Acid hydrolase activity during ecdysis and in cast-off cuticle of *P. ricini*

Molting stages	During ecdysis; total activity/insect			After larval-larval ecdysis; total activity/insect			Cast off cuticle; total activity/cuticle		
	a	b	c	a	b	c	a	b	c
LM_2	2.75	77.10	3.55	1.78	41.87	0.74	0.94	34.38	2.00
LM_3	8.85	338.56	14.94	6.14	228.17	3.30	3.00	101.66	8.33
LM_4	47.27	1686.17	52.96	13.81	1468.24	12.25	31.00	181.50	41.00

LM, Larval-larval molt; *a* acid phosphates activity (μmoles phosphorus released/60 min); *b* acid protease activity (μg tyrosine liberated/3 h); *c* β-glucuronidase activity (μg phenolthalein liberated/h).

lysate is then digested in multivesicular bodies and lysosomes and released into the cytosol[19]. At the time of resorption of molting fluid, appreciable amounts of pigment and products of the cuticular hydrolysate originating from the larval endocuticle and epidermis (but now present in molting fluid) are transferred across the integumentary epithelium and after passing through the hemolymph are taken up through the midgut epithelium into the closed lumen of the midgut[19]. In anurans, a definite mechanism for the excretion of lysosomal enzymes during metamorphosis is known[20]. An anuran tadpole, however, is an open system in the sense that it can take in the nutrients and other things it requires from its environment, and can excrete the waste products into it. An insect larva in molt, on the other hand, is a closed system. It has to meet its nutritional and other demands within the body and the undesirable excretory products formed are also retained within it because of its inability to dispose of them immediately.

The present study reveals that in *P. ricini* the disposal of acid hydrolases is achieved via the cuticle once their function is completed, the modus operandi being different from that in the anuran vertebrates. These observations also suggest that during larval ecdysis the acid hydrolases are probably localized in the molting fluid present between the old and new cuticular layers. However, the lysosomal localization of acid hydrolases has yet to be established. These enzymes probably participate in degradation and digestion of old cuticle. Since they cannot be transported to the hemolymph, they are excreted through the cast-off cuticle.

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2 Sridhar, S., and Bhatt, J.V., J. Insect Physiol. *9* (1963) 693.
3 Hegdekar, B.M., and Smallman, B.N., Can. J. Zool. *45* (1967) 1202.
4 Hegdekar, B.M., and Smallman, B.N., Can. J. Zool. *47* (1969) 45.
5 Pant, R., and Lacy, P.S., Indian J. exp. Biol. *6* (1969) 154.
6 Lockshin, R.A., and Williams, C.A., J. Insect Physiol. *11* (1965) 831.
7 Varute, A.T., and Sawant, V.A., Comp. Biochem. Physiol. *38*B (1971) 211.
8 Varute, A.T., Ph. D. Thesis Shivaji Univ-Kolhapur, India, 1969.
9 Varute, A.T., and More, N.K., Comp. Biochem. Physiol. *38*B (1971) 225.
10 Pant, R., and Lacy, P.S., Indian J. Biochem. *5* (1968) 13.
11 Fishman, W.H., In: Methods of Biochemical Analysis, Vol. 15, 77. Ed. D. Glick. Interscience, New York 1967.
12 Matsushita, S., and Iwami, N., Mem. Res. Inst. Fd Sci. Kyoto Univ. *28* (1967) 75.
13 Radford, S.V., and Misch, P.W., J. Cell Biol. *49* (1971) 702.
14 Agui, N., Yagi, S., and Fukaya, M., Appl. ent. Zool. *4* (1969) 156.
15 Marks, E.P., and Leopold, R.A., Biol. Bull. *140* (1971) 73.
16 Locke, M., and Krishnan, N., Tissue Cell *5* (1973) 441.
17 Kimura, S., Insect Biochem. *6* (1976) 479.
18 Bade, M.L., and Shoukimas, J., J. Insect Physiol. *20* (1974) 281.
19 Jungreis, A.M. in: Advances of Insect physiology, Vol. 14, p. 109. Academic Press, New York 1979.
20 Varute, A.T., and Sawant, V.A., Comp. Biochem. Physiol. *41*B (1972) 675.

