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# Variations in the Phosphate Content and Thiol/Disulphide Ratio of Histones during the Cell Cycle

## STUDIES WITH REGENERATING RAT LIVER AND SEA URCHINS

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1. In regenerating rat liver the phosphate content of the lysine-rich histone F1, but not that of the more arginine-rich histone F3-1, increases during the period of DNA synthesis. 2. The phosphorylation of histone F1 in this 'S period' is decreased by $\gamma$-irradiation, but, like phosphate uptake into DNA, is affected to an even greater extent if the irradiation is given in the presynthetic period. 3. Histones from three species of sea-urchin eggs show similarities to the F2 and F3 groups of histones from mammalian thymus gland. 4. The proportion of thiol to total thiol plus disulphide in acid extracts from sea-urchin eggs varies from less than 20% in mature unfertilized eggs to 59% just before cleavage. 5. The phosphorylated forms of histones F1 and F3 are less effective in decreasing DNA synthesis by DNA polymerase than the non-phosphorylated forms. 6. Oxidation of thiol groups on histone F3-1 does not affect its capacity to decrease DNA synthesis *in vitro*.

The indications that histones F1 and F3 (Johns, 1964) exist in nuclei from rat liver and thymus gland in different states of phosphorylation and thiol oxidation (Ord & Stocken, 1967*a*) promoted a study of how these states might vary during the life cycle of the cell. Two situations have been examined: phosphorylation of histones F1 and F3 in regenerating rat liver and the thiol/disulphide ratio of the total histones extracted by 0·25M-hydrochloric acid from a nuclear fraction of eggs from three species of sea urchin, *Arbacia punctulata*, *Paracentrotus lividus* and *Echinus esculentus*, during the first division after fertilization. The association between phosphorylation of the lysine-rich histone F1 and DNA synthesis was suggested from the marked similarity of inhibition of $^{32}P$ incorporation into DNA and into histone F1 after X-irradiation (Ord & Stocken, 1966*b*). This led to the study of the phosphorylation of histones F1 and F3 during liver regeneration and the finding that the phosphate content of histone F1 increases during the period in which DNA is synthesized ('S period') (Ord & Stocken, 1967*b*).

It is usually accepted (see Frenster, 1965) that no gross differences can be detected in composition or amounts of histones extractable from chromatins with differing capacities for RNA and protein synthesis. Chromatin that is active with respect to uptake of amino acids, however, contains histones with a greater proportion of total thiol plus disulphide as thiol than does chromatin that is more inert synthetically (Ord & Stocken, 1966*a*). Many studies have been made of the variations in acid-extractable and total protein thiol/disulphide ratios during division in fertilized sea-urchin eggs and other dividing cells (see Mazia, 1961), but little information is so far available on the chemistry of the histones in sea urchins, although resemblances to histones from calf thymus (Comb & Silver, 1966) and a low content of histone F1 before gastrulation (Bäckström, 1965) have been reported. Since the proportion of thiol in the thiol plus disulphide of total histones extracted from nuclei from rat liver usually exceeds 80% (M. G. Ord & L. A. Stocken, unpublished work), and since in thymus it is necessary to separate active from inert chromatin to show the higher amount of disulphide in the latter (Ord & Stocken, 1966*a*), it was decided to use sea-urchin eggs to examine possible changes in the proportion of thiol that might occur in histones during the cell cycle. New messenger RNA synthesis probably does not commence in these fertilized eggs before about 4hr. after fertilization (see Comb & Silver, 1966), and the observations of Hilton & Stocken (1966) that DNA-dependent RNA synthesis is more repressed by the disulphide form of histone F3 than by its thiol form suggested that the disulphide form of the histones might predominate in mature unfertilized eggs. This was found to be the case, and the variations in proportion of thiol through the first cell division are reported here.

## METHODS

*Animals.* Partial hepatectomies of rats 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% sucrose to drink and food *ad lib.* after operation. Control rats were sham-operated.

Three species of sea urchins were used: *Arbacia punctulata*, *Paracentrotus lividus* and *Echinus esculentus*. The gonads were removed from the animals and placed in filtered sea water, and the mature eggs were collected by sedimentation after filtration through muslin. At the required times after fertilization the eggs were concentrated by sedimentation at 1230*g* for 10min. and the packed eggs kept at −10° until required. At least six *Arbacia* or *Echinus* or ten *Paracentrotus* were required to give 3ml. of 0·25M-HCl extract. Fertilization was performed at 22–25° for *Arbacia* and *Paracentrotus* and at 10° for *Echinus*.

*Irradiation.* This was provided from a $^{60}Co$ source that delivered 100rads/min. 100cm. from the source. The rats were exposed in individual Perspex cages; the control animals were similarly confined for the period of irradiation.

