The Synthesis and Decay of Histone Fractions and of Deoxyribonucleic Acid in the Developing Avian Brain

By STEPHEN C. BONDY

Division of Neurology, University of Colorado Medical Center, Denver, Colo. 80220, U.S.A.

(Received 8 January 1971)

1. The turnover of cerebral histones and DNA after injection of [4,5-³H]leucine or [methyl-3-³H]thymidine, respectively, was studied in the developing chick. 2. Chromatin was prepared from chick nuclei that had been purified by centrifugation through 1.9M-sucrose. 3. Nuclear proteins were fractionated into three major histone classes, F1 (lysine-rich), F2(b) (slightly lysine-rich) and [F3+F2(a)] (arginine-rich), and a non-histone protein residue. 4. The proportions of the histone classes remained constant throughout the period of development studied. 5. All histone fractions decayed at a similar rate, initially with a half-life of around 5 days, later with a half-life of 19 days. 6. Non-histone proteins from chromatin decayed in a heterogeneous manner with a wide range of half-lives. 7. Short-term labelling studies showed that all histone fractions were synthesized at the same rate. 8. Some non-histone proteins were very rapidly synthesized relative to histones. 9. DNA had a longer half-life than any histone fraction studied. A biphasic exponential decay curve with half-lives of 23 and 50 days was found. 10. It was concluded that the turnover of histones can occur independently of that of DNA and that different histone classes have similar rates of synthesis and decay.

Regulation of gene expression in higher organisms may largely be achieved by template restriction of the DNA genome by histones (Bonner *et al.* 1968). Although neither DNA nor histones are clearly tissue-specific (Neidle & Waelsch, 1964; McCarthy & Hoyer, 1964; DeLange, Fambrough, Smith & Bonner, 1969), they interact with other nuclear components so as to result in a tissue-specific chromatin (Paul & Gilmour, 1968).

The exceptional stability of cerebral DNA (Adams, 1966), in conjunction with a considerable capacity of cerebral chromatin as a template in RNA synthesis (Bondy & Roberts, 1969), makes the relation between cerebral DNA and histone metabolism of considerable interest.

The relative metabolic inertness of cerebral histones was demonstrated by Piha, Cuenod & Waelsch (1966) who found the turnover of radioactively labelled histones in the rat brain to be slower than that of any other protein class studied. Histone decay followed a biphasic exponential course. This suggested either heterogeneity in the stability of histone classes or that the histones of two cell types within the brain were turning over at differing rates. This paper is an attempt to resolve this question by a study of the synthesis and decay of various classes of cerebral histones. This process has been examined in relation to the stability of cerebral DNA. The system used was the developing

avian brain. Injection of radioisotopes into fertile chick eggs very shortly after the start of embryogenesis permitted virtually all molecular species to be radioactively labelled. This approach avoids problems related to the placental barrier and wastage of isotopes that occur with labelling of mammalian fetuses by maternal injection.

EXPERIMENTAL

Administration of label. Fertile chick eggs of White Leghorn strain K137 were incubated at 37.5°C in a forced-draught incubator. After 2 days, the tops of the eggs were washed with ethanol and a small hole was bored in the shell. A 1 in hypodermic needle (no. 26 gauge) was vertically placed into the egg and 100 µCi of either [4,5-³H]leucine (19.7 Ci/mmol) or [methyl-3-³H]thymidine (0.36 Ci/mmol) in 0.1 ml of water were injected into the yolk. The hole was then sealed with wax and the eggs were replaced in the incubator. Hatched chicks were injected intracranially at the base of the skull with 20 µCi of [³H]leucine in 0.02 ml of iso-osmotic (0.14M) NaCl. Radiochemicals were obtained from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.

Preparation of cerebral chromatin and histone fractionation. At various times after injection, brains were dissected out from six to ten decapitated chicks or chick embryos. These brains were then homogenized in 0.32M-sucrose and chromatin was prepared as previously described (Bondy & Roberts, 1969). The resulting pellet was used in the preparation of three major histone classes by a

Scheme 1. Outline of histone fractionation procedure employed.

method based on that of Johns (1964) adapted for small amounts of material. A flow sheet is shown in Scheme 1. The histone nomenclature is that of Johns, Phillips, Simson & Butler (1960).