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DNA methylation in chicken brain and liver

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Summary. Adult chicken brain DNA is subject to a developmental alteration of methylation at *Hpa*II sites flanking 4, 1, 0.8 and 0.7 kilobase sequences. This change is not evident in adult chicken liver DNA and 1-day-old chick brain and liver DNAs, suggesting tissue specificity and age-dependency in DNA methylation.

5-Methylcytosine is the only minor base found in all eukaryotic DNAs so far investigated[1-4]. Although the biological significance of this modification is currently unknown, it has been suggested that methylation may be involved in gene expression, cell differentiation and development[5-7]. It is therefore of interest to compare the degree of DNA methylation in different tissues. This paper describes the analysis of DNA methylation in chicken brain and liver by using high-pressure liquid chromatography (HPLC) and restriction endonucleases.

Materials and methods. The brain and liver tissues were obtained from 6-month-old White Leghorn female chickens (*Gallus gallus* var. *domesticus*) and 1-day-old chicks killed under chloroform anesthesia. To isolate liver tissue depleted of blood, liver perfusion was performed with Hanks' balanced salt solution. The tissues were homogenized and lysed in a solution containing 8M urea, 1% sodium dodecyl sulfate (SDS), 1mM disodium ethylenedi-aminetetraacetate (Na_2EDTA), 1 M sodium perchlorate and 0.24 M phosphate buffer, pH 6.8. After extraction with a mixture of chloroform and isoamyl alcohol (24:1, v/v), the DNA from these tissues was isolated by the hydroxyapatite batch elution technique[8]. The absorbance at 260 and 280 nm was measured to assess DNA concentration and purity. The ratio of A_{260}/A_{280} was between 1.8 and 2.0.

Chick and chicken DNAs digested with enzymes which recog-

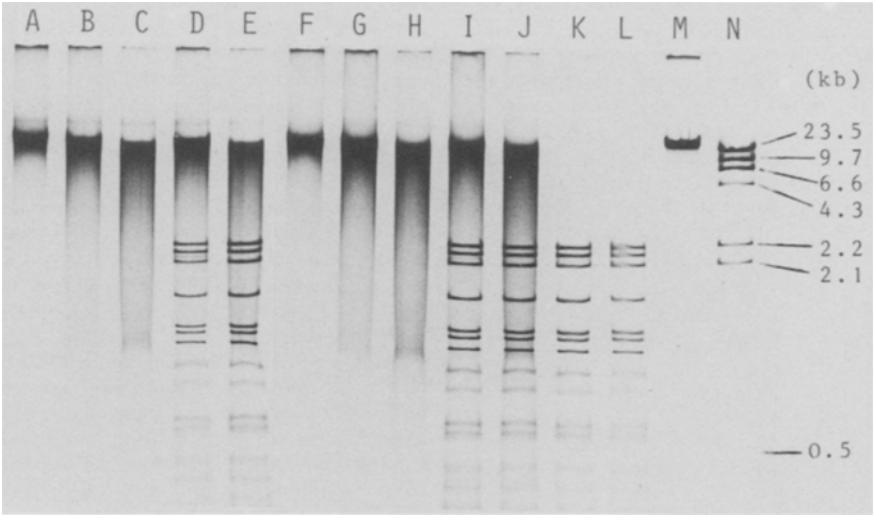

Figure 1. Electrophoretic patterns of 1-day-old chick DNA digested with restriction endonucleases. Track A: uncut chick brain DNA (no enzyme); tracks B and C: chick brain DNA digested with *Hpa*II and *Msp*I, respectively; track D: chick brain and phage lambda DNAs digested with *Hpa*II; track E: chick brain and phage lambda DNAs digested with *Msp*I; track F: uncut chick liver DNA (no enzyme); tracks G and H: chick liver DNA digested with *Hpa*II and with *Msp*I, respectively; track I: chick liver and phage lambda DNAs digested with *Hpa*II; track J: chick liver and phage lambda DNAs digested with *Msp*I; tracks K and L: phage lambda DNA digested with *Hpa*II and with *Msp*I, respectively; track M: uncut phage lambda DNA (no enzyme); track N: phage lambda DNA digested with *Hin*d III.

