In principle, the experiment is simple because the prelumirhodopsin form can easily be removed by heating the sample to room temperature for about 1 minute. At this temperature, this form is converted rapidly to meta I- and meta II-rhodopsin, both of which absorb at shorter wavelengths and should not interfere with an emission at 575 mμ.

Cooling of the sample again to 77°K should allow the 575-mμ fluorescence to be measured again. This proved to be difficult because the recooling process invariably produced a glass with slightly different scattering properties from that formed initially. Therefore, the intensity of the 575-mμ emission could not be related quantitatively to the intensity observed initially. This problem was solved by the addition of a fluorescent internal standard to the rhodopsin solution. Fluorescein amine was chosen for this purpose because its emission which is at 500 mμ would not interfere with the 575-mμ fluorescence and because it was clearly resolved from the scattering background and the peak at 575 mμ. If we keep constant the intensity and resolution of the fluorescein amine emission, we may judge the quantitative behavior of the 575-mμ peak.

In five experiments, we consistently found the intensity of the 575-mμ emission after heating to be that observed immediately after the 440-mμ irradiation and not that found initially. Apparently, the 575-mμ fluorescence is not dependent on the amount of the prelumirhodopsin form present, and thus it probably does not come from an impurity. It is reasonable to assume then that this emission arises from rhodopsin.

If the photoconversion of rhodopsin to prelumirhodopsin is carried out by light of 440-mμ wavelength, Yoshizawa and Wald (*9*) found only a 57-percent conversion. Apparently, there is a photoequilibrium in which the prelumirhodopsin form, which also absorbs at 440 mμ, is driven back to rhodopsin. In measurements of the 575-mμ fluorescence, we find a similar situation; that is, irradiation at 440 mμ apparently reduces the intensity of the 575-mμ emission, but only to a value I', which is of the order of 50 percent of the initial intensity I_0. If we destroy the prelumirhodopsin species formed after a given irradiation, the resultant intensity now at the value I' can be further reduced with irradiation at 440 mμ to a value about half I' or $I_0/4$. We have not been able to record absorption spectra for these irradiated samples; however, the behavior described above qualitatively parallels that found by Yoshizawa and Wald for their rhodopsin samples.

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References and Notes

1. G. Wald, P. K. Brown, I. R. Gibbons, *J. Opt. Soc. Amer.* **53**, 20 (1963).
2. W. A. Hagins, H. V. Zonana, R. G. Adams, *Nature* **194**, 844 (1962); M. Fuortes, *J. Physiol.* **148**, 14 (1959).
3. This may or may not be the ground state of the all-*trans* species.
4. B. Rosenberg, in *Advances in Radiation Biology*, L. G. Augenstein, R. Mason, M. R. Zelle, Eds. (Academic Press, New York, 1966), vol. 2, pp. 193–241.
5. W. A. Hagins and W. H. Jennings, *Discussions Faraday Soc.* **27**, 180 (1959).
6. Cattle retinas were obtained from the Hormel Company. The high-intensity monochromator was made by Bausch and Lomb.
7. G. Wald and P. K. Brown, *J. Gen. Physiol.* **35**, 797 (1952).
8. J. M. Bowness, *Biochim. Biophys. Acta* **31**, 305 (1959).
9. T. Yoshizawa and G. Wald, *Nature* **197**, 1279 (1963).
10. C. A. Parker and R. T. Rees, *Analyst* **85**, 587 (1960).
11. Supported in part by a grant from the AEC [contract AT(11-1)-1627] and by a PHS fellowship to G.L.P. (1-F1-GM-36, 155-01).

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Histone Acetylation in Insect Chromosomes

Abstract. *Acetylation of histones takes place along the salivary gland chromosomes of* Chironomus thummi *when RNA synthesis is active. It can be observed but not measured quantitatively by autoradiography of chromosome squashes. The "fixatives" commonly used in preparing squashes of insect chromosomes preferentially extract the highly acetylated "arginine-rich" histone fractions; the use of such fixatives may explain the reported absence of histone acetylation in* Drosophila melanogaster.

Acetylation of histones does occur on insect chromosomes, and a recent report to the contrary by Ellgaard (*1*) is based on inadequate methods of histone fixation. Ellgaard stated that there is "a definite absence of chromosomal acetylation" and that there is no preferential acetylation of histones in regions of gene activation for RNA synthesis. His conclusions were based entirely upon negative results obtained in the autoradiography of chromosome squashes after excised salivary glands were incubated in the presence of sodium acetate-^{3}H (*1*). If correct, these results would constitute a serious objection to the view that the acetylation of histones is correlated with gene activation for RNA synthesis (*2–4*). However, it is not likely that they are correct.