*Nuclei.* Liver nuclei were prepared in 2·2M-sucrose–5mM-$MgCl_2$–5mM-tris–HCl buffer, pH7·2, as described by Ord, Raaf, Smit & Stocken (1965). They were washed with 5mM-$MgCl_2$–10mM-tris–HCl buffer, pH7·1, and 1mM-HCl before isolation of the histones.

*Preparation of nuclear fraction from sea-urchin eggs.* The nuclear fraction was prepared as described by Tocco, Orengo & Scarano (1963). The eggs were disintegrated by homogenization in 5vol. of 0·25M-sucrose–0·415M-KCl–2·5mM-$MgCl_2$–2·5mM-$CaCl_2$–0·1M-tris–HCl buffer, pH7·2. The extent of cell breakage was checked microscopically; it exceeded 95%. The suspension was centrifuged for 10min. at 1230*g* at 0°. The sedimented residue was redispersed in sucrose medium and again centrifuged. The crude nuclei were then washed twice with 5mM-$MgCl_2$–10mM-tris–HCl buffer, pH7·1, and three times with 1mM-HCl. The first wash with $MgCl_2$–tris removed considerable amounts of protein, and three washes with 1mM-HCl were used to ensure removal of low-molecular-weight nucleotides.

*Extraction of histones.* Histone F1 was extracted by the $HClO_4$ method of Johns (1964) or by a modification of his method 2, which gave phosphate-rich F1 (Ord & Stocken, 1967*a*). This latter procedure was also used with liver nuclei to give phosphate- and thiol-rich histone F3-1, which was usually washed with 5% (w/v) $HClO_4$ to remove associated phosphate-poor histone F1 (Ord & Stocken, 1967*a*). Total histones were extracted from washed sea-urchin nuclei with 0·25M-HCl. Extracts from *Arbacia* were bright orange–red, but much of the pigment could be removed by dialysis against two changes of 50vol. of 50mM-HCl. All extracts from sea-urchin eggs were therefore dialysed in this way.

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

*Thiol and disulphide groups.* Thiol and thiol plus disulphide were estimated by the method of Ellman (1959) as described by Marsh, Ord & Stocken (1964).

*Phosphate determination.* Inorganic phosphate was measured in the original tissue homogenates after deproteinization with 10% (w/v) trichloroacetic acid. The method of Berenblum & Chain (1938) was used. In some experiments with liver, the deproteinized extract was hydrolysed for 15min. at 100° with M-HCl to allow determination of the total acid-labile phosphate in the extracts. Total phosphate in DNA and in proteins F1 and F3-1 was obtained after ashing with $HClO_4$–$H_2SO_4$ [70% (w/v) $HClO_4$–98% (w/v) $H_2SO_4$ (3:1, v/v)]. In the experiments on the effects of histones on DNA polymerase, the total phosphate contents of the proteins were measured by the procedure of Bartlett (1959).

*DNA.* $^{32}P$ incorporation into liver DNA was measured by the method of DeLuca, Rossiter & Strickland (1953). Tissue inorganic phosphate was used as a reference standard for determining the relative specific radioactivities in control and experimental animals.

*DNA polymerase system.* DNA polymerase (EC 2.7.7.7) was obtained from calf thymus by a method developed by Mr G. Bazill & Mr A. P. Philips (personal communication) based on the preparation described by Bollum (1960). The procedure entailed precipitation of the enzyme at pH5·2, treatment with protamine and fractionation with $(NH_4)_2SO_4$. The final preparation was free from deoxyribonuclease (EC 3.1.4.5) activity. The assay system contained (final vol. 0·5ml.) 4–10$\mu$g. of heat-denatured DNA, histone and water to 0·4ml., and 0·1ml. of a reaction mixture containing 2·5$\mu$moles of $MgCl_2$, 5·0$\mu$moles of tris–HCl buffer, pH7·2, 5·0$\mu$moles of potassium phosphate buffer, pH7·2, 0·33$\mu$mole of EDTA, pH7·2, 0·33$\mu$mole of $\beta$-mercaptoethylamine hydrochloride, 8m$\mu$moles each of deoxy-CTP, deoxy-GTP and TTP, 1·4m$\mu$moles of deoxy-ATP, 7·0m$\mu$moles of deoxy-[8-$^{14}C$]ATP (specific radioactivity 28·4mc/m-mole; Schwarz BioResearch Inc., Orangeburg, N.J., U.S.A.) and 100–150$\mu$g. of enzyme. The system was incubated at 37° for 20min., cooled in ice and made 20% (w/v) with respect to trichloroacetic acid. The precipitate obtained by centrifugation was washed three times and dissolved in 0·6ml. of anhydrous formic acid, and 0·5ml. was taken for counting.

