bypassing the shield to attack the body (*11*).

These observations confirm the advantage of a regularly ridged keratinized shield in reducing adhesion and friction. They may be extrapolated to explain the function of the smooth ventral shields of most snakes (as well as many lizards), and they indirectly document the advantages both of regular ecdysis and of more frequent shedding when the skin is damaged (*12*). The reduced friction promotes the effectiveness of lateral undulation, but the shedding of foreign objects is more important to elongate limbless animals that lack alternate methods of grooming. Consequently, the structural interference colors of reptiles lack specific function but are, instead, side effects (*13*).

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10. The caudal pad also enhances the function of head mimicry, probably directed to jungle fowl and other common gallinaceous birds sharing the forest floor [C. Gans, in *Morphology and Biology of Reptiles*, A. d'A. Bellairs and C. B. Cox, Eds. (Linnean Society of London, London, 1976)]. The tail is stuck out and the pointed head is hidden when the snake is exposed on the surface. Eye spots alongside the cloaca, and lines more posteriorly, divert pecks to the caudal cap, which is protected by mud and bone.
11. Observations of snakes in artificial tunnels confirm that these snakes do try to block the opening with the tail when attempts are made to insert straws or similar small objects.
12. Disease states may often be detected by changes in the surface of a snake's skin, which then becomes undulant and dull and may show breaks in the free edges of the scales even before obvious lesions appear. Apparently shedding maintains a "low-energy surface" (*9*).
13. Examination of uropeltid color by itself in ignorance of its origin might well result in the assumption that the characteristics were selectively "neutral." However, the concept of neutrality should be applied to the totality of a character state rather than to a single way of viewing it.
14. We are grateful to many for helping us collect animals in the field as well as for cooperation from the Smithsonian Institution Entomological Research Project in Sri Lanka. Photographs were taken on the scanning electron microscopes (Jeol JFM-U3) of the Royal Ontario Museum (Division of Systematics) and at the Laboratory of Scanning Electronmicroscopy of The University of Michigan (Dr. W. C. Bigelow, director) and printed by D. Bay. S. P. Hollingsworth, R. Hewson, and E. Linn helped with the microscopy. Supported by NSF grant BMS 71 01380.

28 June 1976; revised 8 October 1976

Reconstitution of Chromatin Subunits

Abstract. *The recovery of the subunit structure of chromatin after dissociation and reconstitution is markedly affected by the procedure used. Some procedures give complete regeneration of subunits, but the procedure most commonly used for reconstitution gives poor yields of subunit-containing chromatin.*

Reconstitution of chromatin from its three main constituents, DNA, histones, and nonhistone chromosomal proteins, has been widely used to investigate transcriptional specificity (*1–3*) and chromatin structure (*4–10*). These studies have shown that the reconstituted chromatin may regain many of the properties of native chromatin, including specifically restricted transcriptional potential, thermal denaturation profile, circular dichroism spectrum, x-ray diffraction pattern, nuclease limit digest profile, and ultrastructure. In the case of transcriptional specificity, the presence of endogenous messenger RNA (mRNA) on the chromatin complicates the results (*11*). Nevertheless, when the presence of such contaminating RNA was taken into account, transcriptional specificity was still demonstrable in the reconstituted chromatin (*11*). In this report, the fidelity of various reconstitution techniques in regenerating the subunit (ν body) structure of chromatin (*12, 13*) is analyzed. It is shown that one of the methods commonly used for reconstitution gives poor yields of subunit-containing chromatin. Other regimes, however, give an excellent recovery of chromatin which is indistinguishable from the native starting material with respect to ultrastructure, sedimentation velocity, and nuclease sensitivity.

Chromatin subunit dimer fractions were collected from preparative sucrose gradients of micrococcal nuclease-treated chicken erythrocyte nuclei as previously described (*13*). Erythrocyte chromatin is uniformly heterochromatic, and contains no appreciable protease activity (*14*). After complete dissociation by dialysis into 2.5*M* NaCl, 6.0*M* urea, and $2.5 \times 10^{-4}M$ EDTA at *p*H 8.0, chromatin dimers were allowed to reassociate by gradient dialysis through various reconstitution procedures, the final buffer solution in each treatment being $2.5 \times 10^{-4}M$ EDTA (Table 1). Samples were removed at each stage in the treatment and prepared for electron microscopy by staining with aqueous uranyl acetate (*13*). The final reconstituted products were also analyzed on sucrose gradients both before and after a brief digestion with micrococcal nuclease, and peak fractions were examined with the electron microscope (Figs. 1 and 2). By this method, the proportion of reassociated fragments which regained the sedimentation properties of native dimers could be determined. Nuclease digestion of reconstituted dimers gave two further measures of the fidelity of the reassociation. First, the yield of monomers derived from the splitting of the dimers could be compared to that obtained by digestion of native dimers; and second, the amount of free DNA in the reconstituted chromatin could be monitored, because the conditions were such that naked DNA would be digested, and the breakdown products would appear at the top of the gradient.