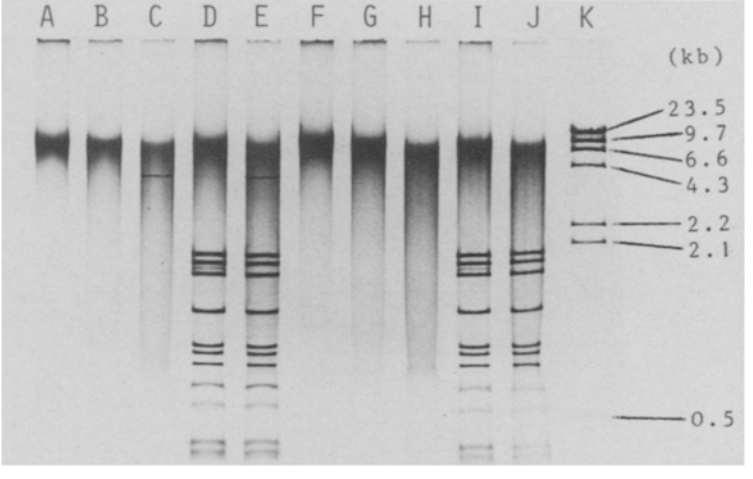

Figure 2. Electrophoretic patterns of adult chicken DNA digested with restriction endonucleases. Track A: uncut chicken brain DNA (no enzyme); tracks B and C: chicken brain DNA digested with *Hpa*II and with *Msp*I, respectively; track D: chicken brain and phage lambda DNAs digested with *Hpa*II; track E: chicken brain and phage lambda DNAs digested with *Msp*I; track F: uncut chicken liver DNA (no enzyme); tracks G and H: chicken liver DNA digested with *Hpa*II and with *Msp*I, respectively; track I: chicken liver and phage lambda DNAs digested with *Hpa*II; track J: chicken liver and phage lambda DNAs digested with *Msp*I; track K: phage lambda DNA digested with *Hin*d III.

nize a sequence containing a CpG doublet were resolved by agarose gel electrophoresis. Restriction endonucleases *Msp*I, *Hpa*II and *Hin*d III were obtained from New England Bio-Labs (Beverly, MA). About 2 to 3 µg of DNA was digested with 10 units of the restriction endonucleases in 20 µl reaction mixture at 37 °C for 60 min according to the conditions recommended by the supplier. The reaction was stopped by adding 5 µl of tracking dye solution consisting of 0.07% bromophenol blue, 7% SDS and 33% glycerol in water. Twenty µl samples of the reaction mixtures were subjected to 1.4% Seakem ME agarose (FMC Co., Rockland, ME) slab gel electrophoresis with a vertical gel electrophoresis apparatus at 100 V for about 3 h until the dye marker reached the bottom of the gel in Tris-borate buffer, pH 8.2, consisting of 89 mM Tris, 2.5 mM Na_2 EDTA and 89 mM boric acid[9]. After staining for 15 min with 0.4 µg of ethidium bromide per ml of water, gels were visualized with shortwave ultraviolet light on a Chromato-Vue transilluminator model C-61 (Ultra-Violet Products, San Gabriel, CA). Photographs were taken with Polaroid type 665 films, using a Polaroid MP-3 land camera with a Kodak 23A Wratten gelatin filter (Eastman Kodak Co., Rochester, NY).

To detect small amounts of 5-methylcytosine, we separated the nucleic acid bases by HPLC, monitoring elution by absorbance at 280 nm, where 5-methylcytosine absorbs maximally. A Shodex HC125 (Showa-Denko Co., Tokyo) column (500 × 2 mm) was packed and equilibrated with mobile phase, 50 mM ammonium formate buffer, pH 2.5. The 5 bases, adenine, cytosine, guanine, 5-methylcytosine, and thymine which were from Sigma Chemical Co. (St. Louis, MO), were dissolved in 0.1 N HCl. Aliquots of this mixture were chromatographed as reference. About 10 µl of a 1mg/ml DNA solution was suspended in 150 µl of 88% formic acid, sealed in vials, and hydrolyzed at 150 °C for 1 h. The hydrolysate was evaporated to dryness under a stream of nitrogen gas, then resuspended in 20 µl of 0.1 N HCl as described by Singer et al.[10]. The hydrolyzed bases were analyzed by HPLC. About 20 µl of sample was applied to the cation exchanger column, and eluted in 50 mM ammonium formate buffer, pH 2.5. The column was run at ambient temperature (22–25 °C) at a pressure of 50 kg/cm^2 (flow rate, 10 ml/h), by use of a Constametric IIG pump (Milton Roy Co., Riviera Beach, FL). The bases were detected by their absorbance at 280 nm measured with a Spectromonitor II (Atto Co., Tokyo). The time of analysis for 5 bases was about 70 min at a flow rate of 10 ml/h under the conditions used.