The conditions employed in preparing the *Drosophila melanogaster* chromosome squashes [treatment with a mixture of ethanol and acetic acid (3 : 1), followed by 45 percent acetic acid (*1*)] are not suitable for studies of histone metabolism; such "fixatives" not only dissolve free histones, but also extract the greater part of the radioactive histone from the chromatin of other nuclear types, such as calf-thymus nuclei labeled in vitro with ^{14}C-acetyl coenzyme A, or rat-liver nuclei labeled in vivo with sodium acetate-(methyl-^{3}H). We have evidence that there is a definite incorporation of radioactive acetate into the chromosomes of another insect species *Chironomus thummi*; the incorporated radioactivity is detectable by autoradiography (even after the poor histone fixation procedures usually employed in the preparation of salivary chromosome squashes).

In studies of histone acetylation in mammalian tissues, it has been observed that the radioactive acetate is largely incorporated into "arginine-rich" histone fractions (*2–4*). In rat liver, for example, more than 60 percent of the newly incorporated acetyl groups appear in the arginine-rich f2al fraction prepared by the method of Johns (*5*). In this protein, the acetyl group is believed to be attached to the nitrogen of the amino-terminal serine residue (*4, 6*). Many acetyl groups also appear in the arginine-rich f3 histone fraction, where they are joined to the protein by both *N*-acetyl and *O*-acetyl linkages (*4*). Both of these arginine-rich histone fractions are soluble in ethanol after treatment with acids, and this solubility is the basis of one of the most important methods for their fractionation (*5*).

When arginine-rich histones are prepared from calf-thymus nuclei and suspended in the mixture of ethanol and acetic acid used in preparing insect chromosome squashes, much of

the protein dissolves; what does not dissolve is readily soluble upon subsequent exposure to 45 percent acetic acid. Thus, free arginine-rich histones would certainly be lost in the first stages of preparing *Drosophila* salivary gland chromosome squashes for autoradiography.

The question remains as to the stability of histone-DNA complexes in such solvents. Would histones be lost from chromosomal "puffs" under these conditions? Robert and Kroeger (*7*) have presented evidence that histones at the "puff" were more susceptible to tryptic digestion and were presumably less protected than histones elsewhere along the chromosome. Thus, histones in "puffing" regions might also be more extractable in the acid "fixatives" used, and would be preferentially lost in preparing the chromosome squash.

We have evidence supporting this view from two types of experiments we carried out on mammalian tissues; both experiments show extensive losses of previously incorporated acetyl groups in the fixatives routinely employed for insect chromosome squashes.

In the first set of experiments, histone acetylation was studied in rat liver in vivo, as follows: 15 minutes after rats had been injected intraperitoneally with ^{3}H-acetate (1.5 mc per 100 g of body weight; specific activity 500 mc/mmole), the liver nuclei were isolated (*8*) and washed with TMS [this solution consists of 0.25*M* sucrose, 0.01*M* tris-HCl (*p*H 8.4), and 4 m*M* $MgCl_2$ (*8*)]. The nuclei were resuspended in TMS and divided into two equal portions for comparative studies of histone retention and extraction. One portion was treated to extract the histones, which were removed in 0.2*N* HCl after a prior washing of the nuclei with 0.01*M* citric acid and 88 percent ethanol containing 0.01 mole of HCl per liter (*2*). The histones were precipitated from the HCl extract with ten volumes of acetone, and the arginine-rich fraction was separated by electrophoresis (*2*). The specific radioactivity of the arginine-rich histone band, and of the residue remaining after histone extraction was measured (Table 1).

A second nuclear portion was treated as recommended for the preparation of chromosome squashes. The nuclei were placed in ten volumes of a mixture of ethanol and acetic acid (3 : 1) for 20 minutes, centrifuged, and then suspended in ten volumes of 45 percent acetic acid for 20 minutes. (Tests of these extracts showed negligible losses of radioactive histone in the ethanol–acetic acid mixture and high levels of radioactivity in the 45 percent acetic acid extract.) The nuclear residue was then treated with 0.2*N* HCl in an attempt to extract the remaining acetylated histones. The latter were precipitated in ten volumes of acetone, weighed, and then purified by electrophoresis (*2*). The specific activity of the separated arginine-rich histone band is shown in Table 1. In addition, the nuclear residue remaining after extraction of the histones was monitored for the presence of radioactive proteins. Although only a small proportion of the total radioactivity remains in liver nuclei after HCl extraction, the insoluble residues were counted directly, and again after treatment with hot 12 percent trichloroacetic acid (to remove nucleic acids and polysaccharides) and extensive washing with an ethanol-ether mixture (3 : 1) and with ether (to remove lipids) (Table 1).

