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Further Studies on Phosphorylation and the Thiol/Disulphide Ratio of Histones in Growth and Development

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1. The proportion of thiol groups in the total thiol + disulphide of histone extracts from fertilized eggs from *Echinus* and *Psammechinus* was increased during periods of structural alterations in the nucleus. 2. The probable start of DNA synthesis in the fertilized eggs coincided with periods of maximum thiol content. 3. Histone extracts from rat liver and regenerating liver were predominantly in the thiol form and no significant variations could be detected during the first 30hr. after partial hepatectomy. 4. An assay system was developed to follow the phosphorylation believed to be associated with the arginine-rich histone F3. Phosphorylation increased by 50% at 1 and 2hr. after partial hepatectomy. The phosphate content also increased during the period of DNA synthesis. 5. The increased phosphorylation found 1hr. after partial hepatectomy was not prevented by actinomycin or prior irradiation. 6. The phosphate content of histone F1 was very high in livers from foetal rats and declined in neonatal rats similarly to the decline in DNA synthesis.

Earlier work (Ord & Stocken, 1968*a*; Stevely & Stocken, 1968) has established that phosphorylation of the lysine-rich histone F1 is associated with replication of DNA in regenerating liver and other animal tissues. It has also been found that mature sea-urchin eggs, in which net protein and nucleic acid synthesis do not occur, have their thiol-containing histone(s) in the disulphide form and that there is a conversion into the thiol form during the cell cycle (Ord & Stocken, 1968*a*,*b*). Sea-urchin eggs do not contain appreciable amounts of histone F1 before differentiation (Bäckström, 1965; Ord & Stocken, 1968*a*); the changes in micro-structure so far detected affect groups in a mixture of histones that behave chromatographically and electrophoretically (Comb & Silver, 1966; Ord & Stocken, 1968*a*) like the F2+F3 group of histones from thymus. In histones from rat thymus the thiol group is mainly associated with the arginine-rich histone F3, which may also be phosphorylated (Ord & Stocken, 1967). We have therefore been interested to investigate more precisely the relation between the thiol changes in sea-urchin histones and the morphologically observable phases of the cell cycle and also to test whether phosphorylation of the arginine-rich histone can be linked in any way to these events. This latter part of the work has been performed with regenerating rat liver.

METHODS

Procedures for the isolation of nuclei, determination of protein, phosphate and thiol+disulphide contents, gel electrophoresis and measurement of radioactivity are as described by Ord & Stocken (1968*a*).

Extraction of histones. The basis for the final procedure is discussed in the text. The washed nuclei were extracted with 5% (w/v) trichloroacetic acid. The pooled supernatant was made 20% with respect to trichloroacetic acid and the precipitate extracted with 5% (w/v) $HClO_4$. Histone F1 was precipitated from the solution in 5% $HClO_4$ by 20% trichloroacetic acid. The protein was redissolved in 250mM-HCl and dialysed for 4hr. against 100vol. of 50mM-HCl and for 15hr. against 100vol. of water at 0°. The nuclear residue remaining after treatment with 5% trichloroacetic acid was extracted with 250mM-HCl to remove the remaining histones. The extract was dialysed as described for histone F1.

ATP content of livers after partial hepatectomy. Livers from sham-operated rats and from animals 1hr. after partial hepatectomy were perfused with cold 0·9% (w/v) NaCl. The livers were homogenized in 10% trichloroacetic acid, centrifuged and the trichloroacetic acid was removed from the supernatant with ether. ATP was measured by the firefly method of Holton (1959).

Nucleotide content of livers after partial hepatectomy. Livers were removed from animals at various times after partial hepatectomy and dropped into liquid N_2. Livers from normal adult rats were used as controls. The livers were extracted with 10% trichloroacetic acid and the nucleotide

content of the extracts was determined after removal of the acid and separation on Dowex 1 (formate form) (Hurlbert, Schmitz, Brumm & Potter, 1954; Potter, Schlesinger, Buettner-Janusch & Thompson, 1957).

Adrenalectomy. Adrenal glands were removed under ether anaesthesia and the rats maintained on 0·9% NaCl soln. The rats were used 4–8 days after operation.

