noted. In regenerating liver and CHO cells (Ord & Stocken, 1968a; Gurley & Walters, 1971), only
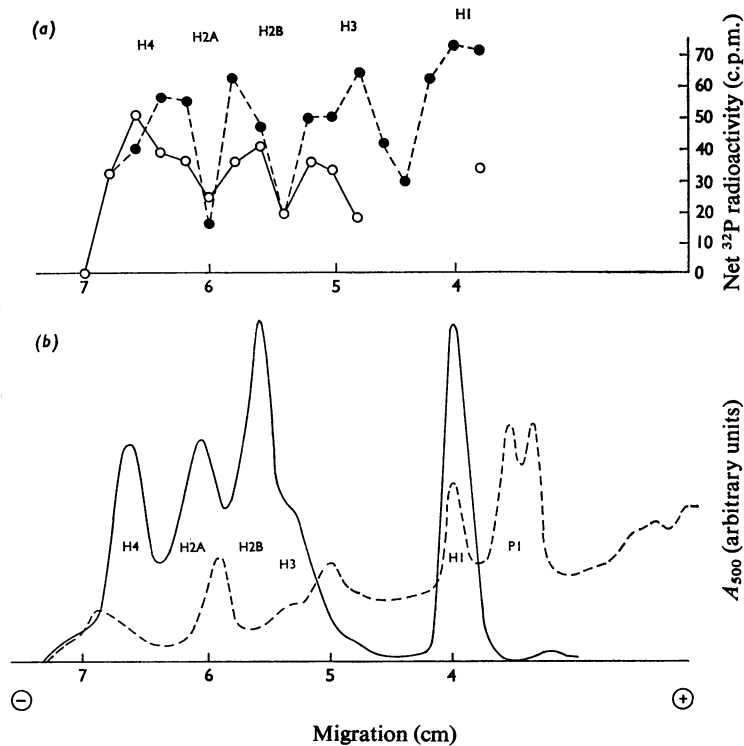

Fig. 3. *Acid/urea-gel electrophoresis of soluble proteins and proteins extracted in 250mM-HCl from rat thymus nuclei incubated with ATP*

Nuclei equivalent to 2.4mg of DNA/ml were prepared and incubated with 2mM-[γ-^{32}P]ATP as described in the text. After separation of the soluble proteins, the nuclear pellet was extracted with 250mM-HCl; 35μg of soluble protein or 10μg of protein from the 250mM-HCl extracts was applied to the gels. (a) Radioactivity measurements (c.p.m./2mm gel slice): ———, 250mM-HCl extract; ----, soluble protein. (b) Vitatron scans: ———, 250mM-HCl extract; ----, soluble proteins. No proteins were present in the 250mM-HCl extract before 3cm, and radioactivity measurements on the protein present in the soluble extract were not made between 0 and 3.6cm. From the scans, non-histone protein P1 migrated as a doublet between 3.3 and 3.8cm, H1 at 3.9–4.2cm, H3 at 4.6–5.3cm, H2B at 5.3–5.7cm, H2A at 5.7–6.4cm and H4 at 6.4–7.2cm.
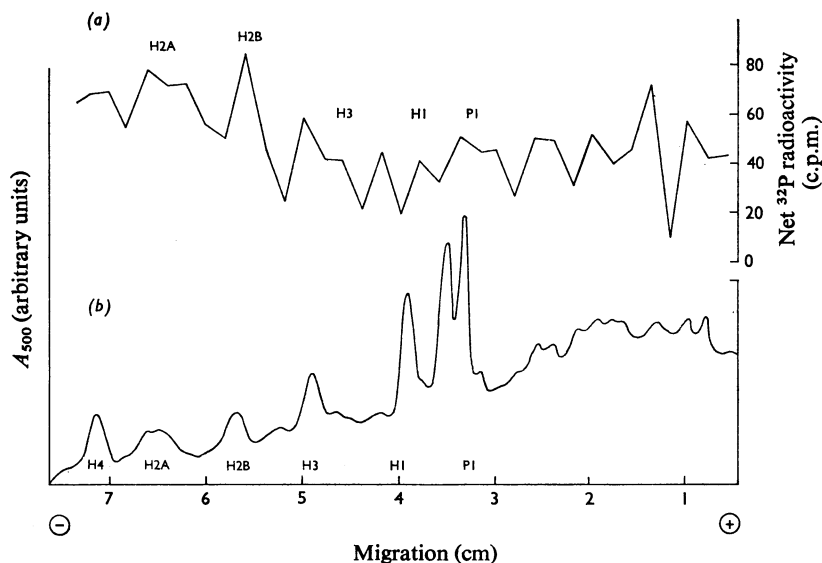
Fig. 4. *Acid/urea-gel electrophoresis of soluble proteins released after incubation of rat thymus nuclei with ATP*
Nuclei equivalent to 2.4 mg of DNA/ml were prepared and incubated with $2 \text{mM-}[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described in the text. Portions (50 μl; 35 μg) of the soluble protein from the 100000g supernatant were applied to the gel, which after electrophoresis was stained with Coomassie Blue, scanned in a Vitatron scanner and the radioactivity measured in 2 mm sections. (*a*) Radioactivity (c.p.m./2 mm section of gel, ±10%); (*b*) Vitatron scan: From these scans, non-histone protein P1 runs as a doublet at 3.3–3.6 cm, H1 (histone 1) about 3.8–4.0 cm, H3 at 4.5–5.0 cm, H2B at 5.6–6.0 cm, H2A at 6.3–6.8 cm and H4 at 7–7.2 cm.