Two main points emerge from this study of radioactive histone distribution: (i) The total recovery of histone is greatly diminished when liver nuclei are treated as the *Drosophila* chromosomes were; and (ii) the specific activity of the arginine-rich histones remaining after such treatment is only half that of the corresponding histone fraction from control nuclei. Since the radioactivity is not in the HCl extract, nor in the residue remaining after HCl extraction, it follows that the radioactive histones were lost during fixation. This was tested directly by analysis of the 45 percent acetic acid extract. After lyophilization to remove the acetic acid, the histones were purified by electrophoresis and counted (Table 1). The results show that this type of fixation would be a major source of error in studies of histone acetylation in the liver, where only one-eighth of the radioactive histone originally present would be retained.

A second test of the effects of the acetic acid–ethanol mixture and 45 percent acetic acid on the retention of acetylated histones was carried out on isolated thymus nuclei. The nuclei were prepared in 0.25*M* sucrose containing 3 mmole of $CaCl_2$ per liter (*9*) and incubated in an isotonic medium containing acetyl coenzyme A labeled with acetate-1-^{14}C (0.5 μc/ml). After 15 minutes at 37°C, the nuclear suspension was divided into three portions and centrifuged. The nuclei were washed with 0.01*M* tris-HCl (*p*H 7.4) containing 3 mmole of $CaCl_2$ per liter to remove the radioactive precursor.

Table 1. Distribution of radioactive acetylated histones in calf-thymus and rat-liver nuclei after exposure to media used in preparing insect chromosome squashes. Treatment No. 1 consisted of fixation in saturated aqueous picric acid; treatment No. 2 consisted of extraction with a mixture of ethanol and acetic acid (3 : 1) followed by 45 percent acetic acid; treatment No. 3 consisted of extraction with 0.2*N* HCl; in treatment No. 4, histones were prepared from the 45 percent acetic acid extract of liver nuclei and purified by electrophoresis.

Treatment	Total nuclear protein			Residue after histone extraction			Histone in HCl extract (mg)	Arginine-rich histones* (cpm/mg)
	Amount (mg)	Specific activity (cpm/mg)	Total activity (cpm)	Amount (mg)	Specific activity (cpm/mg)	Total activity (cpm)		
				Calf thymus				
1	35.4	1570	55800					
2	17.85	1220	21780					5320
3	7.88	400	3150					11100
				Rat liver				
2				3.0	420	1260	1.0	1350
3				3.0	730	2190	4.0	2700
2				8.8	1620			4000
3				12.6	2690			
4								8330

* Specific activity of the arginine-rich histone fraction separated by electrophoresis on cellulose polyacetate (*2*).

The first nuclear portion was treated to extract the acetylated histones, which were removed in 0.2*N* HCl as described above. Histones in the acid extract were precipitated in ten volumes of acetone and purified by electrophoresis before measurement of radioactivity (*2*). The specific activity of the proteins remaining in the nuclear residue was also determined after treatment with hot 12 percent trichloroacetic acid and extensive washing with mixtures of ethanol and ether (3 : 1), ethanol, ether and chloroform (2 : 2 : 1) and ether alone (Table 1).

A second nuclear portion was treated with saturated aqueous picric acid for 30 minutes at 0°C. This fixative was introduced for cytochemical studies of histone localization (*10*), and we have found that it is an effective precipitant of free histones that have been separated by electrophoresis (*2*). For this reason, picric acid fixation has been selected as the standard with which to compare the retention of histones in nuclei treated as the *Drosophila* chromosomes were (*1*). The specific activity and the total radioactivity due to acetylated proteins in the picric acid–treated nuclei are listed in Table 1.

The third nuclear portion was treated at 25°C with 100 volumes of a mixture of ethanol and acetic acid (3 : 1) for 30 minutes, and with 45 percent acetic acid for 30 minutes. The specific activity and the total radioactivity of the remaining proteins were measured. In one experiment, the remaining histones were extracted in 0.2*N* HCl and purified by electrophoresis, and their radioactivity was measured. The specific activity of the arginine-rich fraction, extractable after the treatment with acetic acid–ethanol and 45 percent acetic acid is compared with that of the control histone in Table 1.

More than 60 percent of the total radioactivity is lost from thymus nuclei that have been extracted in the mixture of acetic acid and ethanol and then in 45 percent acetic acid. The histone that does remain and can be extracted in 0.2*N* HCl is only half as active as the histone extractable from control nuclei.

Thus, the results for thymus and liver are in essential accord. The equivalent of the squash technique removes 60 percent of the newly acetylated histone from thymus nuclei and nearly 90 percent of the radioactive histone from liver nuclei. Not all of the radioactive histone is removed, but the arginine-rich fraction that remains has only half the specific activity of the corresponding fraction in the HCl extract of control nuclei.