RESULTS

Changes in the thiol/disulphide ratio in histones during the early cleavage stages in the sea urchin. The experiments were confined to *Echinus* and *Psammechinus*. Work with the former is facilitated by the large number of eggs that may be obtained from a single individual, so that three or four females will provide sufficient material for two or three points at different times after fertilization. *Echinus* is also convenient because of the low temperature to which it is accustomed at the start of the season; at 8° the first furrow takes 3hr. to appear compared with just under 120min. when the temperature has risen to 10° and with 85min. for *Psammechinus* at 12°. The extended time enables a much more detailed study to be made of the thiol changes (Fig. 1).

Different procedures for extraction of the histones were used in the three experiments. In the earlier work with *Echinus* (Fig. 1*a*) the histones were extracted from the washed nuclear fraction with 250mM-hydrochloric acid; in the second run (Fig. 1*b*) the nuclear fraction was extracted with acid–ethanol, and with *Psammechinus* (Fig. 1*c*) the nuclear residue was washed with 5% trichloroacetic acid before extraction with 250mM-hydrochloric acid. The reasons for the different procedures are discussed in connexion with results in regenerating rat liver (see below). The alterations in thiol/disulphide ratios are similar in all three cases. There is an initial rise in the proportion of thiol from a value of less than 10% of the thiol+disulphide; this peak coincides with fusion and is followed by a second, prominent, peak evident by prophase and terminating during telophase. A similar peak occurs in the second division. In *Echinus* at 8° the peak associated with the first mitosis is split; this was not detected in *Psammechinus* at 12° and requires confirmation.

Separation and assay of phosphorylated histones. Histone F3 is usually obtained by the acid–ethanol procedure of Johns (1964). Earlier experiments, however, had demonstrated that extraction of washed rat liver or thymus nuclei with acid–ethanol and dialysis against ethanol for short periods precipitated proteins that contained both thiol and phosphate groups (Ord & Stocken, 1967). The amount of phosphate in the precipitate declined if the time of dialysis against ethanol was increased. Serine (or threonine) phosphate was

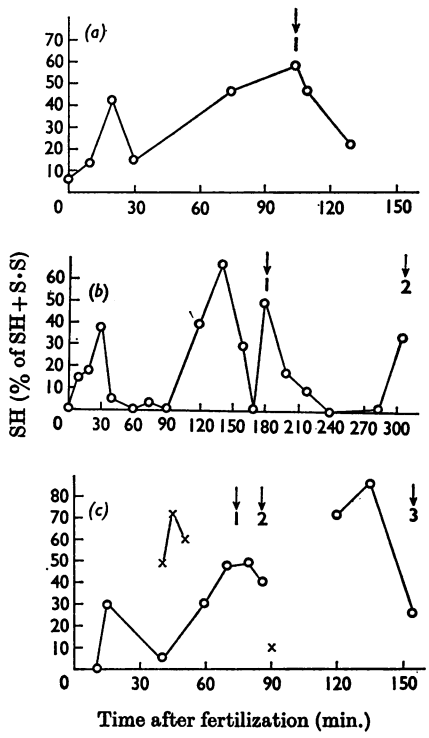

Fig. 1. Variation in thiol proportion of total thiol+disulphide in histones from *Echinus* and *Psammechinus* eggs after fertilization. (*a*) *Echinus* at 10°; total 250mM-HCl extracts. The arrow marks the first furrow. (*b*) *Echinus* at 8°; acid–ethanol extracts. Arrow 1 marks the first furrow; arrow 2 marks the four-cell stage. (*c*) ○, *Psammechinus* at 12°; ×, *Psammechinus* at 14°; 250mM-HCl extracts after washing nuclear residue with 5% trichloroacetic acid. Arrow 1 marks the furrow at 14°; arrow 2 marks the furrow at 12°; arrow 3 marks the four-cell stage at 12°.

detected in the precipitated material after acid hydrolysis and the phosphorus was alkali-labile. The hydrolysis of phosphate in the acid–ethanol-extraction procedure is further illustrated by the decline in alkali-labile phosphate radioactivity from an initial value (taken as 100%) to 91% after 2hr. of dialysis and to 37% after 17hr. of dialysis against ethanol. It is therefore apparent that this procedure, which gives the normal starting material from which to obtain histone F3, causes the hydrolysis of phosphate that is attached to this molecule.