Table 3. *Phosphorylation of histones from thymus nuclei incubated in vitro with $[\gamma\text{-}^{32}\text{P}]ATP$*
Thymus nuclei from normal or irradiated rats were incubated with $2 \text{mM-}[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 20 min at 37°C, at a final concn. equivalent to 12–15 mg of DNA/ml. The reaction was stopped and the proteins isolated as described in the text. ^{32}P transfer is given as mmol/mol of protein. H = histone.

	^{32}P transferred into:		
	Histone 1a	Histone 1b	Non-histone protein P1
Control (6)	29.6 ± 6.5	2.6 ± 1.4	31.4 ± 11.3
+added H1b (0.05 mM)	35.2	19.5, 16.1	23.7
+1.0 M-NaCl	34.3	37.1	30.1
Irradiated (2)	24.1, 23.8	2.7, 0.9	38.3, 17.9
Irradiated + added H1b (0.05 mM)	—	16.7	—

phosphorylation of histone 1 is radiosensitive, and in liver this has been attributed to a radiation-induced delay in the induction of the nuclear cyclic AMP-insensitive protein kinase, which increases in activity before DNA replication (see Ord & Stocken, 1975). In thymus, irradiation *in vivo* causes oxidation of thiol groups on histone 3 and a decrease in the process by which ATP becomes available in the nucleus (Ord & Stocken, 1968b). Dependence of histone-1 phosphorylation on nuclear generation of ATP in thymocytes has been suggested (Dancheva *et al.*, 1974). ATP was determined therefore in thymus nuclei

isolated from rats given 400 rd total body irradiation. Fig. 1 shows that there was a marked decrease in ATP up to 6h, with some slight recovery by 24h after exposure.

