Studies on Histones

6. OBSERVATIONS ON THE BIOSYNTHESIS OF HISTONES AND OTHER PROTEINS IN REGENERATING RAT LIVER*

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Since histones are a major protein constituent of chromosomes it was expected that knowledge of their biosynthesis in relation to the cycle of replication of DNA would throw light on their metabolic behaviour and possibly on their function. Even in resting tissues histones are more active metabolically than would be required for the replication of chromosomes (Brunish & Luck, 1952; Daly, Allfrey & Mirsky, 1952; Smellie, McIndoe & Davidson, 1953; Butler & Laurence, 1960); in tumours, i.e. actively dividing tissues, Busch, Davis & Anderson (1958) and Butler & Laurence (1960) have observed a greater rate of synthesis of histone relative to other proteins. Cytological observations on the time of synthesis of histone in the cell cycle by Bloch & Goodman (1955) suggested that the synthesis of histone and DNA was probably simultaneous. A biochemical examination of this question is attempted in the present investigation with regenerating rat liver, in which a burst of DNA synthesis occurs about 18–20 hr. after partial hepatectomy. The incorporation *in vivo* of [^{14}C]lysine into several types of proteins from subcellular fractions of the liver was studied at various times after partial hepatectomy. Since the absolute level of radioactivity found in any protein is meaningless, because the concentration of the pool from which incorporation occurs is unknown, the experiments are necessarily limited to a comparison of the incorporation into different proteins of the same animal at various times. Owing to the difficulty of handling the fractions obtained from several hepatectomies simultaneously it was not possible to carry out replicate experiments at each period. Nevertheless, the patterns of incorporation obtained are consistent and support each other in giving a general picture of the sequence of incorporation into various proteins.

MATERIALS AND METHODS

Animals

Male Wistar albino rats kept on a mixed diet were starved for 18–20 hr. before death.

* Part 5: Laurence, Simson & Butler (1963).

Lobectomy of the liver (partial hepatectomy) was performed under ether anaesthesia between 9.30 a.m. and 11.30 a.m. unless otherwise stated (Higgins & Anderson, 1931).

Administration of radioactive amino acid

An aqueous solution of uniformly labelled L-[^{14}C]lysine hydrochloride (5 mc/m-mole) (The Radiochemical Centre, Amersham, Bucks.), neutralized with N-KOH, was injected into the right femoral vein or intraperitoneally (33 μC /kg. body wt.). The rat was killed by breaking its neck.

Preparation of subcellular fractions

All operations were carried out at 2–5°.

Tissue suspension. An homogenate was prepared as described by Cohn & Butler (1958) except that the homogenizer tube was moved up and down six to eight times only while the pestle was revolving.

Centrifugal fractionation. The homogenate was centrifuged (no. 40 rotor, Spinco model L centrifuge) at 15000g_{av.} for 10 min. A volume (7.2 ml.) of the supernatant liquid was carefully withdrawn from each tube, pipetted into a fresh centrifuge tube and 0.25M-sucrose (3.8 ml.) added. The microsome fraction was then prepared as described by Cohn & Butler (1958).

Preparation of perfluoro-octanoate pellet. This was done according to the technique of Cohn & Butler (1958) (see Scheme 1).

Preparation of nuclei. The sediment produced by centrifuging the homogenate at 15000g_{av.} was resuspended in 0.25M-sucrose–1.8 mM-CaCl₂ with the aid of a Potter-Elvehjem-type homogenizer (see Scheme 1). The suspension was strained through lint and diluted with 0.25M-sucrose so that a volume of 35 ml. was derived from 2.5–3.0 g. of liver. The suspension was centrifuged at 400g_{av.} for 5 min. in a MSE Minor centrifuge. The cloudy supernatant liquid was pipetted off, and the pink-and-buff-layered sediment resuspended in 2.2M-sucrose with the aid of the homogenizer (Chauveau, Moulé & Rouiller, 1956) so that 10 ml. of suspension was equivalent to approx. 1 g. of liver. The suspension was centrifuged (Spinco model L centrifuge) either at 31000g_{av.} for 1 hr. (SW 25 rotor) or at 105000g_{av.} for 15 min. (no. 40 rotor). The supernatant liquid was decanted and allowed to drain, and the remains of the supernatant pad were wiped off the walls of the tubes. The samples were kept frozen at –20° if not required immediately.

