sometimes after 7 days. No toxic effects have ever been observed with any of the alkenylsuccinic acids. As indicated (*2, 5*), closure of stomata by these compounds usually results in a greater inhibition of transpiration than of photosynthesis.

Opening of stomata can be prevented by inhibiting any of the biochemical reactions concerned with the increase in turgor of guard cells in the light, or by affecting the permeability of their membranes (*7*). The alkenylsuccinic acids which exhibit optimal activity for stomatal closure have also been shown by Kuiper (*8*) to be most effective in increasing the permeability of cell membranes of roots to water. The parallelism suggests that these long-chain unsaturated dibasic fatty acids behave as inhibitors of stomatal opening because of their effects upon the lipid layers of the membranes of the guard cells. A model of the effect of certain herbicides upon the lipids of plant cell membranes has previously been outlined by van Overbeek and Blondeau (*9*). However, the alkenylsuccinic acids are highly specific in their action, and apparently penetrate guard cells more readily than other epidermal cells. Thus these compounds provide an example of still another biochemical approach to the reduction of transpiration in the field and forest through stomatal control.

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Histone Staining with Ammoniacal Silver

Abstract. *Under controlled conditions, ammoniacal silver (A-S) stains the bands of Dipteran salivary gland chromosomes in a precise and selective manner. Such staining is dependent upon the histone content of the band, as shown by the effects of selective extraction and blocking of the histones. The effects of acid extraction and HONO solution on the A-S staining of different bands suggest that specific genes may have particular histones associated with them.*

Histone rather than DNA appears to be the cytochemical covariable in many instances of functional change and cell development (*1*). However, even the chemical definition of histones is still speculative; as yet it has only been demonstrated that they are basic proteins of the nucleus intimately associated with DNA and extractable in dilute acid or acid salt solutions. At a recent symposium on histones, one conclusion reached was that a method is needed that is sufficiently delicate and sensitive to demonstrate whether histones do, indeed, differ in different functional states and stages of development (*2*).

In 1958 we reported that under controlled conditions ammoniacal silver (A-S) stained the nuclear chromatin of a variety of cells (*3*). This was followed by a series of reports in which the A-S staining of nuclear chromatin under different functional conditions was described. Data were presented which suggested that the A-S was staining the histone component of nuclear chromatin (*4*). It was found that A-S stained the nuclear chromatin of formalin-fixed smears of all types of cells tested, including mammals, fowl, invertebrates, and plants. The cytoplasm of such cells was stained faintly or not at all, while nucleoli were unstained. To test further the use of A-S as a cytochemical stain for histones, we decided to apply it to the giant chromosomes of the salivary glands of various Diptera, including those of *Sciara coprophila* (*5*).

The salivary glands were squashed in 45 percent acetic acid, a step which, while not at all essential to the A-S reaction, is essential to good spreading of the chromosomes in question. The coverslip was removed after gentle heating and the preparation was air dried. Where histones were to be extracted before staining, it was found best to extract the larvae being dissected in a drop of 0.25*N* HCl before squashing, and to transfer the glands to a vial with several changes of acid in the cold over a period of 1 hour. The extraction of histone by this method is remarkably complete and cannot be improved by changing the time in the acid or its concentration. The slide to be stained is fixed in 10 percent acetate-buffered neutral formalin for at least 15 minutes, rinsed in five changes of distilled water, placed in the A-S solution, and agitated for 5 to 10 seconds. Longer periods do not increase the staining significantly. It is then washed well in five changes of distilled water and developed in 3 percent formalin for 2 minutes, washed again, and run through the alcohols, cleared in xylol, and mounted permanently. The A-S solution, which should be fresh, is prepared by adding a 10 percent solution of $AgNO_3$ to concentrated NH_4OH until the first permanent turbidity appears (approximately 10 : 1, vol/vol, respectively). The exposure to formalin before staining is an obligatory step whether the material has been previously fixed in formalin or not; only the brief rinsing in five changes of distilled water should intervene.