Phosphorylation of histones in thymus nuclei in vitro: the effects of ATP

To examine further the control of histone phosphorylation and its radiosensitivity, thymus nuclei from normal and irradiated rats were incubated *in vitro* with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Preliminary experiments estab-
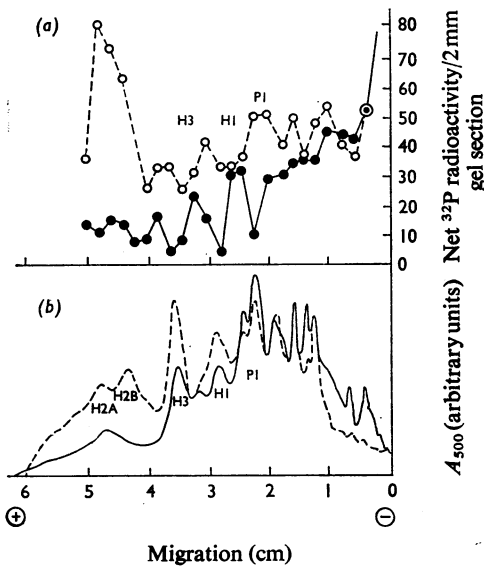
Fig. 5. *Sodium dodecyl sulphate/polyacrylamide-gel elec-trophoresis of soluble proteins, released from rat thymus nuclei incubated with ATP in the presence or absence of added histone 1b*

Nuclei ($\equiv$ 2.4 mg of DNA/ml) were prepared and incubated with $[\gamma$-^{32}P]ATP and histones prepared as described in the text. When added, histone 1 b was present at 0.05 M (1.08 mg/ml). Samples (50 μl; approx. 35 μg of protein in the control samples) were run on the gels. (*a*) ^{32}P radioactivity as c.p.m./2 mm section gel: ●, control; ○, +added H1 b. (*b*) Vitatron scan of gels: ——, control soluble proteins; ----, +added H1 b (histone 1b). Non-histone protein P1 runs as a doublet at about 2.2–2.5 cm, H1 at 2.8–3.0 cm, H3 at about 3.5 cm and the other histones between 4 and 6 cm.

lished that ^{32}P uptake was linear with time for about 20 min and was unaffected by 10 μM-cyclic AMP, which was therefore omitted. The nuclei were not washed before incubation, so that proteins which might be loosely associated with the nuclei would be retained.

If ATP was present in the incubation medium, amounts of proteins released into the supernatant fraction after incubation increased (Table 2, Expts. 1 and 2; Fig. 2). This was particularly evident for the non-histone proteins and histones 1 and 3 (Fig. 2). When the proteins were examined on acid/urea gels, histones 2A, 2B and 3 released into the supernatant fraction migrated slightly less rapidly than those subsequently extracted with 250 mM-HCl (Figs. 3 and 4); ^{32}P radioactivity associated with the supernatant histones also migrated more slowly than the protein peaks detectable by staining. With greater resolution

(Fig. 4), the more slowly migrating element in the non-histone protein P1 doublet appeared to be the more phosphorylated. The phosphorylated component in histone 1 was retarded and histone 3 separated into two or three possible phosphorylated species. Related experiments with liver nuclei indicate that the most slowly migrating component of histone 3 may be an ADP-ribosylated derivative. The doublet of histone 2A may be due to polymorphic forms.

From 1.3 to 1.5 mg of total protein/mg of DNA were recovered from the nuclei, of which 0.3–0.5 mg was released into solution during the incubation (Table 2). Of the remaining 1.0 mg of acid-extractable proteins, 20% were soluble in 5% (w/v) HClO$_4$. Assuming colour equivalence for the staining of the different proteins, densitometer traces indicated that 30% of the protein released into the supernatant fraction from thymus nuclei incubated with ATP was histone, which was about 10% of the total histone content of the nuclei. About 12.5% of the total histone 1 was released, approximately equivalent amounts of histones 2A and 2B (8% of their totals), rather less histone 4, and 20% of histone 3. Almost all of the non-histone protein P1 was released into the soluble fraction. Since quantitative determination of the amount of γ-phosphate transferred *in vitro* (see below) was very low (maximally 0.05 mol of phosphate/mol of protein), the results suggest that incubation preferentially released histones that were already phosphorylated *in vivo*. The bulk of the histones, which were extracted only in acid, were significantly less radioactive (Fig. 3), but significant ^{32}P uptake occurred into protein P1 and into that fraction of histone 1 (1a) which carries ADP-ribose (Smith & Stocken, 1975) (Table 3). All the radioactivity in protein P1 and histone 1a was released as alkali-labile P$_i$.