*Electrophoresis.* Analytical electrophoresis on polyacrylamide gel was performed as described by McAllister, Wan & Irvin (1963) with glycine–acetic acid buffer, pH4·0, at 300v and 4–5mA/tube. The samples were made 5M with respect to urea before being run.

*Radioactivity measurements.* Inorganic [$^{32}P$]phosphate (The Radiochemical Centre, Amersham, Bucks.) was given intramuscularly to the rats 30 or 60min. before death. $^{32}P$ and $^{14}C$ radioactivities were measured by gas-flow counting (Nuclear–Chicago Corp. model D47 instrument; counting efficiencies: $^{14}C$, 17%, $^{32}P$, about 40%). Sufficient counts were reproduced to give an accuracy of ±3%.

## RESULTS

*Phosphorylation of histones F1 and F3-1 in regenerating liver.* Livers regenerating after partial hepatectomy provide a mammalian cell population with some degree of synchrony *in vivo*. Jaffe (1954) has described diurnal variations in the timing of the first wave of mitosis in rat liver; indications of such variations were also present in our results (Table 1). The extent of phosphorylation of histone F1 in the livers of normal and sham-operated rats was greater in animals killed in the afternoons compared with that found in livers from animals killed

Table 1. *Phosphorylation of histone F1 in liver nuclei during the first 30 hr. after partial hepatectomy*

Four to seven rats were used/group. Sham-operated and partially hepatectomized rats were kept in the laboratory after operation; food and 10% sucrose to drink were available *ad lib.* The animals received 100 $\mu$c of inorganic [$^{32}$P]phosphate intramuscularly/100 g. body wt. 30 or 60 min. before death. Nuclei were isolated and histone F1 was extracted in 5% (w/v) $HClO_4$, as described in the text. Specific radioactivities are referred to that of inorganic phosphate in the original homogenate. The numbers of experiments are given in parentheses. Phosphate content is expressed as m$\mu$g.atoms of P/mg. of protein and the means are given ± S.D.

| | | Phosphate content of histone F1 | |
|---|---|---|---|
| Animals killed 9–10 a.m. | Time after hepatectomy (hr.) | Phosphate content | Mean |
| Normal adults (3) | — | 34·7 | 34·7 ± 6·6 |
| Sham-operated (2) | 22–25 | 34·7 | |
| Hepatectomized (2) | 16–20 | 79·3 | 78·8 ± 21·0 |
| (2) | 22 | 93·1 | |
| (4) | 23–25 | 66·7 | |
| Animals killed 2–3 p.m. | | | |
| Normal adults (1) | — | 85·5 | |
| Sham-operated (2) | 4–5 | 56·3 | 54·9 ± 12·1 |
| (2) | 28 | 53·5 | |
| Hepatectomized (2) | 21–24 | 143·5 | 129·0 ± 30·2 |
| (1) | 29 | 101·0 | |

| | | 10 × Rel. sp. radioactivities of $^{32}$P in histone F1 and DNA | | | |
|---|---|---|---|---|---|
| | | 30 min. labelling | | 60 min. labelling | |
| | | Histone F1 | DNA | Histone F1 | DNA |
| Sham-operated, a.m. (4) | 22–25 | 0·36 | 0 | 0·62 ± 0·25 | 0·01 |
| Sham-operated, p.m. (4) | 4–5, 28 | — | — | 0·65 ± 0·10 | — |
| Hepatectomized, a.m. or p.m. | 16–25 | 0·68 ± 0·09 | 0·04 | 1·46 ± 0·49 | 0·10 |

Table 2. *Phosphorylation of histone F3-1 in livers from partially hepatectomized rats 5·5 and 24 hr. after operation*

Conditions are given in Table 1. Histone F3-1 was obtained and washed with 5% (w/v) $HClO_4$ as described in the text. Phosphate content is expressed as m$\mu$g.atoms of P/mg. of protein.

| | | Time after hepatectomy (hr.) | Phosphate content of histone F3-1 | 10 × Rel. sp. radioactivity |
|---|---|---|---|---|
| Normal adults | (1) | — | 79·0 | — |
| Sham-operated | (1) | 5·5 | 98·9 | 0·70 |
| | (3) | 24 | 94·2 | 0·67 |
| Hepatectomized | (1) | 5·5 | 104·0 | 0·73 |
| | (3) | 24 | 91·0 | 1·12 |

between 9 and 10 a.m. This increased phosphorylation did not, however, prevent a further twofold increase in phosphorylation detected in both series of partially hepatectomized rats 16–29 hr. after operation, roughly coincident with the first peak of DNA synthesis. The rise in phosphate content of histone F1 probably reached its height about 22 hr. after partial hepatectomy; the relative specific radioactivity of the phosphate was similarly increased. The greater degree of phosphorylation found in histone F1 from control rats in the afternoon was not accompanied by any change in relative specific radioactivity. No change in either phosphate content or specific radioactivity of histone F1 could be detected in partially hepatectomized rat livers 4–5 hr. after the operation, when accelerated RNA synthesis and protein synthesis were becoming apparent.