Preparation of protein fractions

Histones. The sediment of nuclei was washed three times with 0.14M-NaCl–0.01M-trisodium citrate and centrifuged at 600g_{av.} until the supernatant liquid was almost clear.

The sediment was then extracted three times with 0.25-N-HCl (5 ml.) for 20–24 hr. with mechanical agitation on a rocking apparatus. The suspension was centrifuged as above and the supernatant liquid was drawn off. The three extracts were pooled, dialysed against water on a rocking apparatus and frozen for storage. If they appeared turbid after dialysis they were centrifuged in the Spinco preparative centrifuge (no. 40 rotor) at 26000 g_{av} for 20 min.

Scheme 1. Fractional centrifuging of homogenate and preparation of basic and HCl-insoluble proteins. The percentages refer to the proportions of protein out of the total protein of the subcellular fraction.

Table 1. Composition of histones and of basic proteins of perfluoro-octanoate pellet

Experimental details are given in the text. Amino acids are expressed as moles/100 moles of all amino acids found.

	Histones		Basic proteins,† Perfluoro-octanoate pellet
	Re-generating liver	Calf thymus*	
Aspartic acid	5.0	14.2	7.5
Glutamic acid	9.7	9.7	10.4
Glycine	8.8	8.5	7.5
Alanine	12.5	13.8	8.5
Valine	6.9	6.4	6.8
Leucine + isoleucine	10.8	10.6	11.7
Serine	6.4	5.2	5.3
Threonine	5.4	5.8	4.6
Phenylalanine	1.9	1.9	3.0
Tyrosine	2.0	1.7	2.1
Proline	4.3	5.1	4.8
Histidine	2.4	2.0	2.7
Lysine	14.0	15.6	13.6
Arginine	9.7	9.2	11.5
Basic:acidic ratio	1.78	1.89	1.55

* Johns, Phillips, Simson & Butler (1960).

† Butler, Cohn & Simson (1960).

Perfluoro-octanoate pellet. Pellets were dispersed in water with the aid of a loose-fitting pestle. To a dilute suspension 2N-HCl was added to give a final concentration of 0.2N-HCl; the final volume was 2.5 ml. of suspension for each pellet. A precipitate appeared on the addition of the acid. The suspension was kept in a stoppered tube on a rocking apparatus for about 16 hr.; it was then centrifuged (no. 40 rotor, Spinco model L) at 26000 g_{av} for 20 min. The supernatant liquid was dialysed against water on a rocking apparatus for 4–5 hr. with two changes of water and frozen for storage.

Hydrochloric acid-insoluble residues and cell sap. Samples of cell sap and of the HCl-insoluble residue of nuclei and of perfluoro-octanoate pellet were precipitated and washed three times with 0.4N-HClO₄ containing L-lysine hydrochloride (3 mM). The precipitates were then processed as described by Cohn & Butler (1958) except that a treatment with 5% (w/v) trichloroacetic acid (5 ml. to each sample at 90° for 20 min.) was substituted for the digestion with N-NaOH and subsequent reprecipitation with 6N-HCl.

Radioactivity assays

The dry samples were placed on polythene planchets (1 cm.²) and assayed in a counter in which a ¹⁴C-labelled reference source of specific radioactivity 1 μC/g. produced a counting rate of 550 counts/min. When the amount of sample was insufficient to give infinite thickness the proportion of the maximum radioactivity assayed was ascertained from a saturation curve. At least 1000 counts in duplicate were collected from each sample.

Samples of solutions of histones and basic proteins from perfluoro-octanoate pellet were dried on aluminium planchets under an infrared lamp. The samples, infinitely thin, were assayed in a Nuclear-Chicago flow counter in which samples of ¹⁴C-labelled amino acids of known specific radioactivity produced a counting rate of 850 counts/min./μmc. Total counts of 5000 were collected in duplicate. The protein contents of all samples from nuclei and perfluoro-octanoate pellets were calculated from their nitrogen contents, which were determined by a micro-Kjeldahl method.

