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Variations in the Phosphate Content of Histone F1 in Normal and Irradiated Tissues

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1. The capacity of the histone–DNA complex to act as a template for RNA synthesis is increased by phosphorylation of histone F1. 2. In regenerating liver, DNA synthesis is preceded by phosphorylation of histone F1. 3. Exposure to ionizing radiation *in vivo* decreases and delays the phosphorylation of histone F1 in regenerating liver, and decreases it in the hyperplastic kidney. 4. Histone F1 is phosphorylated to a greater extent in dividing than in resting tissues.

It was reported by Stevely & Stocken (1966) that a 50% increase in the phosphorylation of histone F1 significantly increased the priming ability of a DNA–histone F1 complex in the DNA-dependent nucleoside triphosphate–RNA nucleotidyltransferase (EC 2.7.7.6). Similarly, increasing the phosphorylation of histone F1 increased the priming ability of the complex in DNA nucleotidyltransferase (EC 2.7.7.7) (Ord & Stocken, 1968). The possible physiological function of these effects was indicated by the finding that in partially hepatectomized rats there was a twofold increase in the phosphate content of histone F1 in 16–24hr. after the operation when DNA synthesis was taking place. These results implicating the extent of phosphorylation of histone F1 with metabolic events of cell division have been confirmed and extended.

METHODS

Animals. This Laboratory's strain of Wistar rats was used except for the first of the experiments in which the phosphate content of histone F1 was related to DNA synthesis after partial hepatectomy, when rats were obtained from the Department of Pathology, University of Oxford.

Thymus glands from calf and lamb, and lamb kidneys, were transported to the laboratory on ice.

This Laboratory's strain of gerbils (*Meriones unguiculatus*) was used.

Partial hepatectomies. Partial hepatectomies were performed under ether anaesthesia by the method of Higgins & Anderson (1931). Male rats of about 200g. body wt. were used. They were given 10% (w/v) sucrose to drink and food *ad lib.* after the operation. Control rats were sham-operated.

Irradiation. This was provided from a ^{60}Co source that delivered 125rads/min. at 80cm. from the source. The rats were exposed in individual Perspex cages; the control animals were similarly confined for the period of irradiation.

Nuclei. Nuclei were isolated from rat liver as described by Chaveau, Moulé & Rouiller (1956) by using 2·2M-sucrose–5mM-$MgCl_2$–5mM-tris–HCl buffer, pH7·2.

Nuclei from rat thymus, brain and spleen were isolated in 0·25M-sucrose – 5mM-$MgCl_2$ – 5mM-tris–HCl, pH7·2 (Creasey & Stocken, 1959).

Nuclei from kidney were isolated in 45mM-glucose–85mM-KCl – 8·5mM-NaCl – 2·5mM-$MgCl_2$ – 2·5mM-$CaCl_2$ – 5mM-tris, adjusted to pH7·2 with KOH (Barnes, Esnouf & Stocken, 1956).

Nuclei from ascites tumour cells were isolated in 2·4M-sucrose after hypo-osmotic shock in 5mM-$CaCl_2$ (M. Gronow, personal communication).

Nuclei from calf and lamb thymus were obtained by the method of Allfrey, Littau & Mirsky (1964).

All the preparations of nuclei were washed twice with 10mM-tris–HCl buffer–5mM-$MgCl_2$, pH7·1, and once with 1mM-HCl.

Diffuse and dense chromatin. These were obtained from rat thymus nuclei by the procedure of Frenster, Allfrey & Mirsky (1963). After preparation of the nuclei in the usual way, they were kept for 10min. at 0° with 0·25M-sucrose and then treated ultrasonically for 1min. at 1·5A and 20kcyc./sec. in an MSE Sonicator. The material was centrifuged for 10min. in a Servall centrifuge at 1000*g* to bring down the dense chromatin. The material that sedimented after a further 30min. at 3000*g* was discarded, and diffuse chromatin was sedimented after 60min. at 80000*g* (35000rev./min. in a Spinco model L ultracentrifuge, rotor 40). After two or three washes with 1mM-HCl the histones were extracted from dense and diffuse chromatin in the usual way (see below).

Extraction of histone F1. Washed nuclei were extracted with 5% (w/v) $HClO_4$ to give histone F1 (method 1 of Johns, 1964). The $HClO_4$ extract was made to 20% (w/v) with 100% (w/v) trichloroacetic acid to precipitate the protein, which was redissolved in water and reprecipitated with trichloroacetic acid (20%, w/v) before finally being dissolved in water. Two reprecipitations were sufficient to remove any traces of inorganic phosphate.