Phosphorylation of histones in thymus nuclei in vitro: the effects of exogenous histones

Enzymes for the phosphorylation of non-histone proteins and histones 1 and 2B are retained in human tonsillar lymphocyte nuclei isolated in hypo-osmotic sucrose (see Faragó *et al.*, 1973). High ionic strength or detergent (Siebert *et al.*, 1971; Farron-Furstenthal, 1975) is required if the enzymes are to be dissociated from the chromatin. To confirm that histone kinase(s) were present in thymus nuclei, purified rat liver histones (1 mg/ml) were added to the incubated preparations. Most of the added histones were removed from the soluble phase and bound on to the nuclei (Table 2, Expt. 3) (cf. Johnson *et al.*, 1973; Bonner, 1975). In agreement with the observations of Kaplowitz *et al.* (1971) and Johnson *et al.* (1973), the addition of histone 1b increased the phosphorylation of the soluble non-histone proteins, including protein P1 (Fig. 5). Additional histones 2A, 2B and 3
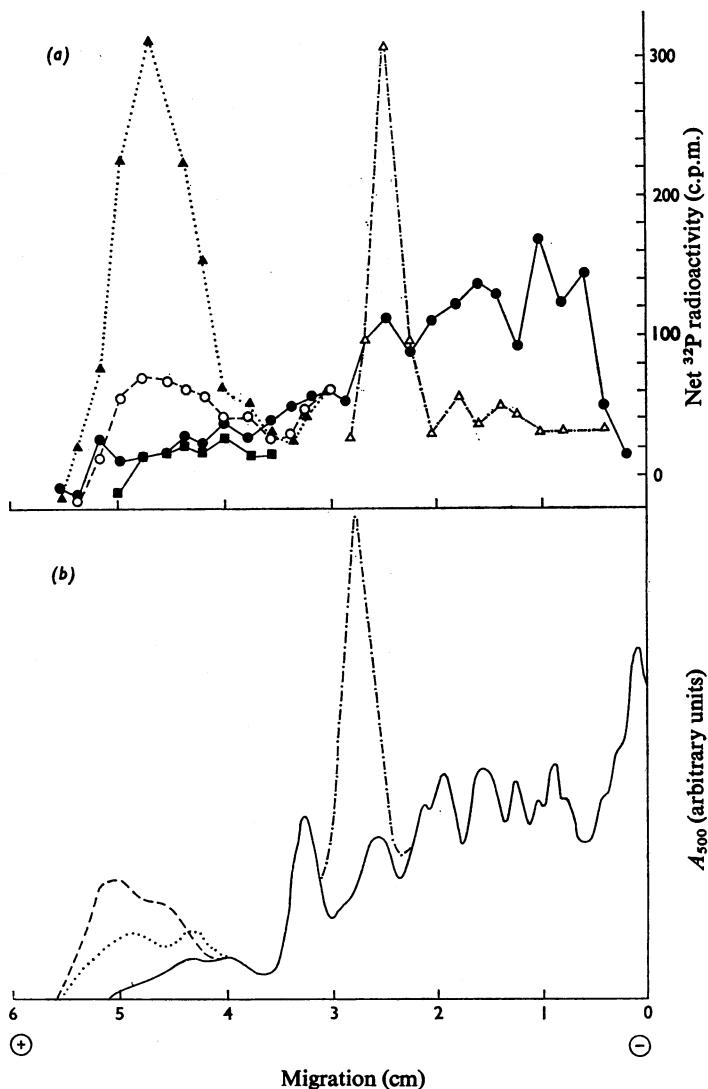
Fig. 6. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of soluble proteins released from rat thymus nuclei during incubation at 37°C for 20 min*