(1) The chromatin was homogenized in an equal volume of cold 10% (w/v) HClO_4 and centrifuged at 25000g for 5 min in a Sorvall RC-2 centrifuge. The precipitate was extracted twice more with 1 ml of 5% HClO_4 .

(2) The combined supernatants from this step were adjusted to 18% (w/v) trichloroacetic acid with 50% trichloroacetic acid and kept for 24 h at 0°C. The resulting precipitate was then centrifuged down and constituted fraction F1.

(3) The precipitate from step 1 was homogenized with 2 ml of ethanol and this was kept for 24 h at 0°C. After centrifugation (25000g, 5 min) the precipitate received two further washes each of 1 ml of 80% (v/v) ethanol.

(4) The combined supernatants from the above step 3 were mixed with 5 vol. of acetone and 0.12 vol. of conc. HCl. The resulting precipitate was centrifuged down and consisted of fractions F2(a) and F3.

(5) The precipitate from step 2 was homogenized in 1 ml of 0.25 M-HCl, centrifuged down and re-extracted with a further 1 ml of 0.25 M-HCl. Acetone (5 vol.) was added to the combined supernatants from this, and fraction F2(b) was centrifuged off. The precipitate that was insoluble in HCl was the residual non-histone fraction N.

Determination of radioactivity of fractions. The precipitated fractions were dissolved in 0.1 M-NaOH and samples taken for protein assay (Lowry, Rosebrough, Farr & Randall, 1951), with bovine serum albumin (Sigma Chemical Co., St Louis, Mo., U.S.A.) as a standard. Radioactivity was determined after neutralization with Beckman Bio-Solv solubilizer no. 2 (Beckman Inc., Fullerton, Calif., U.S.A.). The neutralized protein solution (2 ml) was mixed with 15 ml of standard scintillation solution consisting of a 20% (v/v) solution of Beckman

Bio-Solv solubilizer no. 3 in toluene containing 0.5% 2-phenyl-(5-biphenyl-2-yl)-1-oxa-3,4-diazole and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.).

Radioactivity was measured in a Packard Tri-Carb scintillation spectrometer at an efficiency of 28% and an instrument background of 15 c.p.m.

Assay of DNA. After injection of [^3H]thymidine, cerebral chromatin from the chick was prepared as described above and this preparation from individual chicks was homogenized in 5 ml of cold 5% (w/v) trichloroacetic acid. The resulting precipitate was centrifuged at 3000g for 10 min and extracted once more with 5 ml of 5% trichloroacetic acid and twice with 5 ml of ethanol. The final precipitate was incubated for 2 h at 37°C with 1.25 ml of 0.3 M-KOH, and then the alkali was neutralized with 70% (w/v) HClO_4 and the precipitate centrifuged down at 0°C and 3000g for 10 min. The precipitate, containing DNA, protein and KClO_4 , was heated at 90°C for 20 min together with 2.5 ml of 0.5 M-HClO₄ to hydrolyse the DNA. After the precipitate had been centrifuged off, samples of the supernatant were taken for colorimetric determination of DNA (Burton, 1956) and measurement of radioactivity. Of this supernatant 1 ml was mixed with 15 ml of the standard scintillation solution and radioactivity was determined as described above for protein.

Recovery of DNA during the preparation of chromatin varied between 72 and 77% of original total brain DNA. The proportion of DNA recovered did not vary with the state of maturity of the chick brain.

RESULTS AND DISCUSSION

The relative proportions of the three major histone classes within the chromatin did not vary significantly with the age of the chick (Table 1) and were similar to proportions reported for calf thymus histone by Johns (1964). These results are also in agreement with the report of Agrell & Christensson (1965) on the constant proportion of histone fractions within developing chick brain.