Samples of histone F1 of different phosphate contents were obtained either by extraction from various tissues or after incubation of rat thymus nuclei *in vitro* (Stevely & Stocken, 1966).

The histone preparations were always checked by electrophoresis on acrylamide gel, and no significant contamination could be detected.

Protein. This was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with acid-extracted protein from thymus nuclei as standard.

Phosphate determinations. The total phosphate content of the proteins was measured by the procedure of Bartlett (1959), with 60% (w/v) $HClO_4$ instead of 10N-H_2SO_4 and omission of the H_2O_2.

DNA. Incorporation of ^{32}P into liver DNA was measured by the method of DeLuca, Rossiter & Strickland (1953). Tissue phosphate soluble in 20% (w/v) trichloroacetic acid was used as a reference standard for determining the relative specific radioactivities in control and experimental animals.

Radioactivity measurements. Inorganic [^{32}P]phosphate (The Radiochemical Centre, Amersham, Bucks.) (100μc/100g. body wt.) was given intramuscularly to the rats 30min. before death.

^{32}P and ^{14}C radioactivities were measured by gas-flow counting (Nuclear-Chicago Corp. model D47; counting efficiencies: ^{14}C, 17%; ^{32}P, about 40%). Sufficient counts were recorded to give an accuracy of $\pm 1\%$.

RNA polymerase. RNA nucleotidyltransferase (EC 2.7.7.6) was prepared from *Escherichia coli* (Chamberlain & Berg, 1962) up to the step of fraction 3. *E. coli* was obtained from the Microbial Products section of the Microbiological Research Establishment, Porton, Wilts.

The assay system was that described by Hilton & Stocken (1966). Calf thymus DNA (Sigma Chemical Co., St Louis, Mo., U.S.A.), 0–25μg. in 1·5mM-citrate–15mM-NaCl, and 0–20μg. of histone in 20mM-tris–HCl buffer, pH 8·2, were mixed in a final volume of 0·15ml. of 20mM-tris–HCl buffer, pH 8·2. To each tube was added 0·1ml. of a mixture containing 1μmole of $MgCl_2$, 0·25μmole of $MnCl_2$, 1μmole each of GTP, CTP and UTP, 0·5μmole of [8-^{14}C]ATP (1·0mc/m-mole; Schwartz BioResearch Inc., Orangeburg, N.J., U.S.A.), 3μmoles of β-mercaptoethanol, 10μmoles of tris–HCl buffer, pH 8·0, and 12μmoles of KCl. RNA polymerase (0·01ml. containing 50–60 units in 20–30μg. of protein) was then added and the mixture incubated for 10min. at 37°. The reaction was terminated by rapidly cooling the mixture to 0° and adding 0·2ml. of 4M-NaCl to dissolve the DNA–histone aggregate; 0·3ml. of 10% (w/v) trichloroacetic acid was then added and, after the mixture had stood for at least 5hr. at 2°, the precipitates were collected, washed on Millipore membrane filters and counted in a gas-flow counter.

RESULTS

Effect of phosphorylation of histone F1 on RNA synthesis in model systems. The effect of differing degrees of phosphorylation of histone F1 was studied in the RNA nucleotidyltransferase system described by Hilton & Stocken (1966) with constant histone/DNA ratio 0·7:1. The maintenance of a constant histone/DNA ratio was an important consideration in view of the insolubility of the complex. This point has been fully discussed by Butler & Chipperfield (1967), who concluded that no precise relationship existed between the insolubility of the complex and its priming ability.

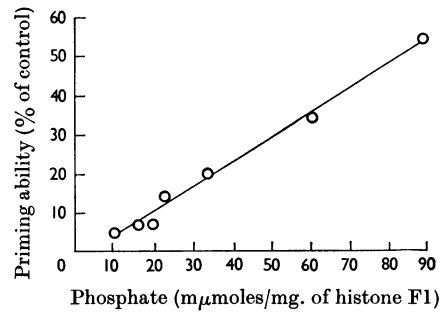

Fig. 1. Capacity of complexes of DNA with histone F1 containing different amounts of phosphate to repress DNA-dependent RNA synthesis. The preparation of the extracts and the polymerase system are described in the text. The priming ability is expressed as a percentage of the activity with DNA alone.

Fig. 1 shows that there is an approximate linear relationship between the phosphate content of histone F1 and the priming ability of the complex.

Phosphorylation of histone F1 in regenerating liver and kidney. The preliminary experiments (Ord & Stocken, 1967*a*, 1968) showed an approximate temporal correlation between the degree of phosphorylation of histone F1 and DNA synthesis in regenerating rat liver. A more detailed analysis at about the time of onset of DNA synthesis (15–19hr. after partial hepatectomy) showed (Table 1) that the increase in phosphorylation of histone F1 precedes the incorporation of ^{32}P into DNA by approximately 1hr.