Nuclei were prepared and incubated with [γ-^{32}P]ATP as described in the text. When added, histones 2A, 2B or 4 were present at 1.0 mg in a final volume of 1.0 ml. The concentration of nuclei was equivalent to 5.0 mg of DNA/ml. After incubation, the preparations were centrifuged at 100 000 g for 15 min to obtain the soluble proteins in the supernatant. About 35 μg of soluble protein was applied to each gel. The gels were scanned in a Vitatron scanner, 2 mm sections were dissolved in H_2O_2 and their radioactivity determined. (*a*) ^{32}P radioactivity (c.p.m./2 mm gel slice): ●, control; ○, change caused by added H (histone) 2A; ■, change caused by added H4; ▲, change caused by added H2B; △, change in the presence of 0.3 M-NaCl. (*b*) Vitatron scans: ——, control nuclei; ...., change produced by added H2B or H4; ----, change produced by added H2A; ·—·—, change in the presence of 0.3 M-NaCl. The scans with added H2B and H4 were identical, as were all other regions of the scans than those indicated. Protein P1 as a doublet ran at 1.8–2.2 cm, H1B at 2.4–2.8 cm, H3 at 3–3.5 cm, and the other histones at 3.7–5.5 cm.

were released into the supernatant fraction in the presence of added histone 1b; these were phosphorylated (Fig. 5). Added histone 1b was itself extensively phosphorylated, including both the small amount

present in the supernatant fraction (Fig. 5) and that extracted in 5% $HClO_4$ (Table 3). The other bound (acid-extractable) proteins showed no increase in phosphorylation. The phosphorylation of added his-

Net ^{32}P radioactivity (c.p.m.)

Control	29	35	55		40	33
+ Added H4	61	1	44		35	37
+1.0 M-NaCl	0	56	22		35	28

(a)

H4 H2A   H3 (SS)

4   2

⊖   ⊕

← Migration (cm)

Control	8	20	21	24
+ Added H2B	10	73	—	—
+1.0 M-NaCl	19	47	15	0

(b)

H2B

4   2

⊖   ⊕
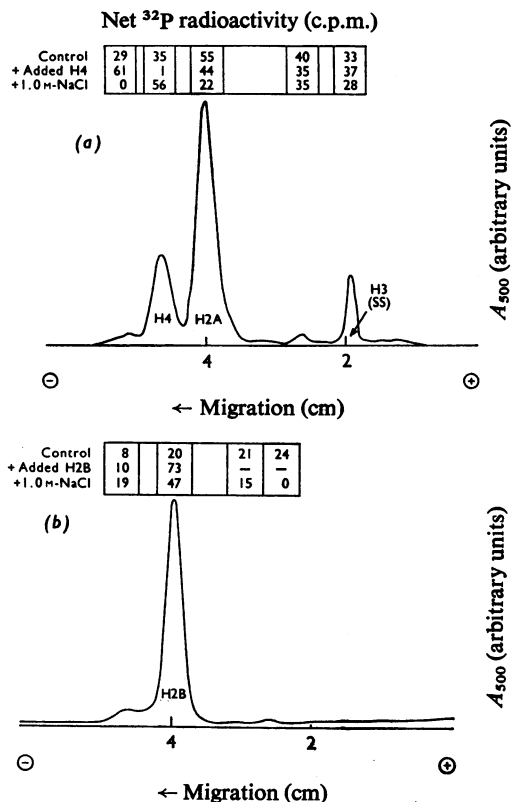
← Migration (cm)

Fig. 7. *Acid/urea-gel electrophoresis of histones extracted from rat thymus nuclei which have been incubated with [γ-^{32}P]ATP and added histone 4 or in the presence of 1.0M-NaCl*
Nuclei equivalent to 2.8mg of DNA/ml were incubated in the presence of 2mM-[γ-^{32}P]ATP and either added H (histone) 4 (1mg/ml) or 1.0M-NaCl. After 20min at 37°C the samples were plunged into ice, and the control preparation and that with added H4 centrifuged at 100000g for 15min. The sample containing the NaCl was adjusted to 0.5M-HClO$_4$ and centrifuged similarly. The histones were separated as described in the text: 24μg of mixed H2A, H3 and H4, or 23μg of H2B, were electrophoresed on acid/urea gels at pH2.9. ^{32}P radioactivity was determined in the bands as shown in the Figures. No 2-mercapto-ethanol was present in the gel system. Vitatron traces are shown from (a) H2A, H3 and H4 and (b) H2B. In (a), the slowest component is H3, migrating in the disulphide form; H2A is at about 4cm and H4 at 4.5–5cm. In (b), H2B is the main component. Net ^{32}P radioactivity in the bands is shown over the appropriate peak. The accuracy was ±10%.

tone 1b was not decreased when nuclei were isolated from irradiated rats (Table 3).