Analyses

The analysis of total amino acids was carried out by the fluorodinitrobenzene method of Levy (1954) and Fraenkel-Conrat, Harris & Levy (1955), with the modifications described by Phillips & Johns (1959).

RESULTS

Scheme 1 summarizes the method of preparation of the subcellular fractions and shows how the basic and hydrochloric acid-insoluble proteins are derived from nuclei and perfluoro-octanoate pellets. Histone amounts to 58% of the protein that could not be washed out from nuclei by 0.14M-sodium chloride–0.01M-trisodium citrate. The histone obtained from regenerating liver nuclei by the technique described above is similar in amino acid composition to that of calf thymus, i.e. it contained 25% of basic and 14–15% of acidic residues (Table 1). Extraction with hydrochloric acid of the perfluoro-octanoate pellet yields basic proteins that

form 29 % of the total protein and have a content of 25 % of basic and 18 % of acidic amino acid residues. The hydrochloric acid-insoluble residue is made up of acidic proteins (P. Cohn, unpublished work) derived from ribosomes and lipoprotein of the endoplasmic reticulum.

Two periods of incorporation of L-[^{14}C]lysine were employed: the amino acid was injected either 2 min. (condition A) or 30 min. (condition B) before the rat was killed. Table 2 shows the specific radioactivities of the different protein fractions obtained under condition A: appreciable incorporation is shown only by the hydrochloric acid-insoluble fraction of the perfluoro-octanoate pellet. This result agrees with previous findings on ribosomes from rat liver (Hultin, 1955; Littlefield, Keller, Gross & Zamecnik, 1955; Cohn & Butler, 1958). When the specific radioactivities of the proteins are studied after an incorporation time of 30 min. (Table 3) an appreciable incorporation of [^{14}C]lysine into all fractions is found. The specific radioactivities of all fractions are higher between 10 and 22.5 hr. than at the other times examined. The incorporation into histone and the hydrochloric acid-insoluble proteins of the nucleus varied relative to each other at different intervals. In contrast, the basic proteins of the perfluoro-

octanoate pellet always had a specific radioactivity well below that of the hydrochloric acid-insoluble fraction.

The specific radioactivities of four fractions [histone, hydrochloric acid-insoluble (of nuclei), basic and hydrochloric acid-insoluble (of perfluoro-octanoate pellet)] relative to their average values at different times after partial hepatectomy until 22.5 hr. are shown in Fig. 1. The relative specific radioactivity of the histone decreases to less than average between 6 and 10 hr. but rises to average and above between 12 and 22.5 hr. On the other hand, both the hydrochloric acid-insoluble fraction of the nuclei and of the perfluoro-octanoate pellet are above average between 1.5 and 12 hr., the maximum radioactivity of the hydrochloric acid-insoluble fraction of the perfluoro-octanoate pellet being at about 6 hr. and that of the nuclear hydrochloric acid-insoluble fraction at about 12 hr. At 18.5 hr. the radioactivity of the nuclear hydrochloric acid-insoluble fraction has returned to average but that of the hydrochloric acid-insoluble proteins of the perfluoro-octanoate pellet shows a second maximum at 18.5 hr. In liver 1.5 hr. after sham operation there is little difference between the amounts of incorporation into any of the fractions.

Table 2. *Specific radioactivities of proteins 2 min. after injection of L-[^{14}C]lysine into partially hepatectomized rats*

Experimental details are given in the text. L-[^{14}C]Lysine was injected into the right femoral vein 2 min. before the rat was killed.

				Specific radioactivity ($\mu\text{C/g.}$)				
Time after operation (hr.)	...	...	...	12	18.5	22.5	25	47
Protein fraction								
Histone				0.16	0.17	0.055	0.079	0.13
Nucleus, HCl-insoluble				0.12	0.19	0.072	0.051	0.030
Perfluoro-octanoate pellet, basic				0.19	0.19	0.071	0.066	0.058
Perfluoro-octanoate pellet, HCl-insoluble				2.38	1.72	0.94	1.55	0.92
Cell sap				0.084	0.066	0.040	0.046	0.036

Table 3. *Specific radioactivities of proteins 30 min. after the injection of L-[^{14}C]lysine into partially hepatectomized rats*

Experimental details are given in the text. L-[^{14}C]Lysine was injected intraperitoneally 30 min. before the rat was killed.