Histone F1 may also be phosphorylated; it is therefore essential to remove histone F1 from any material in which the possible phosphorylation of histone F3 is being examined. Extraction with perchloric acid (5%) is commonly employed for this purpose (Johns, 1964) although other dilute acids

Table 1. *Extraction procedure used to assay phosphate groups in extracts containing histone F1 and histone F3 and thiol groups in histone F3*

Rats were injected intramuscularly with inorganic [^{32}P]phosphate 1 hr. before death. Liver nuclei were isolated and washed as described in the text. They were then extracted twice with 5% (w/v) trichloroacetic acid, with a total of 3 ml. of acid/liver originally taken. The 5% trichloroacetic acid extract was made 20% with respect to trichloroacetic acid by the addition of 100% (w/v) trichloroacetic acid. The precipitate was taken up in 1 ml. of 5% (w/v) $HClO_4$/liver originally used and centrifuged, and histone F1 was precipitated from the supernatant by 20% trichloroacetic acid. The nuclei from which histone F1 had been removed were extracted with 2 ml. of 250 mM-HCl/liver originally used. Half the extract was dialysed as described in the text and the other half precipitated at 20% trichloroacetic acid, redissolved and dialysed.

Extraction with 5% trichloroacetic acid	
Radioactivity in pooled 5% trichloroacetic acid extract (counts/min.)	14740
Radioactivity in supernatant after first precipitation with 20% trichloroacetic acid (counts/min.)	12300
Precipitated radioactivity insoluble in 5% $HClO_4$ (counts/min.)	456
Radioactivity in supernatant after second precipitation with 20% trichloroacetic acid (counts/min.)	0
(Difference) radioactivity in histone F1 (counts/min.)	1984
Radioactivity recovered in histone F1 after dialysis (counts/min.)	1232 (62%)
Protein in original trichloroacetic acid extract (mg.)	2·63
Protein in histone F1 after dialysis (mg.)	2·12 (81%)
Extraction with 250 mM-HCl	
Radioactivity in 250 mM-HCl extract (counts/min.)	19800
Radioactivity after dialysis of extract for 17 hr. at 0° (counts/min.)	13590 (68%)
Radioactivity in supernatant after precipitation of 250 mM-HCl extract at 20% trichloroacetic acid (counts/min.)	350
Radioactivity in precipitated material after re-solution and dialysis for 17 hr. at 0° (counts/min.)	15000 (76%)
Protein in original 250 mM-HCl extract (mg.)	19·1
Protein in extract after dialysis (mg.)	14·3 (75%)
Protein in extract after precipitation and dialysis (mg.)	14·5 (76%)
SH+S·S content of dialysed 250 mM-HCl extract (nmoles/mg. of protein)	39·2
SH+S·S content of precipitated and dialysed 250 mM-HCl extract (nmoles/mg. of protein	40·4
SH content of dialysed material (% of SH+S·S)	85
SH content of precipitated and dialysed material (% of SH+S·S)	70
(SH+S·S)/P ratio of dialysed material	1·25
(SH+S·S)/P ratio of precipitated and dialysed material	1·23

may be used. Trichloroacetic acid (5%) was used here to avoid any possible oxidation of thiol groups on histones and because it was extremely effective in removing bound inorganic phosphate from the nuclei (Table 1). In experiments in which histone F1 was also being studied, the trichloroacetic acid extract was made 20% (w/v) with further trichloroacetic acid and histone F1 was reprecipitated after solution in 5% perchloric acid (Table 1).

The nuclear residue from which histone F1 and bound inorganic phosphate had been removed was then extracted with 250 mM-hydrochloric acid. Only small amounts of inorganic phosphate were now present in the extract. These were usually removed by dialysis against 100 vol. of 50 mM-hydrochloric acid and 100 vol. of water; occasionally the 250 mM-hydrochloric acid extract was precipitated with trichloroacetic acid and the material redissolved and dialysed. Either procedure gave material with identical phosphorus and sulphur contents (Table 1), but the additional precipitation increased the possibility of oxidation and was therefore normally avoided.

