AVOIDANCE OF STAINING ARTIFACTS IN SMEARS OF ECTOTHERM BLOOD

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ABSTRACT.—The destructive effect of unsuspected water contamination in Wright's stain upon the morphology of the blood cells of certain ectotherms was studied. The results agree with those found in mammals. In the frog, the cytoplasm of the red and of some of the white cells became vacuolated and the red cells tended to show changes in shape. In the turtle, similar vacuolization of cells occurred, with no changes in shape of the red or white cells. In *Necturus* there was a tendency toward vacuolization in red and white cells, but no changes in cellular size or shape. Experimental addition of 1 ml of water to the stain produced crenation and marked cytoplasmic vacuolization in the red and white cells of the frog. The vacuolization effects were essentially similar although less marked in *Necturus* and the turtle, but there were no changes in the size or shape of the blood cells. Prior treatment of blood smears in 95 percent methanol reduced all artifacts in the frog and turtle. The optimal treatment for *Necturus* involved drawing blood into 1 percent acetic acid, smearing the blood on slides and then immersing the smear in 95 percent methanol prior to staining.

It has been shown in the human peripheral blood smear that Wright's stain may produce artifacts affecting primarily the red blood cell (Bettigole, 1964). Because of the increasing use of amphibians and reptiles in research, it is of interest to find whether their blood cells, both red and white, are similarly affected by the procedure used to visualize them and, if so, to establish remedial measures. Three common laboratory ectotherms were selected for this purpose, a salamander, frog and turtle.

MATERIALS AND METHODS

Frog and turtle. Blood of *Rana pipiens* and *Pseudemys scripta elegans* was obtained by cardiac puncture in a syringe containing 1.6 percent sodium oxalate, using 1 volume of anticoagulant to 9 volumes of blood (Kaplan, 1956). When heparin was used, it produced a slight tendency toward shrinkage of the red cells in the frog, but not in the turtle. Blood smears were made on slides and stained with Wright's stain.

Commercial Wright's stain is frequently contaminated with traces of water, especially upon standing, and the effect of this contaminant was studied. A number of Coplin jars were filled with 50 ml of Wright's stain. Water was added in specific amounts such that a graded series of two or three solution concentrations was obtained. The differential effect of water contamination could then be visualized. Some jars were filled with commercially obtained Wright's stain with no extraneous increments of water experimentally added.

To test the possible protective effect of prior fixation in alcohol against artifact formation, some slides were fixed for periods varying from 10 seconds to 8 minutes in 95 percent methyl alcohol before staining for 3 to 6 minutes in undiluted commercial Wright's stain. They were rinsed for 1 minute in distilled water for its acidic reaction, then for 1 minute in tap water for its basic reaction and finally dried in air. Other slides were also fixed in methyl alcohol, but they were then immersed in Wright's stain containing varying amounts of added water.

Salamander. It was found on blood obtained by cardiac puncture from *Necturus m. maculosus* that the cells drawn into 1.6 percent sodium oxalate, 3.4 percent sodium citrate or 1 percent heparin tended to be distorted. Heparin produced shrinkage. The oxalate or the citrate used in 1 to 9 volume ratios in isomolar concentrations did not fully prevent coagulation. Therefore, the blood was drawn into 1 percent glacial acetic acid in the ratio of 0.1-0.3 ml of acetic acid to each ml of blood and then smeared at once. Tobias (1936) reported that acetic acid expands red cells. We have observed that it minimizes any shrinkage due to later treatment by other chemicals.

Results

Some typical results are illustrated in Figure 1. The presence of red cell staining artifacts due to water spontaneously occurring in commercially obtained Wright's stain produced cellular changes. In the frog the red cells were found to be prominently vacuolated, with many cells showing a minor tendency to change their shapes. Some of the white cells of the frog also became vacuolated. If the smeared slides are not rapidly dried prior to staining by waving them to and fro in the air, vacuolization of all the blood cells can be artificially produced to a greater or lesser degree.

In the turtle blood which was stained with commercial Wright's stain, the red cells were commonly vacuolated somewhat in excess of the normal, but their shape and size did not appear to be changed. All the turtle white cells showed many vacuoles which coalesced and distorted the normal cytoplasmic architecture.

In *Necturus* blood which was stained with commercial Wright's stain there was the least vacuolization of the three animal bloods examined, although the abnormality was definite. There was no obvious alteration of the shape or size of the red cell. The white cells failed to stain as prominently as they did in water-free stain.

Two or three drops of water added to 50 ml of stain in the Coplin jar produced red and white cell vacuolization in the frog and turtle, the effects being clearly greater than that seen with the undiluted commercial Wright's stain. If the staining solution stood for several days, the aberrations were even more obvious. There was also greater vacuolization in the *Necturus* red cells but the white cells showed no greater changes than those seen in the undiluted commercial Wright's stain. The frog red cells showed a crenation that did not appear in *Necturus* or turtle red cells.