Results and discussion. *Hpa*II and its isoschizomer, *Msp*I, recognize the tetranucleotide sequence 5'-CCGG-3' (C, cytosine; G, guanine) in DNA. *Hpa*II can cleave this sequence when the external cytosine is methylated, but does not cleave when the internal one is modified. *Msp*I on the other hand is able to cleave this sequence when the second cytosine is methylated, but cannot cleave when the first one is methylated[11].

Phage lambda cI857 Sam7 DNA obtained from Bethesda Research Laboratories (Gaithersburg, MD) served as a control of specific enzyme activity, and its *Hin*dIII-fragments were used as a molecular mass reference. When the phage lambda DNA was added to reaction mixtures as an internal control, it was demonstrated that digestion by *Hpa*II and *Msp*I was not being restricted by an inhibitor copurified with DNA.

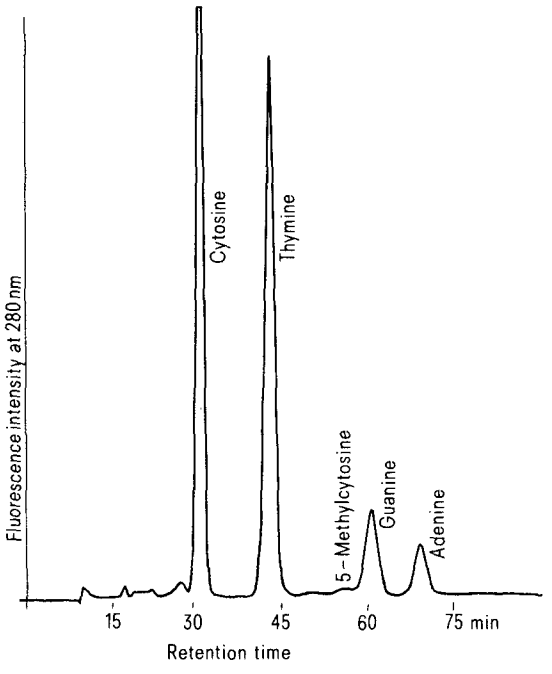

Figure 3. Chromatogram of nucleic acid bases from the brain DNA hydrolysate of 1-day-old chick. Column: Shodex HC-125; eluent: 50 mM ammonium formate, pH 2.5; flow rate: 10 ml/h; pressure: 50 kg/cm^2; detector: ultraviolet, 280 nm.

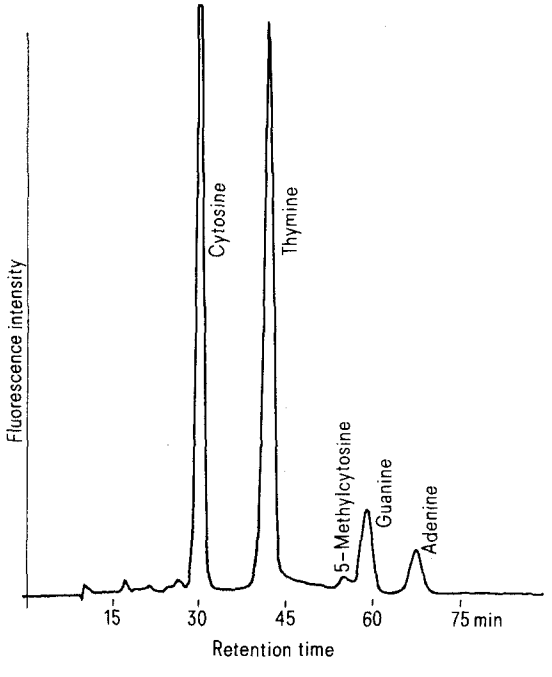

Figure 4. Chromatogram of nucleic acid bases from the brain DNA hydrolysate of adult chicken. Conditions are the same as figure 3.