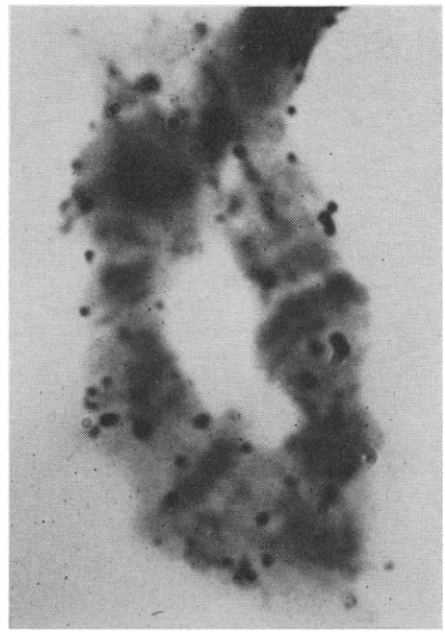

Fig. 1. Autoradiograph of a chromosome of *Chironomus thummi* after 15 minutes of incubation of excised larval salivary glands in the presence of sodium acetate labeled with tritium in the methyl group. The grain distribution indicates that the radioactive acetyl groups are incorporated along the chromosome.

The fact that some acetylated histone does remain probably accounts for the successful detection of acetylated histones in *Chironomus thummi* chromosomes (Fig. 1). In this experiment, excised larval salivary glands were incubated for 15 minutes at 20°C in Ephrussi-Beadle medium containing sodium acetate labeled with ^{3}H on the methyl group (2.5 mc/ml). The glands were washed in nonradioactive medium, and then fixed in a mixture of ethanol and acetic acid (3:1) for a short time, and then in 45 percent acetic acid for 30 minutes. They were squashed and stained in orcein in 47 percent lactic acid, coated with liquid photographic emulsion, and stored in the dark for 2 weeks. The autograph was developed in Kodak D19 and photographed in the light microscope. Figure 1 shows that the uptake of radioactive acetate takes place along the chromosomes of this insect. Simultaneously prepared autoradiographs of ^{3}H-uridine–labeled material also indicated extensive chromosomal RNA synthesis. We have not tested to see whether acetylation would occur preferentially in a "puffing" region, for it is clear that such tests will be significant only if the squash technique can be modified to preserve all of the radioactive histone and not just a fraction of it. Based on extensive experimentation in mammalian systems, meaningful studies of the relation between histone acetylation and RNA synthesis will require careful kinetic analysis of acetyl uptake and "turnover" in particular histone fractions (*2–4*), as well as a knowledge of acetate "pool" sizes.

Finally, we have not stated that "histone acetylation is a necessary prerequisite for the synthesis of RNA's at previously repressed gene loci" (*1*), though this may turn out to be the case. Our statement was "that a change in the structure of the chromatin —brought about by, or coincident with histone acetylation—is a necessary prerequisite for the synthesis of new RNA's at previously repressed gene loci," and that "this aspect of control has, as yet, little direct evidence to support it" (*3*). However, many striking correlations have been observed between histone acetylation and the capacity of the cell nucleus for RNA synthesis (*2–4*). Although these may be coincidental, the correlation has now been seen in enough systems to warrant further and more detailed investigations. The *Drosophila* labeling experiments (*1*) were certainly a step in the right direction, but for the technical reasons outlined above, the negative results do not constitute a serious objection or obstacle to further study of this interesting phenomenon.

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References and Notes

1. E. G. Ellgaard, *Science* **157**, 1070 (1967).
2. B. G. T. Pogo, V. G. Allfrey, A. E. Mirsky, *Proc. Nat. Acad. Sci. U.S.* **55**, 805 (1966).
3. V. G. Allfrey, R. Faulkner, A. E. Mirsky, *ibid.* **51**, 786 (1964); V. G. Allfrey, *Can. Cancer Conf.* **6**, 313 (1964); *Cancer Res.* **26**, 2026 (1966); B. G. T. Pogo, V. G. Allfrey, A. E. Mirsky, *J. Cell Biol.* **35**, 477 (1967).
4. B. G. T. Pogo, A. O. Pogo, V. G. Allfrey, A. E. Mirsky, *Proc. Nat. Acad. Sci. U.S.*, in press.
5. E. W. Johns, *Biochem. J.* **92**, 55 (1964); *ibid.* **105**, 611 (1967).
6. D. M. P. Phillips, *ibid.* **101**, 23 (1966).
7. M. Robert and H. Kroeger, *Experientia* **21**, 326 (1965).
8. A. O. Pogo, V. G. Allfrey, A. E. Mirsky, *Proc. Nat. Acad. Sci. U.S.* **56**, 550 (1966).
9. V. G. Allfrey, V. C. Littau, A. E. Mirsky, *J. Cell Biol.* **21**, 213 (1964).
10. D. P. Bloch and H. Y. C. Hew, *J. Biophys. Biochem. Cytol.* **8**, 69 (1960).
11. Supported in part by a grant (GM-04919) from the USPHS.

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