The dialysed extract obtained in this way contained the total histones present in the nuclei, minus histone F1. This was verified by gel electrophoresis, when it was found that [^{14}C]*N*-ethylmaleimide binding and ^{32}P radioactivity both coincided with the band corresponding to histone F3. Since histones of the F2 group contain negligible cysteine and phosphorus, the thiol + disulphide and phosphorus contents of the dialysed extracts were thought to be due mainly to arginine-rich histone F3. Ideally, phosphorylation of this protein would have been followed by estimation of alkali-labile phosphate in the dialysed extract. With the small amount of material available for the assays, however, considerable errors were introduced if this procedure was followed because the solution contains polypeptides soluble in 20–25% trichloroacetic acid. These polypeptides are precipitated as the phosphomolybdate complex in the Berenblum & Chain (1938) method for distinguishing inorganic phosphate from organophosphates.

Phosphorylation of histone F3 in regenerating rat liver. Earlier work on the phosphorylation of

Table 2. *Oxidoreduction state of histone F3 and the extent of phosphorylation of histone F3 and histone F1 in regenerating rat liver nuclei at various times after partial hepatectomy*

Methods of isolation of the histone are described in the text. Unless otherwise stated [^{32}P]phosphate was injected 30 min. before death. The specific radioactivity is given relative to that of inorganic phosphate in the original homogenate.

	'Histone F3'				Histone F1	
Controls	SH+S·S (nmoles/mg. of protein)	SH (% of SH+S·S)	P (ng.atoms/mg. of protein)	10 × Rel.sp. radioactivity of histone P	P (ng.atoms/mg. of protein)	10 × Rel.sp. radioactivity of histone P
Normal liver (4)	40·8 (38·9–43·8)	85–100	37·7 (31·4–43·0)	—	—	
Anaesthetized rats (3)	38·1 (29·0–43·5)	85–100	45·9 (43·2, 48·5)		45·9 (43·2, 48·5)	
Sham-operated						
1 hr. (1)	39·7	—	45·8	1·05	41·7	0·33
2 hr. (2)	37·8 (34·4, 41·2)	87 (87, 87)	45·3 (39·5, 51·0)	0·70 (0·66, 0·74)	44·7 (39·2, 50·2)	0 22 (0·21, 0·23)
22 hr. (2)*	36·8 (32·0, 41·5)	96 (92, 100)	32·8 (31·6, 34·0)	2·1 (2·0, 2·2)	40·9 (31·0, 50·7)	0·58 (0·52, 0·64)
30 hr. (1)*†	33·0	100	35·8	1·5	61·2	—
Mean	38·9 ± 4·7	—	39·2 ± 6·5	—	43·5	—
Partially hepatectomized						
1 hr. (3)	42·1 (30·3–49·4)	83	59·5 (54·8–67·4)	0·71 (0·63–0·85)	38·2	0·32
2 hr. (2)	47·0 (42·3–51·6)	68 (68, 68)	56·0 (52·9, 59·0)	0·70 (0·68, 0·72)	44·3 (40·6, 48·0)	0·23 (0·16, 0·29)
22 hr. (2)*	49·9 (46·9, 52·9)	90 (79, 100)	81·5 (69·2, 93·7)	1·14 (0·87, 1·41)	73·2 (72·0, 74·4)	1·85 (1·7, 2·0)
30 hr. (1)*†	41·4	90	46·8	1·65	52·0	0·44
Mean 1 and 2 hr.	44·0 ± 8·4	—	58·1 ± 5·7	—	42·3	—

* [^{32}P]Phosphate 1 hr. before death.
† Rats killed in the afternoon.

histones in regenerating rat liver was confined to histone F1, as only with this histone is there a convenient method for quantitative removal of protein, free from other material, under conditions in which the phosphate group does not seem to be hydrolysed. The procedure that had detected phosphate groups on histone F3 (Ord & Stocken, 1967) led only to the isolation of thiol-containing protein, carrying phosphate groups. About 80% of the thiol present in the acid–ethanol extract was not precipitated in the 2 hr. period of dialysis used and it was therefore impossible to examine any variations there might be in the extent to which the thiol-containing histone was phosphorylated.

The present method in which the phosphorylated histone F1 is removed by prior extraction with 5% trichloroacetic acid is thought to give an extract containing all the thiol-containing histone so that changes in its phosphate content represent changes in extent of phosphorylation of the protein.