		Specific radioactivity ($\mu\text{C/g.}$)									
Time after operation (hr.)	...	1.5	6	6	10	12	18.5	18.5	22.5*	47	Sham-operated†
Protein fraction											
Histone		0.92	0.69	0.19	1.26	1.70	2.88	1.39	1.42	1.02	0.60
Nucleus, HCl-insoluble		0.93	1.31	0.46	2.34	2.00	2.36	1.27	1.61	0.41	0.76
Perfluoro-octanoate pellet, basic		0.55	0.92	0.36	0.97	1.08	1.55	0.95	1.27	0.41	0.51
Perfluoro-octanoate pellet, HCl-insoluble		0.77	1.50	0.48	1.81	1.89	2.91	1.92	1.53	1.15	0.78
Cell sap		0.34	0.29	0.17	0.69	0.83	0.77	0.57	0.62	0.62	0.35

* Lobectomy performed at 4.30 p.m.

† The liver was palpated after laparotomy and the rat killed 1.5 hr. after the operation.

Fig. 1. Specific radioactivities of histone and of proteins of HCl-insoluble fraction of nuclei and of basic and HCl-insoluble proteins of perfluoro-octanoate pellet relative to their average values at different times after partial hepatectomy (results from Table 3). ■, Histones; △, basic proteins of perfluoro-octanoate pellet; ●, HCl-insoluble fraction of nuclei; ○, HCl-insoluble fraction of perfluoro-octanoate pellet.

DISCUSSION

It is evident that the biosynthesis of histone is occurring at all times in liver regenerating after partial hepatectomy. This obscures to some extent the synthesis necessary for the increased amount of histone required when cell division occurs. Only about 3–5 % of the remaining liver cells take part in the synchronous wave of DNA synthesis that occurs 18–20 hr. after the operation. The events in these cells must be regarded as being superimposed on the background of cells that do not take part in the first wave of DNA synthesis. The relation of the additional amount of histone to be synthesized to the turnover that is occurring at all times cannot be readily determined, as the size of the pool of L-lysine in the animal is unknown. We cannot say what degree of enhancement of the normal level would be required to accompany the synthesis of new DNA. Nevertheless, when the incorporation of L-[^{14}C]lysine into various protein fractions is compared with the average incorporations into the four fractions examined (Fig. 1), marked differences in the relative rates of incorporation are apparent at different times. It is clear from these curves that partial hepatectomy disturbs the pattern of protein biosynthesis in the

remaining liver cells and that the amount of the disturbance changes with time, the synthesis of histone being initially depressed while the synthesis of hydrochloric acid-insoluble proteins of the nucleus and the perfluoro-octanoate pellet is relatively enhanced. There is also a subsidiary peak of synthesis of hydrochloric acid-insoluble proteins of the perfluoro-octanoate pellet at about 18–20 hr., i.e. coinciding approximately with DNA synthesis.

There is no sharp wave of histone synthesis at the time of onset of DNA synthesis, but after the initial depression histone is being synthesized at average or rather more than average rate over several hours before the onset of synthesis of DNA. It is thus probable that synthesis of the histone required for the new deoxyribonucleoprotein begins during a period before that in which the DNA is formed. Though one period of enhanced synthesis of the hydrochloric acid-insoluble proteins of the perfluoro-octanoate pellet coincides with the period of greatest relative incorporation into histone, there is another such period at a much earlier stage. The fraction is evidently complex metabolically.

SUMMARY

1. The incorporation *in vivo* of L-[^{14}C]lysine into nuclear and microsomal protein fractions in regenerating liver was studied at various intervals after partial hepatectomy.
2. Incorporation into histone was depressed 1.5–12 hr. and equal and above average 12–22.5 hr. after the operation; incorporation into the hydrochloric acid-insoluble fractions of nuclei and of the perfluoro-octanoate pellet was relatively enhanced to varying extents during the whole period.
3. The results make it probable that the synthesis of histone required for cell division begins before the synthesis of DNA.