No change was found in phosphate content of histone F3-1 (perchlorate-washed) either at 5·5 hr. or at 24 hr. after hepatectomy, although the specific radioactivity of the phosphate was increased during the period of DNA synthesis (Table 2). The phosphate content of histone F3-1 did not show any alteration between values obtained in the mornings and in the afternoons.

Since $\gamma$-irradiation decreased $^{32}$P uptake into histone F1 in thymus nuclei to the same extent as it decreased uptake into DNA, whereas incorporation

Table 3. *Effect of γ-irradiation* in vivo *on the phosphorylation of histones from normal and regenerating rat liver*

The animals were irradiated at 100 rads/min. They were injected with inorganic [$^{32}$P]phosphate immediately after exposure and were killed 50 min. later. The histones and DNA were isolated and their specific radioactivities determined as described in the text. Histone F1 was prepared by method 1 of Johns (1964). The relative specific radioactivities were calculated with respect to that of the inorganic phosphate in the original liver homogenate Phosphate content is expressed as mμg.atoms of P/mg. of protein. R/C (%), (irradiated/control) × 100.

| Time of death after hepatectomy (hr.) | Dose of γ-irradiation (rads) | 10 × Rel. sp. radioactivity | | | Phosphate content | |
|---|---|---|---|---|---|---|
| | | Control | R/C (%) | DNA [R/C (%)] | Control | Irradiated |
| Histone F1 | | | | | | |
| Normal adult | 1000 | 1·4 | 100 | — | 31·0 | 28·4 |
| 4 | 500 | 1·7 | 100 | — | 42·8 | 34·5 |
| 24·5 | 500 | 1·5 | 73 | 63 | — | — |
| 23·8 | 1000 | 1·3 | 79 | 55 | 95·5 | 76·8 |
| 23·0 | 250* | 1·6 | 74 | 99 | — | — |
| 24·0 | 500* | 1·0 | 49 | 28 | 58·5 | 54·9 |
| Histone F3-1 (washed) | | | | | | |
| 24·5 | 1000 | 1·5 | 100 | 77 | 69·9 | 68·1 |
| 24·0 | 500* | 0·9 | 97 | 46 | 95·8 | 102·9 |

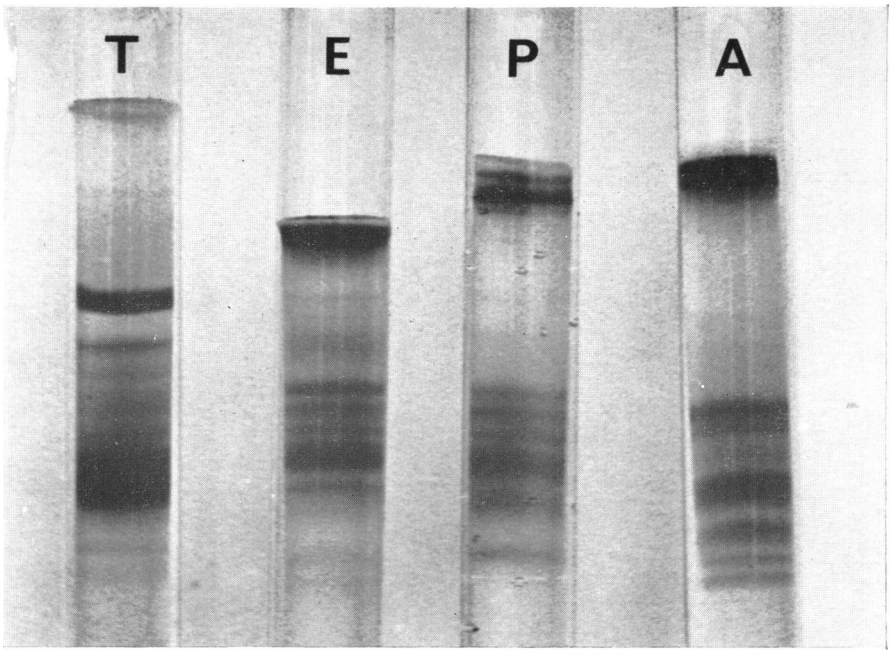
* 250 or 500 rads given 3 hr. after partial hepatectomy.

into histone F3-1 was unaffected (Ord & Stocken, 1966*b*), the effects of ionizing radiation on $^{32}$P uptake into the histones and DNA of regenerating liver were examined. Phosphorylation of histone F1 was unaffected by γ-irradiation in livers from normal adults and in those from partially hepatectomized rats 4 hr. after operation. If the rats were irradiated during the period of DNA synthesis, however, a decrease in specific radioactivity was apparent (Table 3). This was more marked if the irradiation was given 3 hr. after operation and the phosphorylation examined at 24 hr. after operation. The enhanced sensitivity of the phosphorylation of histone F1 if the rats were exposed in the first 6 hr. after operation parallels the greater sensitivity to irradiation of DNA synthesis in livers from partially hepatectomized rats under the same conditions (Holmes & Mee, 1956). As with thymus gland, phosphorylation of histone F3-1 was apparently unaffected even if the rats were exposed 3 hr. after operation and not killed until 24 hr. after operation; uptake of $^{32}$P into acid-labile phosphates was also unaffected with exposures either at 3 or at 23 hr. after operation.