Added histone 2B was extensively phosphorylated. This could be seen both for unbound histone 2B

(Fig. 6), which remained with the supernatant proteins after incubation, and for histone 2B subsequently extracted from the nuclei with acid and electrophoresed on acid/urea gels (Fig. 7b). Added histones 2A and 4 were slightly phosphorylated (Figs. 6 and 7a), again without affecting the phosphorylation of the other histones or protein P1. The addition of histone 4 decreased the solubility and phosphorylation of the other non-histone proteins by 20–50% (control phosphate transfer 8.2 and 13.3nmol/mg of soluble protein; plus histone 4, 5.0 and 6.3nmol/mg). Decreased phosphorylation of low-molecular-weight non-histone proteins from liver nuclei in the presence of added histone 4 was also found by Johnson *et al.* (1973).

Phosphorylation of added non-histone proteins by thymus nuclei in vitro

Entry of non-histone proteins is frequently postulated as a regulatory factor in nuclear behaviour. Retention of these proteins is selective, since a number of relatively low-molecular-weight non-histone proteins such as cytochrome c and lysozyme (Bonner, 1975) can enter nuclei but are not retained. Similar experiments to those reported with added histones were therefore performed with lysozyme, cytochrome c or the nuclear non-histone protein P1 (Smith & Stocken, 1973)/HMG-1 (Shooter *et al.*, 1974). In agreement with experiments in oocytes (Bonner, 1975), neither lysozyme nor cytochrome c (0.8mg of added protein/ml) was bound to thymus nuclei (2mg of DNA/ml), nor were either of these two proteins phosphorylated. By contrast, when the non-histone chromosomal protein P1/HMG-1 was added, it was phosphorylated (Figs. 8 and 9). This protein binds non-specifically to histones (Smith & Stocken, 1973) and DNA (Shooter *et al.*, 1974). The presence of protein P1/HMG-1 in the incubation mixture caused histones to be released from their nuclear binding, especially histones 2A and 3. These released histones were phosphorylated. No difference was detected between protein P1 obtained from rat liver and protein HMG-1 from calf thymus (a gift from Dr. E. W. Johns, Chester Beatty Research Institute, Royal Cancer Hospital, London) in their capacity to release bound histones from the incubated nuclei, and their actions were proportional to the amount of protein added (Fig. 9). When small amounts of protein P1 were present, phosphorylation was especially evident in the more slowly migrating of its two components (Fig. 9).