Long-term histone labelling. The radioactivity of histone classes at various times after injection of [^3H]leucine was determined (Fig. 1). As I was interested in the rate of disappearance of radioactivity rather than in isotope dilution due to subsequent histone synthesis, results are expressed as c.p.m./fraction (the points represent the fractions from one chick brain) rather than c.p.m./mg of histone. All three histone fractions decayed exponentially between day 11 of incubation and 1 day after hatching, with similar half-lives (4.0 days for fraction F1, 5.3 days for fraction F2 and 4.9 days for fraction [F2(a)+F3]).

Between 1 and 14 days after hatching, the rate of decay of all fractions decreased. Whether this was due to decreased histone metabolism concurrent with maturation (Gaitonde & Martenson, 1970) or to heterogeneity of decay of histones in various cell types is not known.

Table 1. Proportions of various histones in chick brain chromatin

Chromatin was prepared from purified nuclei of six to ten chick brains from each age group. Histone fractions were prepared as described in the Experimental section.

Histone class	Age of chick				
	Days of incubation		Days after hatching		
	11	14	1	4	15
			(% Composition)		
F1 (lysine-rich)	19	17	23	21	21
F2(b) (slightly lysine-rich)	30	26	31	32	26
F2(a)+ F3 (arginine-rich)	51	57	46	47	53

Fig. 1. Radioactivity remaining in chick brain histone fractions at various times after injection of 100 μ Ci of [3 H]leucine into fertile chick eggs incubated for 2 days. Details are given in the Experimental section. Δ , Fraction F1; $\circ$, fraction F2(b); $\bullet$, fraction [F2(a)+F3]; $\blacktriangle$, histone fraction N (0.1 $\times$ values). Arrow indicates time of hatching.

Non-histone residual proteins (N) decayed in a non-exponential manner, some components having half-lives as short as 1.8 days. This is similar to the characteristics of the non-histone nuclear proteins of the rat described by Piha *et al.* (1966). The high initial specific radioactivity may reflect the high metabolic activity of some proteins within this fraction (Bustos-Valdes, Deisseroth & Dounce,

1968; Burdman, Haglid & Dravid, 1970). The wide range of decay rates is indicative of the heterogeneity of the residual proteins. This fraction is tissue specific (Dravid & Burdman, 1968) and may determine chromatin specificity (Gilmour & Paul, 1969).

The specific radioactivities of the chick histones were calculated and corrected for their different leucine contents (Murray, Vidali & Neelin, 1968) (Table 2). These values were similar for all classes of histone throughout the experiment. This indicated that most histones may be synthesized from a common precursor pool at the same rate and that they have essentially the same stability.

Short-term histone labelling. The rate of histone synthesis in newly hatched chicks was studied by intracisternal injection of [3 H]leucine, followed 1 h later by preparation of histone fractions (Table 3). Again, at this time-point, rates of synthesis expressed on a basis of specific radioactivity of protein leucine were very similar for all histone classes. As in the embryos, these classes are synthesized at identical rates. This short-term experiment suggests that there is no considerable histone fraction with an unusually fast turnover rate that might not have been detected in the long-term decay studies. Dick & Johns (1969) also reported identical synthetic rates for five major histone fractions of rat thymus.

The high specific radioactivity of the non-histone nuclear proteins indicates the high metabolic activity of some of these proteins. Although the purity of the histone fractions was not checked by amino acid analysis, the finding that the specific activity of all fractions was similar on a leucine basis, in both long- and short-term studies, suggests that these fractions were not significantly contaminated with highly labelled non-histone protein.

Turnover of DNA. Parallel studies on DNA decay were made by injection of [3 H]thymidine into 2-day incubated eggs. The specific radioactivity of thymidine was low to avoid the possibility of radiation damage to cerebral DNA (Merits & Cain, 1969).

DNA decayed with a half-life of 22.6 days up to

Table 2. *Specific radioactivity of histone fractions from cerebral chromatin at various times after injection of 100 μ Ci of [3 H]leucine into 2-day-old chick embryos*

For details see the Experimental section.