*Sea-urchin histones.* As preliminary experiments had indicated that in nuclei from adult livers the thiol plus disulphide content of the total histone extract in 0·25 M-hydrochloric acid was predominantly present as thiol, it was unlikely that any change in the thiol/disulphide ratio would be detectable through the first cell division after partial hepatectomy, where only 5–10% of the cells divide. Fertilized sea-urchin eggs were therefore used, since they are virtually synchronous during the first division, and since they also provide a starting population of mature eggs in which a very dramatic increase in nucleic acid and protein synthesis is initiated by fertilization. As reported by Comb & Silver (1966) the histones have striking similarities to those from thymus (Plate 1). Comb & Silver (1966) found that their material from *Lytechinus variegatus* co-chromatographed with a peak from calf thymus corresponding to the fraction IIb described by Murray (1964), and that some lysine-rich histone might have been present. Bäckström (1965) found very little lysine-rich histone in *Paracentrotus* before it hatched. This is confirmed by our results; extracts from *Arbacia* and *Paracentrotus* showed only traces of material running in the position of histone F1. Rather more histone F1 may have been present in extracts from *Echinus*. Only quantitative differences (Plate 1) were apparent between the patterns from the three species we examined, and they all showed a prominent band that only just moved into the gel, and that was not present in extracts from rat thymus nuclei. No differences were detectable in the patterns obtained from any of the three species at various times through the first division.

The low concentration of histone F1 indicated by the electrophoretic pattern was confirmed by using both the perchlorate and the acid–ethanol methods of Johns (1964) with mature unfertilized eggs from *Paracentrotus*. The acid–ethanol method also showed the presence of proteins corresponding to histones F2a, F2b and F3, but these proteins have not yet been further characterized.

*Thiol/disulphide ratio and phosphate contents of*




EXPLANATION OF PLATE 1

Tubes T, E, P and A show the patterns obtained after electrophoresis of 0·25 M-HCl extracts from washed nuclei of rat thymus, *Echinus*, *Paracentrotus* and *Arbacia* respectively. About 80 $\mu$g. of protein was applied to each gel. The front band in the extract from *Paracentrotus* ran 4·2 cm.

Table 4. *Thiol plus disulphide and total phosphorus content of 0·25 M-hydrochloric acid extracts from the nuclear fraction from developing sea-urchin eggs*

Sedimented and washed eggs were fertilized at 22–26°. Cleavage was at 45 min. after fertilization. The nuclear fraction was obtained by the method of Tocco *et al.* (1963) and washed and extracted as described in the text. Thiol plus disulphide content is expressed as mμmoles/mg. of protein. Phosphate content is expressed as mμg.atoms of P/mg. of protein. SH (%), (thiol/thiol plus disulphide) × 100

| Time after fertilization (min.) | *Arbacia* | | | *Paracentrotus* | | |
|---|---|---|---|---|---|---|
| | SH+S·S content | SH (%) | Phosphate content | SH+S·S content | SH (%) | Phosphate content |
| 0 | 122 | 12 | — | 141 | 16 | — |
| 10 | 130 | 23 | 106 | 147 | 27 | 107 |
| 30 | 138 | 19 | 80 | 140 | 10, 19 | 110 |
| 45 | 99 | 44 | 63 | 96 | 6 | — |
| 60 | 100 | 37 | 71 | — | — | — |
| 65 | — | — | — | 127 | 14 | — |

Table 5. *Thiol plus disulphide and total phosphorus content of 0·25 M-hydrochloric acid extracts from the nuclear fraction from developing eggs of* Echinus

Sedimented and washed eggs were collected and fertilized at 10°. Cleavage was at 110 min. after fertilization. The nuclear fraction was obtained by the method of Tocco *et al.* (1963) and washed and extracted as described in the text. Thiol plus disulphide content is expressed as mμmoles/mg. of protein. Phosphate content is expressed as mμg.atoms of P/mg. of protein. SH (%), (thiol/thiol plus disulphide) × 100.

| Time after fertilization (min.) | SH+S·S content | SH (%) | Phosphate content |
|---|---|---|---|
| 0 | 97 | 6 | 184 |
| 10 | 92 | 13 | 108 |
| 20 | 107 | 42 | 113 |
| 30 | 86 | 14 | 145 |
| 75 | 98 | 40 | 92 |
| 110* | 76 | 59 | 75 |
| 110† | 74 | 47 | 92 |
| 130 | 84 | 22 | 130 |

* Eggs just showing cleavage.
† Eggs in cleavage.