Phosphorylation of histones in thymus nuclei in vitro: effects of ionic strength

Since nuclear structure is profoundly affected by increases in ionic strength, NaCl was added to the incubated preparations. As expected, histone 1 was
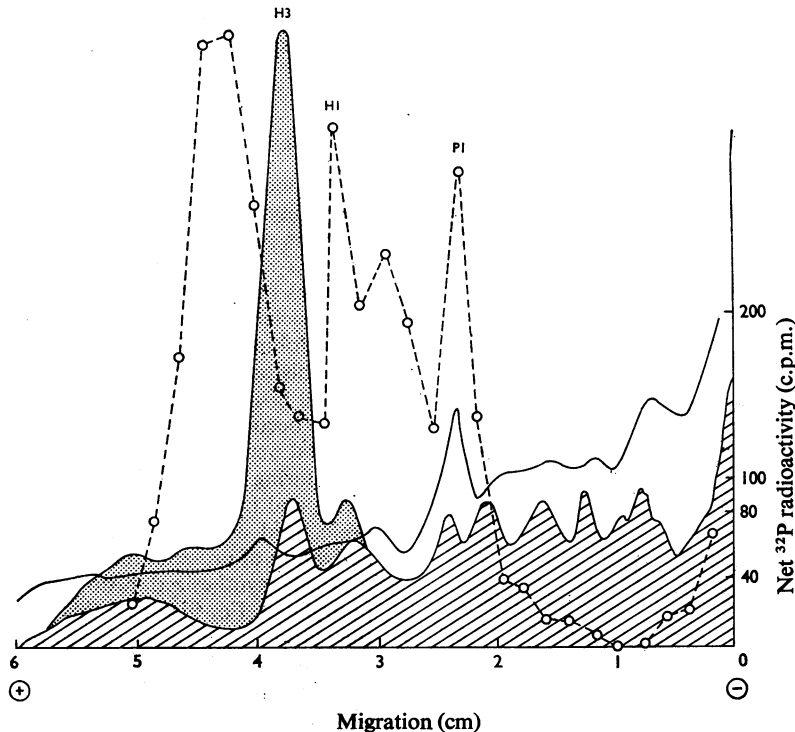
Fig. 8. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of soluble proteins from thymus nuclei incubated in vitro with [γ-³²P]ATP in the presence and absence of protein P1*

Methods of incubation and preparation of soluble protein as described in the text. Nuclei equivalent to 2mg of DNA/ml were incubated with or without liver protein P1 (0.62mg/ml). Sections (2mm) of gel were taken for determination of the radioactivity down the gels. ■, Scan of soluble proteins from control nuclei. ▨, Scan of soluble proteins from nuclei incubated with protein P1. ——, Radioactivity of soluble proteins from control nuclei. ○, 0.5×radioactivity of soluble proteins from control nuclei+added protein P1. Protein P1 migrated between 2 and 2.5cm, H1 between 3 and 3.5cm, H3 at 3.5–4.0cm and the other histones between 4.5 and 5.5cm.

released from the nuclei as the concentration of NaCl increased, and was extensively phosphorylated (Table 3), in spite of the decreased phosphorylation of the soluble, non-histone proteins (Fig. 6; cf. Dastugue *et al.*, 1974). Addition of NaCl caused the suspension to gel, but at 1.0M-NaCl it was evident (Fig. 7*b*) that histone 2B was accessible to the phosphorylating enzyme(s) and there was a slightly increased phosphorylation of histone 4. Histone 2A was not additionally phosphorylated in NaCl up to 1.0M, and was not rapidly phosphorylated by the nuclear enzymes in thymus under any conditions used in these experiments.

Discussion

Prefential release, into the hypo-osmotic incubation medium, of phosphorylated histones supports mechanisms postulating histone phosphorylation as

a physiological factor weakening histone–DNA interactions. The amounts of histones 2A and 2B released were approximately equal, but the amounts of histones 3 and 4 were not, perhaps because of the greater phosphorylation of histone 3 and the firmness with which histone 4 can be bound. Factors influencing the phosphorylation include the following.

(1) Constraints limiting access of endogenous nuclear proteins to endogenous kinases

These constraints may be affected by altering the ionic strength or by exposing nuclei to certain proteins, such as protein P1, histone 1 or histone 4, which influence nuclear protein binding. It is not known whether alteration in ionic strength occurs in nuclei during the cell cycle, but entry of non-histone proteins into nuclei is an early event in cells promoted into growth (Baserga *et al.*, 1975). In some tissues,
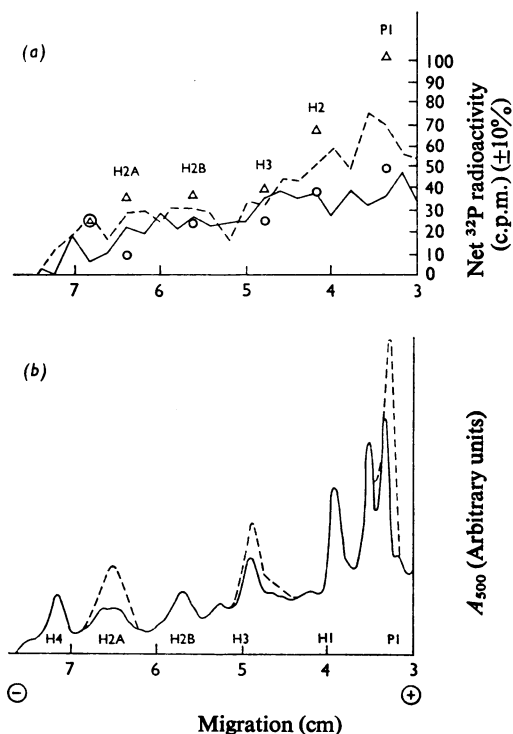
Fig. 9. *Acid/urea-gel electrophoresis of soluble proteins released from thymus nuclei incubated in the presence or absence of non-histone protein* P1

