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Phosphorylation of Rat-Thymus Histone

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Ord & Stocken (1966) have shown that [^{32}P]-phosphate injected into rats is incorporated into the histones of the thymus gland. It has now been found that incorporation also takes place *in vitro*.

Rat-thymus nuclei from six to eight rats of 120–140g. body wt. were prepared as described by Ord, Raaf, Smit & Stocken (1965). These were suspended in 10ml. of the isolation medium (0·25M-sucrose–5mM-$MgCl_2$–5mM-tris–HCl, pH 7·2) to which was added 0·8ml. of 0·9% NaCl containing 100mg. of glucose/100ml. followed by 150μc of inorganic [^{32}P]phosphate (specific activity 50c/mg. of P). At the end of the incubation period the nuclei were washed three times with 15ml. of medium and the histone f1 was isolated by method 1 of Johns (1964).

Reproducible incorporation into histone f1 was obtained. The uptake was rapid, 50% of the activity obtained after 2hr. incubation being present in the histone after 20min.

This uptake of [^{32}P]phosphate was not inhibited by the addition of either actinomycin or puromycin to the incubation medium in concentrations of 100μg./ml., which indicates that the phosphorylation is not related to protein synthesis but is occurring on the preformed histone.

It was also found that after a 1hr. incubation period the phosphorus content of histone f1 increases from 20–25 to 35–45mμg.atoms of P/mg. of protein. This increase was followed over a 2hr. period in conjunction with a [^{32}P]phosphate uptake time-course and both were found to give the same pattern. Results are shown in Table 1.

Analytical electrophoresis on polyacrylamide gels showed that the histone fraction f1 isolated from these experiments *in vitro* gave the same strong single band as material from preparations *in vivo*.

The nature of the ^{32}P incorporation in these studies *in vitro* was investigated. Ord & Stocken (1966) have shown that ^{32}P incorporated *in vivo* is present in the protein as serine phosphate. A 3mg. sample of histone fraction f1 from a ^{32}P-incorporation experiment *in vitro* was hydrolysed for 3hr. at 100° in 6N-HCl, and after removal of the HCl *in vacuo* over KOH the hydrolysed material was dissolved in water and examined by ascending paper chromatography with 2-methylpropan-2-ol–butan-2-one–water-aq. NH_3 (sp.gr. 0·88) (4:3:2:1, by vol.) (Fink, Cline & Fink, 1963). This system gives clear separation of serine and serine phosphate. The ^{32}P had the same R_F value as the serine phosphate marker. The eluate from the spot showing ^{32}P activity was hydrolysed in 12N-HCl *in vacuo* for 17hr. The solution was evaporated to dryness, dissolved in trimethylamine, treated with 1-fluoro-2,4-dinitro[^{14}C]benzene and the DNP-amino acids

Table 1. *Specific activities and phosphorus contents of histone fraction f1 after incubation* in vitro

The conditions of incubation are given in the text.

Expt. no.	Addition	Time of incubation (min.)	Specific activity (counts/sec./mg. of protein)	Phosphorus content (mμg.atoms of P/mg. of protein)
1	None	0		20·8
	None	60		41·7
2	None	0	13	25·0
	None	60	125	47·6
	Actinomycin (100μg./ml.)	60	158	43·5
3	None	60	162	35·7
	Puromycin (100μg./ml.)	60	179	38·5
4	None	0	0	25·0
	None	10	115	30·6
	None	20	168	31·3
	None	40	251	37·1
	None	80	295	41·0
	None	120	321	41.3

run with unlabelled markers in the two-dimensional thin-layer chromatography system described by Randerath (1963). An extract of the DNP-serine spot was found to have ^{14}C activity, thus showing that serine phosphate was present in the eluate from the paper chromatogram and that the ^{32}P was originally present as serine phosphate.

By using this incubation method *in vitro* some histone fraction f1 was obtained with 34·2mμg.-atoms of P/mg. of protein; this was compared with histone fraction f1 with 22·4mμg.atoms of P/mg. of protein and differences were found in the priming ability of the histone f1-DNA complexes in the RNA-polymerase system described by Hilton & Stocken (1966). By using [^{14}C]ATP the $V_{max.}$ for the material of higher phosphorus content was 900 counts/min. incorporated in 10min. whereas the material with the lower phosphorus content incorporated 665 counts/min. in 10min. This suggests that changes in the level of phosphorylation may be an effective means of modifying the repressing ability of this histone.

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The Role of Thiol Groups in the Modification of the Template Activity of Histone–Deoxyribonucleic Acid Complexes

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Stedman & Stedman (1951) originally proposed that histones might act as repressors of genetic transcription, but ready acceptance of this idea is difficult since there appears to be a lack of specificity between these closely related proteins. More recently, however, systems have been described that suggest that modifications in histones, either by *N*-terminal acetylation (Allfrey, Faulkner & Mirsky, 1964) or by their interaction with polyanions (Frenster, 1965), may convey some of the specificity required of a genetic repressor, and this is further supported by the finding of RNA associated with histones (Huang & Bonner, 1965). The presence of thiol groups in histones was reported by Marsh, Ord & Stocken (1964) and Phillips (1965). Ord & Stocken (1966) have also shown that a 50mM-HCl extract of diffuse chromatin contains a higher proportion of its sulphur in the thiol form than does a similar extract of the dense chromatin. Evidence is now presented showing that modification of thiol groups alters the degree of repression produced by the arginine-rich histones.

Calf-thymus nucleohistone was prepared by the method of Zubay & Doty (1959) and histone f3 was extracted by method 2 of Johns (1964). The nucleohistone pellet from 20g. of calf thymus was blended in 200ml. of ethanol and after centrifugation the pellet was extracted in a high-speed blender for 3min. with 40ml. of acid–ethanol (ethanol–1·25N-HCl; 4:1, v/v). The extract was dialysed overnight at 2° against 1l. of ethanol and the precipitated f3 fraction dried *in vacuo*.

Thiol groups were determined by the method of Ellman (1959) in 0·02M-tris–HCl buffer, pH 8·2, and disulphide was measured as SH after reduction in 4M-urea with $NaBH_4$ (5mg./mg. of protein) (Moore, Cole, Grundlach & Stein, 1958).

In six preparations of fraction f3 the total thiol content ranged between 38 and 51mμmoles of SH group/mg. of protein. Only one preparation contained disulphide groups (4%) and in all cases the thiol groups reacted with *N*-ethylmaleimide without treatment of the protein with urea.

To ensure that no disulphide groups were present reduction was carried out by treating the protein in 0·02M-tris–HCl buffer, pH 8·2, with 0·3M-β-mercaptoethanol for 30min. at 37° or 2hr. at room temperature. The β-mercaptoethanol was removed