*sea-urchin histones through the first cell division.* In all three species the thiol/total thiol plus disulphide ratio in the 0·25 M-hydrochloric acid extract from mature unfertilized eggs was less than 0·2 (Table 4). This contrasts with rat liver and thymus, where 80% or more is present as thiol. After fertilization there might be a slight transient rise in the proportion of thiol, but in *Arbacia* and *Echinus* there was a further increase to 44–59% of the total thiol plus disulphide by first cleavage. Because of the longer time to division in *Echinus* it was possible to examine the histones from eggs that were only just showing indentations in their cell membranes before division (Table 5). The proportion of thiol then was the highest found; 15–20 min. after cleavage the proportion as thiol was again lowered. This fall in proportion of thiol after division may explain the results with *Paracentrotus*, since experiments with this species preceded those with *Echinus*, and in the earlier work the eggs were not used until cleavage was fully established.

The total thiol plus disulphide content of the dialysed extracts from the crude nuclei was higher than those found in rat tissues, and a roughly equimolar amount of phosphate was also present. In all three species there was a fall in thiol plus disulphide and phosphate content/mg. of protein at cleavage. The significance of this and the nature of the thiol- and phosphate-containing components awaits the characterization of the histones.

*Effects of phosphorylation and thiol oxidation on the actions of histones F1 and F3 on DNA polymerase.* The experiments of Stevely & Stocken (1966) and Hilton & Stocken (1966) respectively have shown that decreasing the phosphate content of histone F1 or the proportion of thiol in histone F3 increases the capacity of these histones to repress DNA-dependent RNA synthesis. The high proportion of disulphide in the total histone extract from mature unfertilized sea-urchin eggs is consistent with the absence of nucleic acid and protein synthesis at this time. After fertilization DNA synthesis commences, still without RNA being made, and it was therefore decided to examine the effects of altering the thiol/disulphide ratio in histone F3-1 on the actions of this histone on the DNA polymerase system. Since in regenerating liver DNA synthesis is accompanied by an increase in phosphorylation of histone F1, the effects of phosphorylation of histones F1 and F3 on the polymerase system were also studied.

Perchlorate-washed histones F3-1 and F3-2 (Ord

Table 6. *Effect of histones F3-1 and F3-2 on DNA polymerase*

Preparation of the histones and conditions for the assay are given in the text. Histones F3-1 and F3-2 were obtained from chicken livers, and contained 174·5 and 32·6 mμg.atoms of P/mg. of protein respectively. In Expt. 1, 10 μg. of DNA was present throughout; the order of the addition of the components to the assay mixture was DNA, water, histone, substrates + enzyme. In Expt. 2 the order of addition was the same as in Expt. 1 but the histone/DNA ratio was kept constant at 2:1, by wt. The figures in parentheses in Expt. 2 give values obtained when the order of addition was DNA, histone, water, substrates + enzyme.

| Expt. 1 Histone present (μg.) | Deoxy-[$^{14}$C]ATP incorporated (counts/100 sec.) Histone F3-1 | Histone F3-2 |
|---|---|---|
| 0 | 567 | 567 |
| 10 | 776 | 342 |
| 15 | 666 | 40 |
| 20 | 470 | 21 |

| Expt. 2 DNA present (μg.) | DNA alone | | |
|---|---|---|---|
| 4 | 614 | 201 (14) | 37 (< 10) |
| 8 | 2280 | 799 (25) | 29 (< 10) |
| 12 | 2740 | 1097 (62) | 32 (< 10) |
| 16 | 2810 | 1258 (99) | 41 (< 10) |

Table 7. *Effects of thiol oxidation on the action of histone F3-1 in the DNA polymerase system*

The enzyme system was described in the text. Histone F3-1 contained 36·7 mμg.atoms of P and 46·4 mμmoles of thiol plus disulphide/mg. of protein. It was reduced by treatment with β-mercaptoethanol followed by removal of the excess of thiol by passage through Sephadex G-25 and elution with 0·02 M-tris–HCl buffer, pH 8·2; oxidation was performed by bubbling $O_2$ for 24 hr. through 0·02 M-tris–HCl buffer, pH 8·2, in which the histone was suspended in dialysis tubing. The order of addition of the components was: DNA, histone, water, substrates, enzyme.