These procedures were controlled by staining similar preparations by the Feulgen reaction, both before and after extraction, and also by the alkaline Fast Green method of Alfert and Geschwind (*6*) for histones. In addition, duplicate preparations were blocked with HONO before staining with A-S. The HONO solution is prepared by mixing equal parts of a 10 percent trichloroacetic acid (TCA) and a 10 percent sodium nitrite solution. After exposure to formalin and rinsing

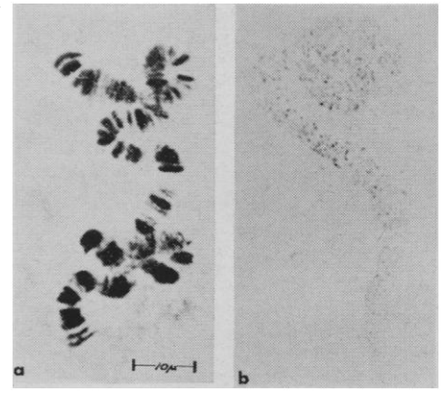

Fig. 1. Chromosome IV, of *Sciara coprophila*, stained with A-S. (*a*) Control; (*b*) extracted in cold, dilute HCl. While extraction of histone removes ability to stain with A-S, an intact banded structure is disclosed by Feulgen staining.

in water, the slides are placed in the HONO solution for 5 to 15 minutes, then washed, again exposed to formalin, rinsed, and stained as already described.

It was found that the A-S procedure stained the chromosome bands distinctly (Fig. 1*a*). Each band stained by A-S corresponded with similar bands in preparations stained by the Feulgen reaction and by the alkaline Fast Green method. In fact, one can readily perform a Feulgen stain on a preparation previously stained with A-S. Such doubly stained chromosomes clearly demonstrate the coincidence between the A-S and Feulgen-stained bands. The interband areas were not stained, or at least not obviously so, with A-S, alkaline Fast Green, or by the Feulgen technique. However, the cytoplasm was stained noticeably with both A-S and alkaline Fast Green.

After extraction with cold HCl, the A-S staining is reduced or absent. Yet the Feulgen stain remains positive, showing that the decreased silver staining is not due to changes in DNA. Attempts to stain acid-extracted chromosomes with alkaline Fast Green were generally unsuccessful. The successive extraction of both histone and DNA almost completely demolishes the chromosomes. We were able to discern the ghosts of such chromosomes by phase microscopy and to stain them with Light Green SF. Such ghosts lacked any discernible band structure.

Inhibition of A-S staining by extraction with acid supports the view that A-S stains the histone component of the bands. This view is greatly strengthened by the restoration of A-S staining after incubating extracted chromosomes in histone solutions. Duplicate slides of the extracted chromosomes were immersed in a 0.1 percent solution of calf thymus histone (*7*) in 0.9 percent saline for 30 minutes. The slides were then rinsed in saline and placed in 10 percent neutral formalin for 10 to 15 minutes, rinsed in distilled water, and stained with A-S. The staining was similar to that of the control preparations (Fig. 2*a*). The bands were precisley stained while the interband regions appeared unstained. Aside from a somewhat more brownish cast, the bands were indistinguishable from those of the untreated chromosome.

It was observed that some of the bands consistently retained the ability to stain with A-S longer than others during extraction in cold acid (Fig. 2*b*). Exposure to HONO also inhibited A-S staining. It was particularly interesting that exposure to HONO for 15 minutes or less also had a differential effect on the A-S staining of different bands (Fig. 2*c*). Some bands appeared to be resistant to HONO as well as to acid extraction, other bands appeared sensitive to both, while still others appeared to differ in their sensitivity to HONO and acid extraction (Fig. 3, *a* and *b*).

The differential effect of HONO on the chromosomal bands was not observed after staining with alkaline Fast Green. While HONO inhibited alkaline Fast Green staining, such inhibition tended to affect all bands similarly.

The data obtained in this study confirm our previous observations of staining with A-S and the effect of acid extraction and histone incubation. Marinozzi has recently reported on the use of silver staining of nuclear proteins as an aid in studying the ultrastructure of the nucleus. While his procedure is quite different from ours, it also seems to be related to the histones (*8*).