No differences were found (Table 2) in the thiol + disulphide and phosphate contents of the extracts from normal livers, from livers removed from rats under ether anaesthesia or at any of the times at which sham-operated animals were used. The constancy of the data indicate the reproducibility of the procedure. Over 80% of the thiol + disulphide was in the reduced form. The extent of phosphorylation of histone F1 is very similar to that reported by Ord & Stocken (1968*a*).

New types of RNA can be detected by hybridization 1 hr. after partial hepatectomy in mice (Church & McCarthy, 1967). Both at 1 and 2 hr. after partial hepatectomy in these rats, the phosphate content of the extract containing histone F3 was increased, without significant changes in its thiol + disulphide content (Table 2). An increase was also found at 22 hr. at the peak of DNA synthesis. The first wave of mitosis reaches its height at 30 hr. At this time the phosphate content was normal, but this may have been because of a failure to isolate nucleoprotein from cells actually in mitosis. At 2 hr. after operation there might be an increase in disulphide content of the histone F3 extract, but at the other periods the percentage of thiol was normal.

In an experiment with rats that had been

Table 3. *Effects of adrenalectomy with or without cortisol administration on the phosphorylation and oxidoreduction state of histones from normal and regenerating rat liver*

Animals were adrenalectomized 4–8 days before use and were subsequently maintained on 0·9% (w/v) NaCl. Unless otherwise stated they were given [^{32}P]phosphate 30 min. before death. The specific radioactivity is given relative to that of inorganic phosphate in the original homogenate.

	'Histone F3'				Histone F1	
	SH+S·S (nmoles/mg. of protein)	SH (% of SH+S·S)	P (ng.atoms/mg. of protein)	10 × Rel.sp. radioactivity of histone P	P (ng.atoms/mg. of protein)	10 × Rel.sp. radioactivity of histone P
Whole liver (4)	37·7	74 (66–88)	49·4	0·8 (1·3*)	40·9	0·4 (0·3*)
Cortisol-treated, whole liver (2)	34·3	78	53·4	0·8	39·0	0·5
Sham-operated (1)	47·0	72	39·8	1·0	47·6	0·3
Partially hepatectomized						
1 hr. (1)	38·4	84	41·0	0·6	40·1	0·3
22 hr. (1)	36·3	81	43·3	1·8*	48·0	2·5*

* [^{32}P]Phosphate given 1 hr. before death.

Table 4. *Effects of γ-irradiation* in vivo *or actinomycin on the phosphorylation of histone F1 and histone F3 in normal and regenerating rat livers*

Rats received 350 or 225 μg. of actinomycin D/100 g. body wt. intraperitoneally. Controls refer to unirradiated rats or to those without actinomycin.

			Time after hepatectomy (hr.)		P (ng.atoms/mg. of protein)			
					'Histone F3'		Histone F1	
γ-Irradiated	Exposure (rads)	Actinomycin D (μg.)	Exposure	Death	Control	Treated	Control	Treated
Normal	1000	—	−1	0	—	—	31·0	28·4
Normal	500	—	−0·5	0	39·2	53·7	—	—
Partially hepatectomized	500	—	−0·5	1	59·5	118·0	—	—
Partially hepatectomized	500	—	3	4	—	—	42·8	34·5
Partially hepatectomized	500	—	3	22	69·2	68·1	72·0	58·1
Actinomycin-treated								
Normal	—	350	−0·5	0	39·2	65·1	43·5	43·0
Partially hepatectomized	—	350	−0·5	1	59·5	73·5	—	—
Normal	—	225	−0·5	1	40·2	41·9	—	46·4
Partially hepatectomized	—	225	−0·5	1	60·6	66·2	34·9	—

adrenalectomized 4–8 days earlier (Table 3) there was no increase in the phosphate content of the extract containing histone F3 1 hr. after partial hepatectomy. There was also no increase in the phosphate contents either of histone F1 or of the extract containing histone F3 at 22 hr. after hepatectomy, which is about the peak of the first wave of DNA synthesis in normal partially hepatectomized rats. The relative specific radioactivities of the phosphate groups of both proteins were increased, especially that of histone F1. Ferrari & Harkness (1954) found that adrenalectomy did not prevent regeneration after partial hepatectomy, but the process was slowed, the mitotic index at 24 hr. after operation in their adrenalectomized rats being only 43·5% of that in the intact animals.