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The Adsorption of Corticosteroids to Particulate Preparations of Rat Liver

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Previous experiments have shown that plasma cortisol entered rat liver by diffusion and was rapidly metabolized (Berliner, Keller & Dougherty, 1961; Bellamy, Phillips, Jones & Leonard, 1962). It was also found that some unchanged cortisol was adsorbed to the particulate fraction of the tissue (Bellamy *et al.* 1962). There is a possibility that particle-bound steroids are resistant to the action of enzymes and that the persistence of small amounts of intracellular adsorbed cortisol is responsible for some of the long-term biochemical changes that follow injections of cortisol. In view of the evidence that liver may be a target organ for corticosteroids (Chiu, 1950; Winternitz, Dintzis & Long, 1957; Goodlad & Munro, 1959; Ashmore, 1960; Long, Smith & Fry, 1960; Harding, Rosen & Nichol, 1961; Goldstein, Stella & Knox, 1962) the interactions of cortisol and corticosterone with the particulate fractions of this tissue have been examined in more detail.

EXPERIMENTAL

Extraction and estimation of ¹⁴C-labelled steroids. The materials and methods used have been described by Bellamy *et al.* (1962).

Fractionation of liver homogenates. Liver from a freshly killed rat (1.5 g.) was homogenized in 15 ml. of 0.9% NaCl and made up to 30 ml. with 0.9% NaCl. The homogenate (5 ml.) was centrifuged at 600g for 2 min. and the supernatant solution pipetted off. The sedimented tissue (fraction 1) was washed three times by resuspension in 0.9% NaCl (4 ml.) and the washings were added to the first supernatant solution. The combined solutions were centrifuged at 8000g for 2 min. and the supernatant

solution was pipetted off. The sedimented tissue (fraction 2) was washed three times as fraction 1 above. The supernatant solution and washings were finally centrifuged at 25000g for 5 min. to sediment all the particulate matter (fraction 1A). In some experiments 0.25 M-sucrose solution was used in place of 0.9% NaCl. All the operations were carried out with the temperature as close to 0° as possible, a refrigerated centrifuge (MSE) being used.

Extraction and chromatography of tritiated steroids. About 1 g. wet wt. of tissue was cut into small pieces and refluxed for 2 hr. with 100 ml. of chloroform-methanol mixture (2:1, v/v) containing 50 µg. of unlabelled cortisol, cortisone and corticosterone. The extract was evaporated to dryness on a rotary flash evaporator, dissolved in 100 ml. of water and extracted three times with 100 ml. of chloroform. The combined chloroform extracts were evaporated to dryness as described above and dissolved in a mixture of 5 ml. of light petroleum (b.p. 40–60°) and 5 ml. of ethyl acetate. The solution was put through a column of 2 g. of silica gel prepared according to Bush & Sandberg (1953). The column was washed successively with 20 ml. of ethyl acetate-light petroleum (1:1, v/v), followed by 9.9 ml. of this mixture containing 0.1 ml. of methanol. The corticosteroids were eluted with a mixture of 6.0 ml. of ethyl acetate-light petroleum (1:1, v/v) and 4.0 ml. of methanol.

The solvent was evaporated off, the residue dissolved in 0.2 ml. of ethyl acetate and spotted $\frac{3}{8}$ in. from the end of a strip (1 in. × 8 in.) of Whatman no. 1 filter paper. The paper strip was sprayed lightly with the bottom phase of the B5 solvent system (Bush, 1952) and the chromatogram developed with the upper phase ascending the paper. Unlabelled cortisol, cortisone and corticosterone were located on the paper by ultraviolet absorption. The strip was then cut into half-inch pieces, which were left in contact with 5 ml. of ethanol overnight. After removal of the pieces of paper the ethanol was evaporated off and the residue was dissolved in phosphor solution and the radioactivity determined (Bellamy *et al.* 1962).