The radiosensitivity of the phosphorylation of histone F1 in thymus gland and in regenerating liver was shown earlier by measurements of the relative specific radioactivities of histone F1 phosphate in the tissues taken from rats killed at 4 and 24hr. after total-body exposure to γ-irradiation (Ord & Stocken, 1968). These results have been confirmed in regenerating liver by measuring the ^{31}P content of histone F1 at the time when DNA synthesis is near its peak (Table 1), when there is a 20–30% decrease compared with the control rats that have not been irradiated.

After unilateral nephrectomy there is an increased synthesis of DNA in the remaining kidney, and Wachtel, Phillips & Cole (1966) showed a depressed uptake of ^{3}H-labelled thymidine into DNA when young rats were exposed to total-body irradiation just before the operation. This effect on DNA synthesis in kidney parallels the well-known effect in regenerating liver, and it is clear (Tables 1 and 2) that the same situation holds for the phosphorylation of histone F1 in the two tissues after total-body γ-irradiation with 990 rads.

Phosphorylation of histone F1 in tissues with

Table 1. *Phosphorylation of histone F1 in regenerating liver*

For experimental details, see the text. The numbers of rats used were: Expt. 1: three rats per hepatectomized group, one sham-operated rat per control group; Expt. 2: four rats per hepatectomized group, three sham-operated rats per control group; Expt. 3: four rats per group.

Expt. no.	Time after hepatectomy (hr.)	Phosphate content of histone F1 from hepatectomized rats (mμmoles/mg. of protein)	Phosphate content of histone F1 from control rats (mμmoles/mg. of protein)	Rel. sp. radioactivity of DNA of hepatectomized rats	Rel. sp. radioactivity of DNA of control rats
1	15	60		0·9	
	15·5	54		0·7	
	16	42	55	0·8	1·0
	16·5	97·4		1·0	
	17	87·6		1·1	
	17·5	100·4		1·2	
	18	140		3·3	
	18·5	110·3		3·3	
	19	128·7	54	3·2	0·6
2	16	39	39	0·8	0·7
	16·5	37		1·0	
	17	52·3		0·9	
	17·5	61·3		0·9	
	18	60·6	41	1·3	0·9
	18·5	66·1	41	1·4	0·8
3	17	51·1 38·0*		0·8 1·1*	
	18	57·5 47·3*		3·1 0·6*	
	19	67·0 47·3*		4·3 1·6*	

* These rats received 990 rads of total-body γ-irradiation in 8 min. at 12 hr. after hepatectomy.

Table 2. *Phosphate content of histone F1 obtained from various tissues*

Tissue	Phosphate content (mμmoles/mg. of protein)
Rat thymus dense chromatin	10
diffuse chromatin	90
Rat kidney cortex	50
cortex from unilaterally nephrectomized rats	71 / 43*
Rat liver	35
regenerating liver	75
Ascites tumour 8 days	52
5 days	87
Chicken erythrocytes	12
Rat thymus	22
Rat spleen	24
Rat brain	24
Gerbil liver	42
Gerbil kidney cortex	55–60
Calf thymus	19
Lamb thymus	21
Lamb kidney cortex	33

* These rats were given 990 rads of total-body γ-irradiation at 90 min. after nephrectomy and killed 24 hr. later.

differing mitotic rates. It seems from the above results that some connexion exists between DNA synthesis and the extent of phosphorylation of histone F1 and, if this is so, it might be expected that tissues with different mitotic rates would show differences in the phosphate content of histone F1.

Because of the known diurnal variation in the phosphorylation of histone F1 (Ord & Stocken, 1968), the tissues were taken from rats killed between 10 a.m. and 12 noon.

Table 2 shows some support was given to the notion that DNA synthesis and the phosphorylation of histone F1 are interdependent. The histone F1 of the more active (diffuse) chromatin from thymus is considerably more phosphorylated than that from the inactive chromatin; in regenerating liver it is more phosphorylated than in resting liver and in the hyperplastic kidney it is more phosphorylated than in the normal kidney. The nucleated chicken erythrocyte, which is completely 'switched off' so far as DNA and RNA synthesis is concerned, has a content of 12 mμmoles of phosphate/mg. of protein, which approximates to that found in the inactive dense chromatin. We also examined ascites-tumour cells, which were taken at 5 days

after inoculation, when they were in the logarithmic phase of growth, and also at 8 days, when they had passed through this phase. The histone F1 of the rapidly growing culture is more phosphorylated than that from the ascites cells that have passed their maximum growth rate.