Cortisol given to adrenalectomized rats 1 hr. before death did not increase the phosphorylation of either histone (Table 3). These findings suggest that, although liver regeneration is faster in the intact rats, the action of corticosteroids may be more concerned with the facilitation of the response to partial hepatectomy than with its initiation.

Actinomycin given to the rats 30 min. before operation, in doses sufficient to block 80% of RNA synthesis in the liver (Revel & Hiatt, 1964), did not prevent the increase in phosphate content of the extract containing histone F3 1 hr. after partial hepatectomy (Table 4). Indeed, with the higher

Table 5. *Adenine nucleotide content in regenerating rat livers 1–24 hr. after partial hepatectomy*

Contents for liver from 6–24 hr. after operation were derived from separation of the nucleotides on Dowex 1 (formate form) columns as described in the text, and are expressed as μmoles/g. wet wt. of liver. Four to six rats were used/group and two or three experiments were carried out at each time-period. The values at 1 hr. after partial hepatectomy were obtained from perfused livers by the firefly method. Six rats were used in each group and the results are expressed as μmoles of ATP/μmole of deoxyribose. Nutritional state: f=fed; s=starved, after hepatectomy.

Time after operation (hr.)		Adenine nucleotides				ATP/AMP ratio
		AMP	ADP	ATP	Total	
0	f	0·35	0·85	1·60	2·80	4·6
6–8	s	0·95	1·19	0·76	2·90	0·8
6–8	f	0·65	1·67	1·29	3·60	2·0
21–24	f	0·42	0·93	1·45	2·79	3·5
Sham-operated	s	—	—	0·32	—	—
1	s	—	—	0·24	—	—

dose of actinomycin, the phosphate content of the extract from livers removed at operation was already increased.

γ-Irradiation had rather similar effects (Table 4). A dose of 500 rads 30 min. before operation caused an increased phosphate content of the extract containing histone F3 both in the lobes removed at operation and still more 1 hr. later. Whole-body irradiation produces an immediate, though transient, rise in ATP content in the liver (Forssberg & Hevesy, 1953; Sherman & Forssberg, 1954). The increased phosphate content of the extract from the intact irradiated rats might be due to this, but the irradiation, which decreases new RNA synthesis (Welling & Cohen, 1960), did not prevent the rise in phosphate content of the extract.

If the irradiation was given 3 hr. after operation and the phosphate content examined at 22 hr., the phosphate content of an extract containing histone F3 was not different from the value for unirradiated rats, although that in histone F1 was decreased. These findings agree with earlier specific-radioactivity measurements (Ord & Stocken, 1968*a*).

Adenine nucleotide contents in livers after partial hepatectomy. A number of observations have been made on concentrations of intermediates in the liver shortly after partial hepatectomy. The most striking effect is the fall in liver glycogen content. This occurs at the earliest time examined and was obvious in our extracts 1 hr. after operation. At 6 hr. a rise in amino acid nitrogen is very marked, and this rise is not prevented by adrenalectomy (Ferrari & Harkness, 1954). An increase in liver lipid is noted by 24 hr.; this is very much less in adrenalectomized rats.

The rapidly changing supply of intermediates in the livers of intact rats after partial hepatectomy complicates the interpretation of data on ATP content (Table 5). Attempts were made, by the provision of sucrose solutions for drinking, to keep up the glycogen content; these were only partly successful, although at 24 hr. after operation liver glycogen was considerably restored. The ATP/AMP ratio falls, as might be expected from the fall in glycogen content. The ATP contents after partial hepatectomy do not suggest that increased utilization of phosphate for phosphorylation of histone F3 and for RNA synthesis is occurring because of increased availability of ATP.

Phosphorylation of histones F1 and F3 in livers of foetal and neonatal rats. Studies on the phosphorylation of histones F1 and F3 were prompted by the apparent relationship between an increased phosphate content of histone F1 and periods of DNA synthesis. A further situation in which DNA synthesis is prominent is in liver during foetal growth. Pregnant rats 15–21 days after conception were used. Their foetuses weighed 4–6 g. The livers of the pregnant females served as controls; their values for phosphate contents of histones F1 and F3 and for ^{32}P incorporation into DNA were within the normal range. The phosphate content of histone F1 from the livers of the foetal rats was about 100 ng.atoms of phosphorus/mg. of protein (Fig. 2) and decreased sharply during the first week after birth. The fall paralleled the decrease in ^{32}P uptake into DNA occurring at the same time.