Treatment of chromatin with 2.5*M* NaCl and 6.0*M* urea at *p*H 8.0, which is widely employed in reconstitution studies (*1–3*), dissociates all the histones (*10*) and most of the nonhistone proteins. In experiments with rat liver chromatin, it has been estimated (*15*) that 3 to 5 percent of the protein remained bound to DNA under similar conditions. In this study, the dissociated chromatin cosedimented with purified DNA on sucrose gradients, and appeared as linear fibers (2.5 nm in diameter) in the electron microscope (*16*). The DNA-histone complexes which formed as the dissociated chromatin was dialyzed to EDTA could be classified according to their ultrastructure as follows: native-like dimers consisting of two 8- to 10-nm spherical subunits interconnected by a 2.5-nm diameter fiber of DNA; particles with one subunit plus a 40- to 50-nm "tail"; particles consisting of one subunit but little or no tail; particles which had formed no subunits; and overlapping or aggregated material. Table 1 shows the distribution of particles obtained from the dimer regions of sucrose gradients when the various reconstitution procedures were used. Examination of the material ap-

plied to the gradients gave similar results, although aggregation made scoring less reliable.

When dialysis from high concentrations of salt and urea initially removed the urea and then, in a second step, removed the salt, the yield of native-like dimers was similar to that of untreated controls (Table 1 and Fig. 1). This reconstituted material also showed the same sedimentation profile as native dimers and, moreover, brief digestion with micrococcal nuclease gave a similar yield of monomers (Fig. 2). In contrast, if the two-step reassociation procedure was reversed, with the first dialysis being to 6.0*M* urea, less than one third as many dimer-like structures were recovered (Table 1 and Fig. 1). Sucrose gradient profiles of this material showed a single broad band spanning the monomer and dimer positions (Fig. 2). This band was merely lowered on treatment with nuclease, indicating that the conversion of dimer to monomer had not occurred, and confirming the absence of native subunit structure. Thus the dialysis sequence from urea plus salt to urea and then to EDTA produced reconstituted chromatin which did not resemble the native material in ultrastructure, sedimentation properties, or sensitivity to nuclease. About the same amount of chromatin was rendered soluble by the digestion treatment in all cases, indicating that the amount of histone-protected DNA was independent of the method of reconstitution (Fig. 2).

The recovery of particles with one subunit and a 40- to 50-nm "tail" was considered to be the result of the successful reconstitution of only one of the two dimer subunits. The yield of this type of particle was highest when the final dialysis was from 2.5*M* NaCl (Table 1). Particles with typical monomer ultrastructure and little or no tail were assumed to be contaminating monomers; they formed about 15 percent of the control population (Table 1).

These results are consistent with the well-documented effects of urea and salt on chromatin (*17*), and the recent evidence that specific histone-histone interactions are fundamental to the chromatin repeating unit (*18*). Removal of the urea from chromatin dissociated in salt plus urea would allow histones to interact nonionically with each other, but not bind to the negative phosphate groups of the DNA. On removing the salt, the histone complexes could bind to DNA, reforming the native subunit structure. With the reverse two-step dialysis, in which the dissociated chromatin was first taken to 6*M* urea, the histones would bind to DNA, but not form nonionic bonds with each other. Under these conditions, histones would be expected to bind randomly to free DNA. Upon removal of the urea, histone-histone bonding would be permitted, but since the histones would no longer be in the correct spatial order or position, proper complex formation, and hence DNA folding leading to subunit formation would not occur. In view of these arguments, it is surprising that the recovery of native-like dimers was as high as 10 percent under these conditions (Table 1). Whether this result indicates that there is considerable freedom in the way histones can be arranged on DNA to form subunit-like structures, or whether some factor or factors allowing the specific positioning of histones on DNA remain partially operational in 6*M* urea, are important questions that merit further study.