Histone ...	Sp. radioactivity (c.p.m./ μ g of protein)			$10^{-3} \times$ Sp. radioactivity (c.p.m./ μ mol of leucine)		
	F1	(F2b)	F2(a)+F3	F1	F2(b)	F2(a)+F3
Age of chick						
11 days incubated	62	88	133	177	168	202
14 days incubated	47	67	82	134	127	125
1 day after hatching	6.9	15.4	20	19.6	29.2	30.5
15 days after hatching	3.6	7.2	11.5	10.3	13.6	17.5

Table 3. *Specific radioactivity of histone fractions prepared from cerebral chromatin 1h after intracisternal injection of 20 μ Ci of [3 H] leucine into each of seven 4-day-old chicks*

Leucine contents of chick histone fractions were taken from Murray *et al.* (1968). Details are given in the Experimental section.

Fraction	Leucine content (mol %)	Sp. radioactivity	
		(c.p.m./mg of protein)	(c.p.m./ μ mol of leucine)
F1	4.6	5370	15290
F2(b)	6.9	8780	16670
F2(a)+F3	8.6	10400	15840
N (non-histone residue)	—	22730	—

4 days after hatching, after which the half-life was calculated at around 50 days (Fig. 2). These results suggest that cerebral DNA does not decay as rapidly as do cerebral histones. This low rate of DNA decay at a time when DNA synthesis is proceeding rapidly in the chick brain (Margolis, 1969) may be related to a decreasing ratio of total histone to DNA during maturation (Table 4). This decrease has been observed by Dingman & Sporn (1964) and may reflect increasing expression of the cerebral genome as differentiation proceeds.

The relation between DNA and histone metabolism has not yet been clearly resolved. Some workers report that turnovers of DNA and histones are totally interdependent (Robbins & Borun, 1967; Gallwitz & Mueller, 1969) and take place together at the same rate (Byvoet, 1966; Murthy, Pradhan & Sreenivasan, 1970). Other studies suggest that histones turn over at different rates (Stellwagen & Cole, 1969) and that histone synthesis can continue in the absence of DNA synthesis (Flamm & Birnstiel, 1964; Sadgopal & Bonner, 1969). Our results indicate that although all histones may turn over as a unit, this proceeds at a much faster rate than DNA turnover. We have no

Fig. 2. Radioactivity remaining in chick brain DNA at various times after injection of 100 μ Ci of [3 H]thymidine into fertile chick eggs incubated for 2 days. Details are given in the Experimental section. Arrow indicates time of hatching.

Table 4. *Histone and DNA content of cerebral chromatin*

Details are given in the Experimental section.

Age of chick	Total histone (μ g/brain)	DNA (μ g/brain)	Histone DNA ratio
14 days incubated	154	151	1.02
1 day after hatching	261	360	0.72
14 days after hatching	280	400	0.70

evidence for the type of histone conservation reported for mouse mastocytes by Hancock (1969).

No evidence for a rapidly turning-over histone within the F1 group was found, although this fraction has been reported to contain tissue-specific constituents (Kinkade, 1969; Nelson & Yunis, 1969; Bustin & Cole, 1968; Panyim & Chalkley, 1969) and rapidly turning-over constituents (Stellwagen & Cole, 1969; Gurley & Hardin, 1970). The biphasic decay of both histones and DNA may

reflect cellular heterogeneity within the brain or may be due to reutilization of radioactive precursors. The radioactivity within the perchloric acid-soluble supernatant pool was not significant throughout the decay studies, and as the radioactivity in histones and DNA remained high, the likelihood of significant precursor reutilization was small (cf. Mori, Yamagami & Kawakita, 1970). However, this possibility cannot be totally ruled out as radioactive breakdown products may be rapidly reincorporated into protein without contributing to a major extent to the radioactivity within the acid-soluble pool.

The independence of histone and DNA turnover suggests a means by which de-repression of the cerebral genome can occur after cessation of neuronal division in the mature brain.

I thank Mrs Beatrice S. Morelos for expert technical assistance. This work was in part supported by Grant no. GB-26037 from the National Science Foundation, Grant no. 1R01 NS09603 from the National Institutes of Health and Grant no. 70-487 from the Foundations' Fund for Research in Psychiatry.