DNA from 1-day-old and adult animals were both extremely resistant to *Hpa*II compared to *Msp*I irrespective of whether brain or liver was examined (figs. 1 and 2). This diminished cleavage by *Hpa*II relative to *Msp*I is evidence of methylation at the inner cytosine residue in this sequence. Although an *Msp*I site would be expected to occur in every 529 nucleotide pairs on the basis of the formula of Nei and Li[12] by assuming that the chicken genome has a G-plus-C content of 41.7 mol%[13], a discrete band of about 4 kilobase was seen in *Msp*I cleavage patterns of chicken brain DNA only. Shorter fragments, 1, 0.8 and 0.7 kilobase were also seen in lanes C and E of figure 2. These findings suggest the reiterative presence of unmethylated sequences both termini of which are flanked by methylated *Hpa*II sites in the chicken brain DNA, supporting the non-random distribution of methylated cytosine in genes[14]. For further characterization of these sequences, the 4 kilobase fragment and also the other fragments are now being investigated by molecular cloning. The data obtained here also demonstrate the tissue-specific and age-dependent patterns in DNA methylation, which were not evident in mouse brain and liver DNAs[15].

Figures 3 and 4 present typical chromatograms of the samples of formic acid-hydrolyzed DNAs isolated from brains of chick and of chicken, respectively. Cytosine, thymine, 5-methylcytosine, guanine, and adenine were eluted in that order. The HC-125 cation exchanger HPLC provided excellent resolution of these bases. It is evident that the total amount of 5-methylcytosine is quite similar in both brain tissues examined. The chromatograms of the liver DNAs were almost the same as those of the brain DNAs. It could be concluded that chicken brain DNA is subject to a developmental alteration of DNA methylation at the *Msp*I sites because the chicken brain DNA digested with *Msp*I generated discrete bands which were not evident in adult chicken liver DNA and 1-day-old chick DNA. The data suggest tissue specificity and age dependency in DNA methylation.

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1 Hall, R.H., The Modified Nucleosides in Nucleic Acids. Columbia Univ. Press, New York 1970.
2 Shapiro, H.S., in: Handbook of Biochemistry, p.H89. Ed. H.A. Sober. CRC Press, Cleveland 1970.
3 Vanyushin, B.F., Tkacheva, S.V., and Belozersky, A.N., Nature *255* (1970) 948.
4 Wyatt, G.R., Biochem. J. *48* (1951) 584.
5 Ehrlich, M., and Wang, R.Y.-H., Science *212* (1981) 1350.
6 Razin, A., and Riggs, A.D., Science *210* (1980) 604.
7 Sobieski, D.A., and Elden, F.C., Nucl. Acids Res. *9* (1981) 6001.
8 Britten, R.J., Povich, M., and Smith, J., in: Annual Report of the Carnegie Institution, p.400. Carnegie Instiution, Washington, D.C. 1968.
9 Peacock, A.C., Bunting, S.L., and Queen, K.G., Science *147* (1965) 1451.
10 Singer, J., Stellwagen, R.H., Roberts-Ems, J., and Riggs, A.D., J. biol. Chem. *252* (1977) 5509.
11 McClelland, M., Nucl. Acids Res. *9* (1981) 5859.
12 Nei, M., and Li, W.-H., Proc. natl. Acad. Sci. USA *76* (1979) 5269.
13 Kriby, K.S., Biochim. biophys. Acta *36* (1959) 117.
14 Razin, A., and Friedman, J., in: Progress in Nucleic Acid Research, vol.25, p.33. Ed. W.E. Cohn. Academic Press, New York 1981.
15 Harasawa, R., Ozaki, A., and Mitsuoka, T., Jpn. J. vet. Sci. *45* (1983) 275.

0014-4754/84/040390-03$1.50 + 0.20/0