The other instance in which a poor yield of dimers was obtained was when dimers were dialyzed into 6*M* urea, and then back to EDTA (Table 1), but in this case, the sucrose gradient profiles before and after digestion were very similar to those of the untreated controls. This suggests that after unfolding in urea, a variety of histone-histone interactions are possible as the urea is removed, many of which might yield an "improperly" folded structure. An alternative explanation involving the migration or exchange of histones in 6*M* urea is unlikely, because the sucrose gradient profiles were unaffected. Further, we have found that dimers can be digested with micrococcal nuclease in the presence of 6*M* urea, and still give the same yield of monomer (*16*). It has previously been shown that the nuclease digestion pattern obtained from chromatin is relatively independent of the extent of folding of the DNA (*19*).

Table 1. Distribution of particle types after various reconstitution regimes. Samples were taken from the dimer region of sucrose gradients (Fig. 2), prepared for electron microscopy (Fig. 1), and micrographs enlarged to × 200,000 for scoring. Abbreviations: S = 2.5*M* NaCl, U = 6.0*M* urea, and E = 2.5×10^{-4}*M* EDTA, *p*H 8.0.

Reconstitution procedure	Particles scored (No.)	Particle types observed (%)				
		Dimer o–o	Monomer plus tail o–	Monomer o	No subunits	Unscored aggregates
Control	343	40	7	17	17	19
S + U → S → E	711	37	17	12	19	15
S + U → U → E	511	10	8	5	65	12
S + U → E	377	47	12	15	10	16
S → E	311	33	8	18	30	11
S → U	249	17	17	6	59	8

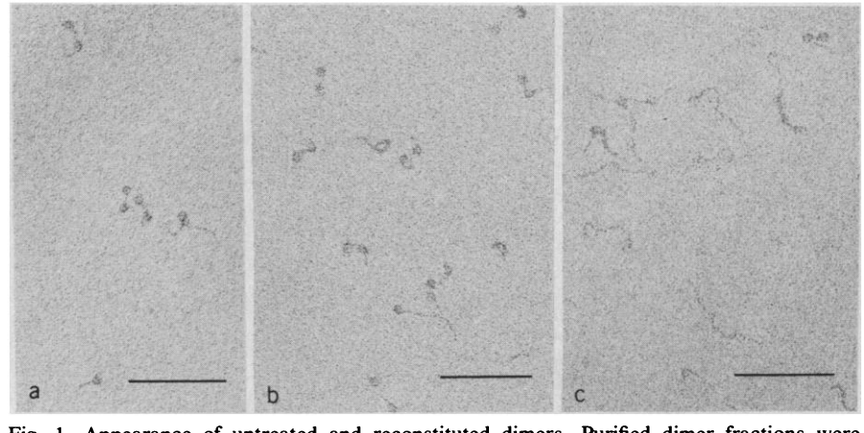

Fig. 1. Appearance of untreated and reconstituted dimers. Purified dimer fractions were adjusted to 50 μg/ml, and dialyzed against 2.5*M* NaCl, 6.0*M* urea, 2.5×10^{-4}*M* EDTA, *p*H 8.0 for several hours; subsequent gradient dialyses were performed as follows: the sample was placed in a dialysis bag in the gradient starting solution, and the bag suspended in the same solution in a 10-ml vial fitted with inflow and outflow ports, and a stirbar. A reservoir containing 1000 ml of the gradient ending solution was connected to the inflow line, and allowed to enter the vial at a rate of about 1 ml per minute with constant stirring. The gradient was allowed to run overnight in the cold. Samples were placed on glow-discharged carbon-coated grids, stained with 2 percent aqueous uranyl acetate, and examined with a Philips EM200 electron microscope. (a) Control, untreated. (b) Salt + urea → salt → EDTA. (c) Salt + urea → urea → EDTA. Scale bars, 100 nm.

When the above experiments were performed on monomers rather than dimers, yields of spherical particles were higher than obtained with dimers, but when the final dialysis was from urea, the characteristic ultrastructure with an electron opaque center was usually absent. This was probably because in the case of monomers, many folding patterns would create an approximately spherical, subunit-like structure. The recovery of dimers is therefore a better indication of true reconstitution since both the ultrastructure and nuclease digestion patterns are more definitive.