Marked increases in liver weight occur in neonatal rats 1–4 weeks after birth and during this time there are many alterations in the pattern and concentration of liver enzymes. The phosphate content of the extract containing histone F3 in 18–21-day-old foetal rats was not different from controls, but at 8 days after birth during the period of rapid development in the liver, the phosphate content of the extract might be slightly increased (Table 6).

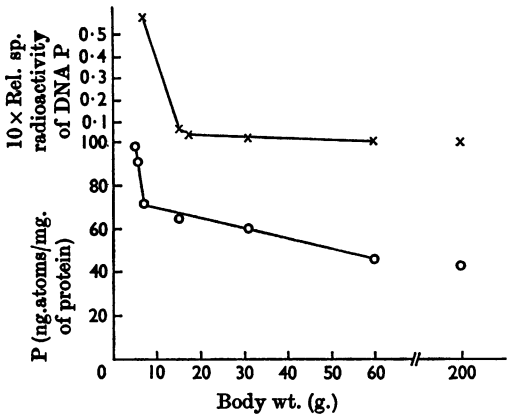

Fig. 2. ^{32}P incorporation into DNA and phosphate content of histone F1 in livers of very young rats. The specific radioactivity of DNA is given relative to that of the inorganic phosphate in the original liver homogenate. ×, ^{32}P uptake into DNA; ○, phosphate content of histone F1.

Table 6. *Phosphorylation of histone F3 in young rat livers*

Extracts containing histone F3 were prepared as described in the text. Foetuses were obtained from pregnant females at 18–21 days; the 8-day-old rats weighed 15g.

	'Histone F3'		
	SH+S·S (nmoles/mg. of protein)	SH (% of SH+S·S)	P (ng.atoms/ mg. of protein)
Controls (Table 2)	38·9±4·7	>85	39·2±6·5
Pregnant females	43·8	87	36·4
Foetuses	41·3	79	43·0
8-day-old rats	37·2	—	46·8

DISCUSSION

Thiol–disulphide interconversions. Two roles may exist for the thiol groups on histones. Thiol may be the required form to allow structural changes to occur in chromatin as at fusion or during mitosis. The thiol form may also or alternatively be required before information transfer from DNA can occur. The results here show that in sea-urchin eggs thiol groups are present on histones during periods of structural alterations in the nucleus. Simmel & Karnofsky (1961), Hinegardner, Rao & Feldman (1964) and Zimmerman & Silberman (1967) have shown that thymidine uptake occurs in *Arbacia*, *Strongylocentrotus* and *Echinarachinus* from fusion to prophase, and in telophase at the first and second divisions. No data for *Echinus* or *Psammechinus* are available, so that it is not known whether thymidine uptake continues from fusion at about 30min. to prophase (120min. with *Echinus* at 8°; 50min. with *Psammechinus* at 12°). Nevertheless initiation of synthesis at fusion and at telophase (furrow) coincides with peaks in the proportion of thiol and the completion of synthesis by the end of telophase (cleavage) is associated with increasing disulphide. The histones therefore are present in the thiol form at times when thymidine uptake probably starts but conversion into the thiol form may be associated with structural changes involved in fusion and mitosis rather than exclusively with synthesis of DNA.

In liver the thiol form of histones normally predominates and there was no detectable difference between normal livers and livers at various times up to the first division after partial hepatectomy. Livers from adrenalectomized rats showed slightly lower proportions of thiol than livers from intact rats. Cortisol administration did not produce any detectable increase in histone thiol 1hr. after injection; in intact liver nuclei, cortisol *in vitro* increased the reactivity of thiol groups (Sekeris, Beato, Homoki & Congote, 1968). If metabolically inert chromatin is isolated from liver or thymus nuclei, a higher proportion of disulphide is present compared with metabolically active chromatin (Ord & Stocken, 1966). Vidali & Neelin (1968) have suggested that in bird erythrocytes the thiol histone is appreciably oxidized. The results with animal tissues, taken together with those from the sea-urchin eggs, are consistent with a need for the histone(s) to exist in thiol form to allow nucleic acid synthesis.