It would be of interest to know if the increased phosphate content of the total histone F1 is due to an increase in the number of molecules of histone F1 phosphorylated, or to an increase in the number of phosphate groups carried by a few molecules. It is not easy to obtain a clear-cut answer to this question, and all we have achieved so far is to separate histone F1 from rat thymus gland into phosphate-poor and phosphate-rich fractions (Fig. 2) on a column of Whatman DE50 DEAE-cellulose that had been pretreated by the method of Peterson & Sober (1956). Mano & Lipmann (1966) used gradient elution to separate fish-roe protamines with high phosphate content, but, when applied to the histone F1 phosphate, this method was less satisfactory than a two-step elution with 50mM-tris–hydrochloric acid buffer, pH7·2, followed by the same buffer containing 0·5M-sodium chloride. The data indicate that two phosphate groups are present on a protein of molecular weight about 20000 and that this comprises about 10% of the total protein. If it is assumed that the low phosphate content of the first fraction is due to a mixture of non-phosphorylated and mono-phosphorylated histone F1, then this latter fraction would roughly correspond to a quarter of the total histone F1 present.

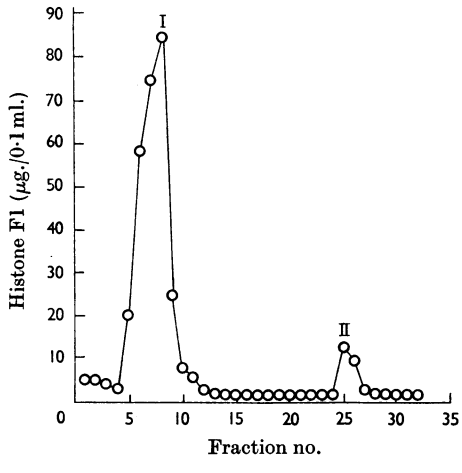

Fig. 2. Fractionation of histone F1 on DEAE-cellulose. Histone F1 (50mg.) in 23ml. of water was applied to a column (1·5cm.×24cm.) of Whatman DEAE-cellulose DE50 that had been pretreated by the method of Peterson & Sober (1956). Elution was with 100ml. of 50mM-tris–HCl buffer, pH7·2, followed by 100ml. of 50mM-tris–HCl–0·5M-NaCl, pH7·2. Fractions (5ml.) were collected. The starting material contained 20·9mμmoles of phosphate/mg. of protein. Peak I contained 9·5mμmoles of phosphate/mg. of protein and peak II 80mμmoles of phosphate/mg. of protein. The solvent change was made at fraction 20.

DISCUSSION

The results presented here are further evidence for the view that histones are intimately concerned in the transfer of genetic information. They also add support to the suggestion that the immediate biochemical damage caused by ionizing radiation to the cell is to be found in the nucleus (Ord & Stocken, 1967*b*).

Umaña, Updike, Randall & Dounce (1964) studied the incorporation of [1-^{14}C]leucine into nuclear proteins of regenerating liver and concluded from the histone:DNA ratio, which reaches a maximum of about 2·8 at 18hr. after partial hepatectomy, that a certain value of this ratio must be reached before DNA synthesis can commence. This view does not appear to agree with the finding *in vitro* that increasing the histone:DNA ratio decreases the competence of DNA in the DNA-dependent DNA polymerase system. Alteration of the microstructure of histone F1 by phosphorylation increases the ability of the DNA–histone complex to act as template (Ord & Stocken, 1968), and, since we have now found that phosphorylation *in vivo* precedes the onset of DNA synthesis, it seems that modification of the structure may be more significant in the dividing cell than changes in the relative amounts of histone and DNA. This suggestion is supported by the concentrations of phosphate found in dividing and non-dividing tissues. It must be pointed out, however, that, except in tissues where a considerable time had elapsed between death and analysis, we always found some phosphorylation of histone F1. This indicates that a certain amount of phosphorylation is normally present but that a considerable increase is required before division can take place. It is this enhanced phosphorylation that is radiosensitive, since it has been shown by Ord & Stocken (1968) that the phosphorylation of histone F1 is unaffected by irradiation in livers of normal rats but is inhibited in the partially hepatectomized animals. In the regenerating liver exposure to ionizing radiation during the first 12hr. after operation delays but does not prevent DNA synthesis (Holmes & Mee, 1956). In our experiments exposure to 990 rads not only decreased the extent of phosphorylation but also delayed the rather sharp increase in phosphorylation that takes place in control rats at about 17hr.

It therefore seems reasonable to conclude that in

the tissues so far studied one of the prior requirements for DNA synthesis and division is an increased phosphorylation of histone F1.

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