These findings have a number of implications with respect to chromatin structure and function. If it is confirmed that chromatin retains tissue and cell-cycle specificity (*2*) and potential transcriptional activity (*3, 11*) after reconstitution regimes which preclude the formation of many chromatin subunits, it may be inferred that the native repeating structure is not required for transcriptional control to be operative. Similarly, the nonhistone constituents which have been implicated in transcriptional control (*2, 3*) cannot themselves be dependent on a specific DNA-histone superstructure (*20*). However, it is clear that more work to define the relationship between reconstitution of native-like structure and transcriptional specificity is required to verify these inferences. It can also be concluded that a number of other properties of chromatin which are reportedly unaffected by reconstitution from urea, including circular dichroism spectrum, and binding of certain "reporter" molecules (*3*), are not dependent on the native subunit structure of chromatin. Other features of reconstituted chromatin are, however, influenced by reconstitution from urea: the recovery of x-ray diffraction maximums was severely inhibited by reconstitution from urea (*5*), and a similar loss of native structure has been described when thermal denaturation was used to monitor the binding of histones H3 and H4 to DNA (*6*). In both these studies, the complete omission of urea was recommended, and, indeed, in previous work in which the reconstitution of chromatin subunits was assayed with the electron microscope, no urea was used (*8*). In cases where reconstitution with histone and nonhistone is desired, urea may be necessary as a solvent for some nonhistones, but here it may be preferable to use simultaneous dialysis from salt and urea (Table 1), or to remove the urea first. Under these conditions, nonspecific histone aggregation may occur in high salt concentrations, but this can be avoided if sufficiently low concentrations of histone are employed (*21*). In the present study, chromatin was used at a concentration of 50 μg/ml, which represents a concentration of histones well below the $10^{-5}M$ level at which aggregation is reported to become significant (*21*).

Note added in proof: A recent study of the behavior of nonhistone proteins during reconstitution of chicken erythrocyte chromatin has shown that a major protein remains associated with the DNA in 2*M* NaCl plus 5*M* urea (*22*).

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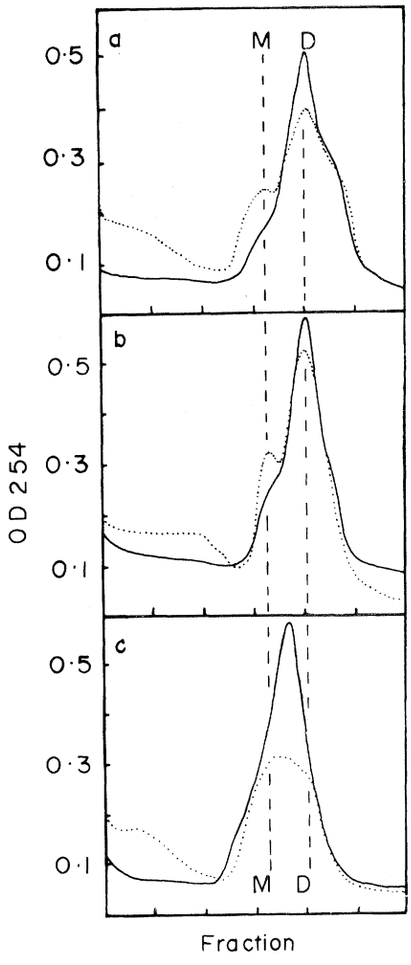

Fig. 2. Sucrose gradient profiles of control and reconstituted dimers. After the final gradient dialysis to $2.5 \times 10^{-4}M$ EDTA, *p*H 8.0, a portion of each sample was layered onto 5 to 20 percent linear sucrose gradients, and centrifuged at 35,000 rev/min for 16 hours in an SW 41 rotor (Beckman). The remainder of each sample was treated with micrococcal nuclease (Sigma). The digestion mixture, containing 50 μg of chromatin, 5 units of enzyme, 2 m*M* $CaCl_2$, and 20 m*M* tris-HCl, *p*H 8.0, was incubated at 37°C for 5 minutes, the reaction stopped by cooling and adding EDTA (*p*H 8) to 2.5 m*M*, and the samples analyzed on sucrose gradients. Gradients were monitored for absorbance at 254 nm, and peak fractions prepared for electron microscopy as described (see Fig. 1). (a) Untreated dimers. (b) Salt + urea → salt → EDTA. (c) Salt plus urea → urea → EDTA. *M* and *D* indicate subunit monomer and dimer positions, respectively. The shoulder to the right of the dimer peak is contaminating trimer. Solid line, without nuclease treatment; broken line, after nuclease digestion.

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23. Supported in part by NSF grant BMS 74-1196. I thank L-L. Y. Frado who participated in the early part of this investigation.

30 July 1976; revised 13 October 1976