The nature of the present test material makes our previous observations more significant since the bands of salivary gland chromosomes are generally considered to be gene loci. Therefore, the finding that the A-S demonstrates differences in the properties of histones associated with different bands offers

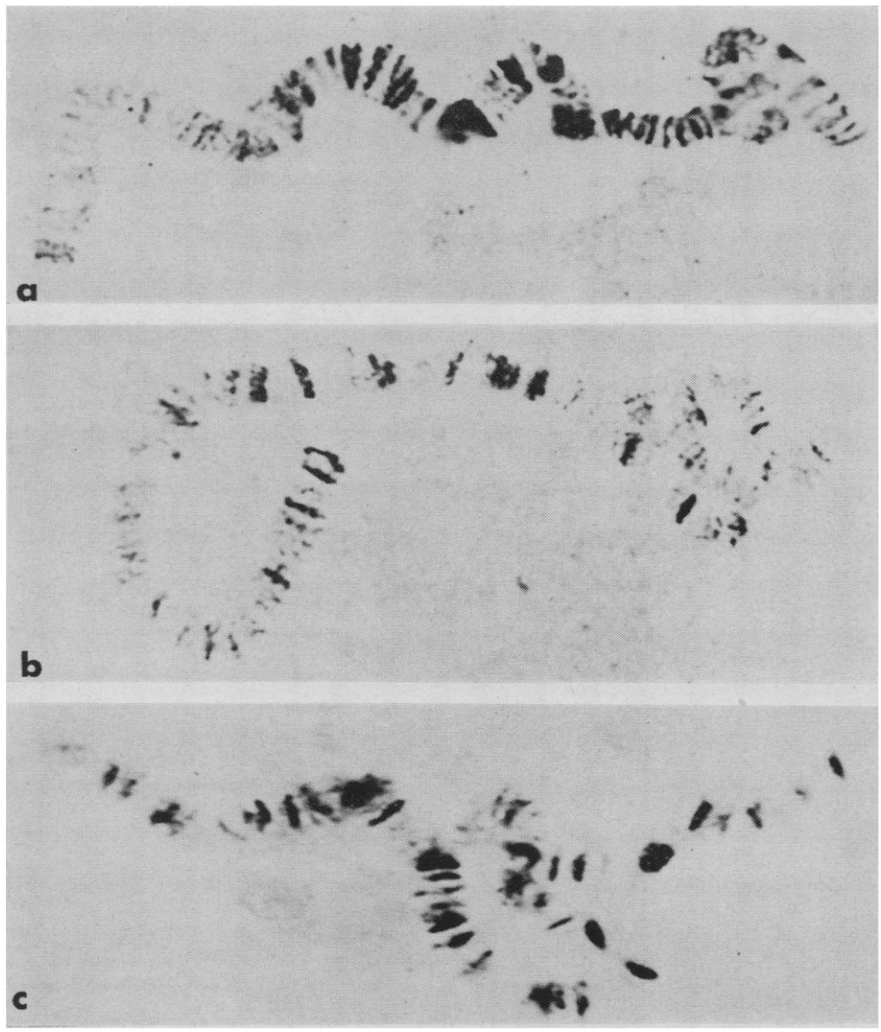

Fig. 2. Chromosome IV, of *Sciara coprophila*, stained with A-S. (*a*) After incubation in calf thymus histone following acid extraction; note similarity to control preparation in Fig. 1. (*b*) After blocking with HONO; note variable effect on different bands. (*c*) After partial extraction with acid; note variation in acid solubility of histone in different bands.

the possibility of testing the thesis that histones function as gene modifiers.

Comment should also be made regarding the comparative features of the alkaline Fast Green and A-S staining procedures. As already stated, staining with alkaline Fast Green requires extraction of DNA, whereas staining with A-S requires only treatment with formalin. It would appear that alkaline Fast Green combines with those bonds of histone molecules which are ordinarily combined with DNA. In contrast, the A-S reaction involves non-DNA-bound histone groups; that is, groups which would, presumably, be free to react in the cell nucleus. Differences between the reaction of A-S and alkaline Fast Green with histones are also demonstrable when isolated histones are studied. As reported previously (*9*), A-S stains lysine-rich histones more avidly than arginine-rich histones. In contrast, alkaline Fast Green stains lysine and arginine-rich histones with almost equal intensity. The affinity for reactive sites not bound to DNA in the lysine-rich fractions of histones may account for the ability of A-S to discriminate more subtle differences in cell function and differentiation.