| Histone/DNA ratio | Deoxy-[$^{14}$C]ATP incorporated (counts/100 sec.) 77% SH | 50% SH | 25% SH |
|---|---|---|---|
| 0·4 | 2050 | 1908 | 2020 |
| 0·8 | 1570 | 1299 | 1570 |
| 1·2 | 603 | 569 | 787 |
| 1·6 | 167 | — | 210 |

& Stocken, 1967*a*) were prepared from chicken livers or rat thymus. Histone F3-1 had three to five times more phosphate and two to three times more sulphur than had histone F3-2. Two types of

Table 8. *Effect of phosphorylation on the action of histone F1 in the DNA polymerase system*

The DNA polymerase activity was measured as described in the text. Phosphate-poor and phosphate-rich samples of histone F1 were obtained from calf thymus by Mr W. S. Stevely (unpublished work). They contained 19·4 and 52·0 mμg.atoms of P/mg. of protein respectively. DNA (10 μg.) was used throughout. The order of addition was DNA, histone, water.

| Histone present (μg.) | Deoxy-[$^{14}$C]ATP incorporated (counts/100 sec.) Phosphate-poor histone F1 | Phosphate-rich histone F1 |
|---|---|---|
| 0 | 2020 | 2020 |
| 2 | 1827 | 2200 |
| 4 | 479 | 2010 |
| 6 | 37 | 1589 |
| 8 | 6 | 1420 |

experiment were performed, the first in which a constant amount of DNA was present but the amount of histone varied, and the second in which the histone/DNA ratio by weight was kept constant but the amount of DNA altered. As in experiments with RNA polymerase (Dr J. Hilton, unpublished work), the order of addition of the components affected the extent by which the histones depressed DNA synthesis (Table 6), but histone F3-2 was consistently more effective than histone F3-1 in decreasing DNA synthesis. Comparison between the results with histone F3 (Table 6) and those with histone F1 (Table 8) showed that a greater proportion by weight of histone F3 to DNA was required to decrease DNA synthesis than of histone F1; similar observations have been made by Billen & Hnilica (1964) and Gurley, Irvin & Holbrook (1964). Oxidation of the thiol groups of histone F3-1 (Table 7) indicated that this did not affect the capacity of this histone to decrease DNA synthesis *in vitro*. Neither in these experiments nor in those with histone F1 was any precipitate formed on the addition of the histones to the heat-denatured DNA or after the addition of the substrates. Increasing the phosphate content of histone F1 (Table 8) decreased its capacity to decrease DNA synthesis.

## DISCUSSION

The results reported here have provided additional evidence for our suggestion (Ord & Stocken, 1967*a*) that changes in the phosphorylation or oxidation–reduction state of histones F1 and F3-1 might accompany variations in the synthetic activity of cell nuclei. Considering first the experiments on the phosphorylation of histone F1 during liver regeneration, studies with this lysine-rich

histone are greatly facilitated by the solubility of this protein in 5% (w/v) perchloric acid. This gives a preparation of histone F1 containing both phosphorylated and non-phosphorylated forms, in which the phosphate is known to be present as serine phosphate (Ord & Stocken, 1966*a*). Mr W. S. Stevely (unpublished work) has shown that, although incubating nuclei *in vitro* can yield a number of phosphorylated proteins (Stevely & Stocken, 1966), the number of phosphorylated forms existing *in vivo* is probably more restricted. In some experiments phosphate-rich histone F1 was obtained from regenerating liver at the time of DNA synthesis; the phosphate content of this material was apparently unchanged. This, taken together with W. S. Stevely's observations, suggests that the increase in phosphorylation of histone F1 during the period of DNA synthesis is due mainly to an increase in the number of molecules of histone F1 phosphorylated, rather than to an increase in the number of phosphate groups/molecule.

Ingles & Dixon (1967) have reported the reciprocal observation; the extent of phosphorylation of protamine in trout testis declines with maturation of the sperm. It is clear, however, that phosphorylation of histone F1 is not exclusively associated with DNA synthesis. In liver the phosphate content of histone F1 varies through the day. The radioinsensitivity of phosphorylation of histone F1 in normal liver and up to 4hr. after hepatectomy suggests that this process must be distinguished from the radiosensitive increase in phosphorylation of histone F1 in regenerating liver coincident with DNA synthesis. It is tempting to speculate that the radioinsensitive phosphorylation might be associated with RNA synthesis; we have been unable to find data on possible diurnal variations in rates of protein or RNA synthesis in rat liver, though changes in enzyme activity have been reported (Potter, Gebert & Pitot, 1966). Since the phosphorylation of histone F3-1 is unaffected by irradiation it is unlikely either that the inhibition of phosphate uptake into histone F1 and DNA is due to an absence of high-energy phosphates, or that all new histone synthesis that occurs during the 'S period' is decreased by exposure to $\gamma$-irradiation, with a resulting shortage of sites for phosphate uptake. It seems more probable that the stimulus from partial hepatectomy provides a signal leading to a number of co-ordinated responses, including increased phosphate uptake into histone F1 and the initiation of DNA synthesis. The sensitivity of these two processes and of RNA synthesis (Welling & Cohen, 1960) to irradiation within 6hr. of operation suggests that it is the signal for co-ordinated response or its reception that is the radiosensitive factor.