In frog blood 1 ml or even less of water added to 50 ml of Wright's stain produced more marked distortion than a single drop did. There were severe red cell crenation and considerable cytoplasmic vacuolization of both red and white cells. All these effects worsened with increasing dilution. When the frog smears were immersed in diluted Wright's stain that had been standing

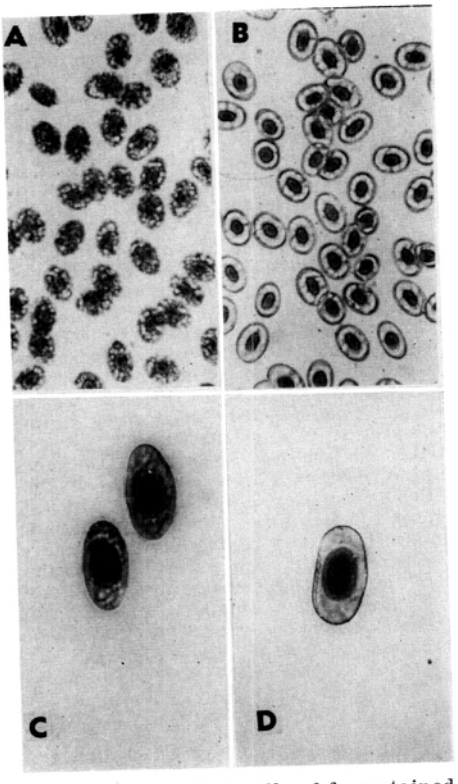

Figure 1. A. Red cells of frog stained in Wright's stain to which 1 drop of water was added. x 965. B. Red cells of frog treated in methanol prior to staining. x 1,020. C. Red cells of *Necturus* stained in Wright's stain to which 1 drop of water was added. x 1,075. D. Red cells of *Necturus* drawn into acetic acid, smeared and immersed in methanol prior to staining. x 1,200.

several hours, the deleterious effect upon the cells was even more severe.

In turtle and *Necturus* blood addition of 1 ml of water to 50 ml of Wright's stain produced greater vacuolization of cells than that seen in undiluted commercial Wright's stain. The shape and sizo of the red cells were not affected. The white cells of both animals were vacuolated.

The drawn blood of the three test animals was then treated to try to eliminate any of the effects of water dilution upon the final appearance of the stained cells. The optimal treatment for the frog was found to involve immersion of dried smears of oxalated cardiac blood in 95 percent methanol for at least 2 minutes, in commercial Wright's stain for 3 minutes, in distilled water for 1 minute, and in tap water for 1 minute. Crenation and cytoplasmic changes were prevented. The white cells were readily differentiated and their cytoplasmic morphology was normal.

The optimal treatment for the turtle involved immersion of dried smears of oxalated cardiac blood in 95 percent methanol for about 2 minutes, in commercial Wright's stain for 6 minutes, in distilled water for 1 minute and in tap water for 1 minute.

The optimal treatment for *Necturus* involved drawing blood by cardiac puncture into a syringe containing 1 percent acetic acid (0.1-0.3 ml of acid/ml of blood), smearing this blood quickly, and immersing the smear in 95 percent methanol for 15-30 seconds, in commercial Wright's stain for 3 minutes, in distilled water for 1 minute and in tap water for 1 minute. In *Necturus*, if the smear was fixed in methanol for more than 1 minute, the red cells typically took on an undesirable greenish hue.

In all instances immediate staining of freshly prepared smears produced the greatest contrast.

Discussion

If commercial Wright's stain were always completely free of water, some of the common artifacts appearing in stained smears of blood cells would be eliminated at the outset. Usually, however, the stain seems to absorb enough water upon prolonged standing to produce aberrations. The usual test of this is to put a drop of the stain upon oven-dried filter paper; if the periphery of the circular spot on the paper appears fuzzy and a pink halo arises around the blue spot, water is present.

The use of methanol prior to staining eliminates free water and produces a more uniform diffusion of the staining fluid, thus minimizing differential concentrations and perhaps variations in pH. The procedure is not new for mammals (Bell, 1950).

The data indicate that the known human blood cell sensitivity to water in Wright's stain extends down into amphibian and reptile blood. Among the ectotherms tested, the frog appears to be the most sensitive, reacting similarly to man in this regard. The cells of the turtle and *Necturus* are more resistant in that order.

The techniques used in this study are grossly qualitative, but they have been found to be reproducible among various unrelated animal colonies sampled.

Literature Cited

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