REFERENCES

- Adams, D. H. (1966). *Biochem. J.* **98**, 636.
- Agrell, I. P. S. & Christensson, E. G. (1965). *Nature, Lond.*, **207**, 638.
- Bondy, S. C. & Roberts, S. (1969). *Biochem. J.* **115**, 341.
- Bonner, J., Dahmus, M. E., Fambrough, D., Huang, R. C., Marushige, K. & Tuan, D. Y. H. (1968). *Science, N.Y.*, **159**, 47.
- Burdman, J. A., Haglid, K. & Dravid, A. R. (1970). *J. Neurochem.* **17**, 669.
- Burton, K. (1956). *Biochem. J.* **62**, 315.
- Bustin, M. & Cole, R. D. (1968). *J. biol. Chem.* **243**, 4500.
- Bustos-Valdes, S. E., Deisseroth, A. & Dounce, A. L. (1968). *Archs Biochem. Biophys.* **126**, 848.
- Byvoet, P. (1966). *J. molec. Biol.* **17**, 311.
- DeLange, R. J., Fambrough, D. M., Smith, E. L. & Bonner, J. (1969). *J. biol. Chem.* **244**, 5669.
- Dick, C. & Johns, E. W. (1969). *Biochim. biophys. Acta*, **174**, 380.
- Dingman, C. W. & Sporn, M. B. (1964). *J. biol. Chem.* **239**, 3483.
- Dravid, A. R. & Burdman, J. A. (1968). *J. Neurochem.* **15**, 25.
- Flamm, W. G. & Birnstiel, M. L. (1964). *Expl Cell Res.* **33**, 616.
- Gaitonde, M. K. & Martenson, R. E. (1970). *J. Neurochem.* **17**, 551.
- Gallwitz, D. & Mueller, G. C. (1969). *J. biol. Chem.* **244**, 5947.
- Gilmour, R. S. & Paul, J. (1969). *J. molec. Biol.* **40**, 137.
- Gurley, L. R. & Hardin, J. M. (1970). *Archs Biochem. Biophys.* **136**, 392.
- Hancock, R. (1969). *J. molec. Biol.* **40**, 457.
- Johns, E. W. (1964). *Biochem. J.* **92**, 55.
- Johns, E. W., Phillips, D. M. P., Simson, P. & Butler, J. A. V. (1960). *Biochem. J.* **77**, 631.
- Kinkade, J. M. (1969). *J. biol. Chem.* **244**, 3375.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- McCarthy, B. J. & Hoyer, B. H. (1964). *Proc. natn. Acad. Sci. U.S.A.* **52**, 915.
- Margolis, F. L. (1969). *J. Neurochem.* **16**, 447.
- Merits, I. & Cain, J. (1969). *Biochim. biophys. Acta*, **174**, 315.
- Mori, K., Yamagami, S. & Kawakita, Y. (1970). *J. Neurochem.* **17**, 835.
- Murray, K., Vidali, G. & Neelin, J. M. (1968). *Biochem. J.* **107**, 207.
- Murthy, L. D., Pradhan, D. S. & Sreenivasan, A. (1970). *Biochim. biophys. Acta*, **199**, 500.
- Neidle, A. & Waelsch, H. (1964). *Science, N.Y.*, **145**, 1059.
- Nelson, R. D. & Yunis, J. J. (1969). *Expl Cell Res.* **57**, 311.
- Panyim, S. & Chalkley, R. (1969). *Biochemistry, Easton*, **8**, 3972.
- Paul, J. & Gilmour, R. S. (1968). *J. molec. Biol.* **34**, 305.
- Piha, R. S., Cuenod, M. & Waelsch, H. (1966). *J. biol. Chem.* **241**, 2397.
- Robbins, E. & Borun, T. W. (1967). *Proc. natn. Acad. Sci. U.S.A.* **57**, 409.
- Sadgopal, A. & Bonner, J. (1969). *Biochim. biophys. Acta*, **186**, 349.
- Stellwagen, R. H. & Cole, R. D. (1969). *J. biol. Chem.* **244**, 4878.