The experiments with mature unfertilized eggs from *Paracentrotus* and the electrophoretic patterns obtained with all three species of sea urchin confirm that only traces of lysine-rich histone are present in eggs at this time (Bäckström, 1965), so that the increase in phosphorylation of histone F1 found in regenerating liver is not obligatorily associated with DNA synthesis. The well-known change in basic proteins from protamine in sperm to a wider spectrum including lysine-rich histones in diploid cells also emphasizes the importance of lysine-rich histones in many differentiated cells. Lysine-rich histones prepared by perchlorate extraction and subsequent dialysis are more effective on a weight basis than arginine-rich histones obtained by acid–ethanol methods in repressing replication or transcription from DNA (but see Hnilica, 1967). As differentiation proceeds, with the ensuing switching off of considerable portions of the genome, the economy of the cell may be increased by the appearance of lysine-rich histones as repressors, whose actions can be controlled through phosphorylation.

In assessing the constancy of the phosphorylation of histone F3-1 in liver and the interpretation of the changes in thiol/disulphide ratio in the extracts from the sea-urchin eggs, the nature of the thiol-containing protein(s) must be considered. The process for the isolation of histone F3-1 was developed to give a histone fraction enriched in phosphate and thiol; it is not homogeneous (Ord & Stocken, 1967*a*). Mauritzen, Starbuck, Saroja, Taylor & Busch (1967) and Phillips (1967) have shown that the arginine-rich histone complex from calf thymus contains a number of different proteins. The sulphur is associated with component(s) having an arginine/lysine ratio slightly greater than 1:1 and a phenylalanine content of just under 3 moles/100 moles of amino acid residues (Mauritzen *et al.* 1967). Very similar analyses have been obtained by Dr J. Hilton (unpublished work) for a thiol-rich component of histone F3 from calf thymus. These proteins were obtained by Dr J. Hilton by methods that lead to a loss of phosphate. It is therefore still uncertain whether the phosphate is attached to the protein containing the thiol groups, and also whether the extent of phosphorylation can be varied. Our procedure may isolate only thiol-containing protein that is extensively phosphorylated; forms with lower amounts of phosphate may be missed.

In acid extracts from rat liver and thymus, the thiol content of histone F3-1 accounts for a considerable proportion of the total thiol plus disulphide of the extracts. Similar analyses are not yet available for the extracts from the sea-urchin eggs, so that we cannot say definitely that the changes in thiol/disulphide ratio during the first division are due to changes in the arginine-rich

thiol-containing histone. The prominent slow-moving component shown on electrophoresis could be due to a thiol-containing protein that is not present in extracts from rat thymus. However, the patterns obtained by Mauritzen *et al.* (1967) with histones from foetal calf thymus showed a thiol-containing slow-moving component, and Dr J. Hilton (unpublished work) has evidence that oxidation of the arginine-rich thiol-containing protein decreases its electrophoretic mobility. It is possible therefore that the slow-moving material in the extracts from the sea-urchin eggs represents an F3-1 type of histone in its disulphide form.

The variations in thiol/disulphide ratio through the first cell division in sea-urchin eggs indicate that acid-extractable proteins from the nuclear fraction of the eggs must be included in the group of thiol-containing proteins (Sakai, 1967) that show alterations in thiol/disulphide ratio during the cell cycle. The significance of the increased proportion of thiol just before cleavage and the exact timing of the changes await further investigation. It is clear though that mature unfertilized eggs have a high proportion of their histone thiol in the oxidized form, and that this situation is restored soon after cleavage. The failure of oxidation to affect the capacity of histone F3-1 to decrease DNA synthesis in the polymerase system *in vitro* does not necessarily reproduce the situation *in vivo*. In the isolated system heat-denatured rapidly cooled DNA was used and the contrast between the increased repression by oxidized histone F3 of RNA polymerase and its inability to augment the suppression of DNA synthesis could be due to the use of single-stranded DNA in the latter system.

If the situation at fertilization of amphibian eggs (Gurdon, 1967) is relevant to that in sea urchins it is likely that the mature unfertilized egg contains the enzymes necessary for DNA synthesis and that it is the substrates or coenzymes that must be adjusted, as a result of the fertilization process, before division can proceed. In liver after partial hepatectomy the enzymes necessary for DNA synthesis are not available but must be induced or activated. The different histone changes in the two cases studied may reflect this difference in initial state, but in both instances the changes required could be brought about by simple enzyme reactions after alterations in coenzyme balance or phosphate availability.

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