Phosphorylation of histones. If it be accepted that for information transfer to take place a dissociation of histone from DNA is an essential prerequisite, an obvious way to achieve this would be to alter charge interactions. This could be effected by the introduction of competing anions to form a complex with the histones (Frenster, 1965) or by alterations in the basicity of the histones or by both of these means. The simplest basic proteins associated with DNA are the protamines, which contain a high proportion of arginine but also, in those whose primary structures have been elucidated (Ando & Suzuki, 1967), serine. Variations in the extent of the phosphorylation of protamine have been reported during the maturation of trout sperm (Ingles & Dixon, 1967). The arginine-rich histone F3 contains a more complex mixture of amino acids, and for this protein, wider modifications in micro-structure are possible. Acetylation, methylation (Allfrey, Faulkner & Mirsky, 1964), oxidoreduction and phosphorylation have all been reported. A detailed study of phosphate content in histones in cleavage stages in sea-urchin eggs has not yet been

made, but the histones carry phosphate groups (Ord & Stocken, 1968*a*) and these are found in acid–ethanol extracts after immediate precipitation with acetone, suggesting their similarity to the F2a + F3 group of histones.

The continuing synthesis of RNA in mammalian cells may require the existence of cysteine-containing histones in their thiol form (Hilton & Stocken, 1966). New RNA synthesis might then necessitate additional controls. Pogo, Pogo, Allfrey & Mirsky (1968) have reported increased acetylation of histones 0–4hr. after partial hepatectomy. The present results suggest that increased phosphorylation of arginine-rich histone also occurs very early in regenerating rat liver and the lack of inhibition when subjected to prior irradiation and to actinomycin treatment indicates that phosphorylation is independent of new messenger RNA and protein synthesis.

The results also confirm earlier indications that the kinases for histones F1 and F3 are different. Increased phosphorylation of histone F3 after partial hepatectomy is observed 15hr. before the increased phosphorylation of histone F1. A further increase in phosphorylation of histone F3 is observed at the same time as the increased phosphorylation of histone F1, during the synthetic period.

Moreover phosphorylation of histone F3 does not show the diurnal variations found for histone F1 (Table 2). The rapidity of onset of phosphorylation of histone F3 in partially hepatectomized rats, with no indications of increased ATP content, suggests that the kinases for histone F3 may be specifically stimulated by partial hepatectomy either allosterically or by a more favourable potential cofactor/inhibitor balance.

The increased phosphorylation of histone F1 detectable just before DNA synthesis is extremely radiosensitive (Ord & Stocken, 1968*a*; Stevely & Stocken, 1968); it is likely that the increase is part of a co-ordinated response to new messenger RNA formed very early after hepatectomy. The changes in enzyme complement early in liver regeneration can be delayed by irradiation in the first few hours after hepatectomy (Holmes & Mee, 1956; Welling & Cohen, 1960), in contrast with the increased phosphorylation of histone F3, which may therefore be due to changes initiated in the cytoplasm.

Implicit in this discussion is a different function for lysine- and arginine-rich histones and some specificity of interaction between these histones and DNA *in vivo*. Most experimental evidence of interactions between DNA and isolated histones has been interpreted as showing no indications of such specificity (Johns & Butler, 1964), but a little information has accumulated suggesting preferred association between polylysine and deoxyadenosine–thymidine-rich regions in DNA (Leng & Felsenfeld, 1966; Cohen & Kidson, 1968). The helical structure of deoxyribonucleoprotein is unaffected by selective removal of histone F1 but profoundly altered by removing the F2a–F3 group of histones (Bradbury, Crane-Robinson, Goldman, Rattle & Stephens, 1967). Nothing is known about nucleotide sequences in DNA in eukaryotic cells required for initiating DNA replication or RNA transcription, although pyrimidine clusters have been implicated for the latter in viral and microbial systems (Kubinski, Opara-Kubinska & Szybalski, 1966). It is not usually suggested that histones correspond to repressors in micro-organisms, but it is possible that selective modification in the microstructures of the different histones may allow different regions of the DNA to be available for information transfer.

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