The addition of this information to our previous findings regarding the A-S staining of cells and histone electropheragrams leaves little doubt that A-S stains histones in a preferential manner and that the procedure is of value in cytochemical investigations. The dipteran salivary chromosomes may prove to be useful for the investigation of the biological function of histones. We submit that our findings suggest that different genes may have different histones.

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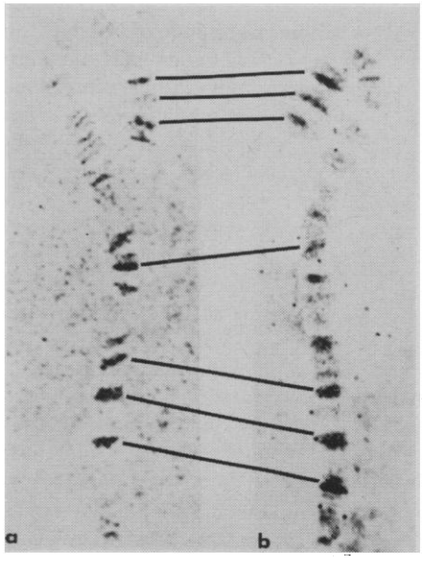

Fig. 3. Chromosome II, of *Sciara coprophila*, stained with A-S. Band-for-band comparison after (*a*) blocking and (*b*) partial extraction demonstrates differences in histone composition among different loci of the same chromosome. Feulgen staining discloses that DNA components of the bands are unaffected by either treatment.

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Autoradiographic Distribution of Radioactive Sodium in Rat Kidney

Abstract. *Autoradiographs of kidney sections from rats injected with sodium-22 showed two areas of relatively high concentration of the radioisotope. The first area appeared in the outer medulla; the second appeared as an area of increasing concentration toward the papilla. Autoradiographs of sections labeled with chloride-36 appeared essentially the same. Autoradiographs of sections labeled with inulin-$C^{14}OOH$ showed an increased concentration in the inner medulla. These results may have a bearing on the counter-current multiplier hypothesis for the production of concentrated urine.*

In 1951 Hargitay and Kuhn (*1*) described in detail their counter-current hypothesis for the production of a concentrated urine in the mammal. It was proposed that the loop of Henle functions as part of a counter-current multiplier system, yielding a progressively increasing gradient of solute concentration in the tubular fluid and surrounding interstitium from cortex to papilla. As the urine in the collecting ducts passed through the region of high solute concentration, it was proposed that water was withdrawn to form a concentrated urine. In confirmation of the general hypothesis, micropuncture studies by Gottschalk and Mylle (*2*) showed that urine in the proximal convoluted tubule was isotonic with plasma, that early distal tubular fluid was hypotonic with plasma, and that fluid in the tip of Henle's loop had the same osmolarity as fluid from the adjacent collecting ducts. From these and other considerations, it was suggested that sodium is actively transported into the interstitial fluid, taking with it chloride ions to maintain electrical neutrality, and thereby causing the pattern of increasing solute concentration. The actual site for active sodium transport has been attributed to the whole length of the ascending limb of Henle's loop (*3*), to the thick ascending part of the loop (*4*), and to both the descending and ascending limbs of the loops of Henle (*5*). Water supposedly does not move with the sodium and chloride in these areas during antidiuresis; therefore, the areas of active transport must be impermeable or less permeable to water. The uncertainty in delineating the actual site of active sodium transport is due to the difficulty in obtaining samples of urine from the outer medulla, the zone of the kidney containing the thick ascending parts of Henle's loops.

Table 1. The concentration of Na^{22} and stable sodium, and the specific activity of Na^{22} in the cortex, outer medulla, and inner medulla of rat kidney. Values represent the mean ± standard error of the mean of six rats.

Tissue sample	Na^{22} (% dose/ g wet tissue)	Stable Na (meq/ g wet tissue)	Specific activity (% dose Na^{22}/ meq stable sodium)
Cortex	.35±.04	.096±.008	3.6±0.4
Outer medulla	.54±.04	.121±.007	4.0±0.4
Inner medulla	.87±.08	.198±.021	3.7±0.4