Nuclei (2.4mg of DNA/ml) were incubated for 20min at 37°C with 2mM-[γ-^{32}P]ATP and non-histone protein P1/HMG-1. A portion (50μl) of supernatant (35μg of soluble protein in the controls) was examined electrophoretically, and then stained with Coomassie Blue. Proteins are identified as in Fig. 5. (*a*) ^{32}P radioactivity/2mm gel slice: control soluble protein (——) and soluble proteins from nuclei incubated with added 100μg of protein P1/ml (————); ○, radioactivity obtained with 50μg of protein P1/ml; △, radioactivity obtained with 200μg of protein P1/ml. (*b*) Vitatron scans of soluble proteins: ——, from control nuclei; ————, from nuclei to which 100μg of protein P1 or HMG-1/ml had been added (identical curves).

such as liver, histone biosynthesis probably precedes that of DNA (Ord & Stocken, 1975; Smirnova & Rodionov, 1975), so causing transient imbalance in the histone/DNA ratio and potentially influencing nuclear protein binding and thus phosphorylation.

(2) ATP availability

In tissues such as liver, control through ATP availability is not normally operative, but in lymphoid tissue (Cross & Ord, 1971, and above) it may

occur and also be the basis of the radiosensitivity of the phosphorylation of histones 1 and 4 in thymus *in vivo*.

(3) Protein kinase activity

Histone phosphorylation will be affected by the specificity and activity of the available protein kinases. Nuclear protein kinases include both cyclic AMP-dependent and -independent enzymes with preferences for either histones or non-histone proteins. The nuclear histone kinases found in thymus and liver (Johnson *et al.*, 1973) preferentially phosphorylated histones 1 and 2B and were relatively inactive against histones 2A and 4. Phosphorylation in interphase occurs both when transcription is promoted and at the start of replication. Increased phosphorylation during transcription can only occur in the nucleus, and in liver, where the change is easily detectable (Ord & Stocken, 1975), it is associated with activation of protein kinase by cyclic AMP.

Newly synthesized histones are generally phosphorylated. For histone 4 this takes place initially in the cytoplasm (Ruiz-Carillo *et al.*, 1975), consistent with the low activity of the nuclear enzyme against this protein. The high activity of the nuclear kinase for histones 1 and 2B indicates that phosphorylation of these newly synthesized molecules can occur in the nucleus.

(4) Amounts of protein kinase

Increased phosphorylation of histones in actively replicating cells is partly due to increased amounts of cyclic AMP-insensitive histone kinase, which in nuclei from regenerating liver phosphorylated histone 1 rather than a mixture of histones 2A+4 (Siebert *et al.*, 1971; see also Farron-Furstenthal, 1975). This increase may account for the increased phosphorylation of 'old' histones, especially histone 1, detectable before biosynthesis of 'new' histones occurs (see Ord & Stocken, 1975).

(5) Activity of protein phosphatases

Any variation in protein phosphorylation implies a significant role for protein phosphatases in the regulation of chromatin function. Experimental evidence for this in relation to histone phosphatases is still fragmentary (Thrower *et al.*, 1975).

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