

# Phloretin Inhibition of Water Transport in Turkey and Duck Erythrocytes is pH Dependent

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## Abstract

Phloretin, a natural product found in root bark of apple trees, has a long history of being used to study cell membrane transport. Numerous reports indicate that phloretin has little effect on the transport of water in mammalian erythrocytes. However, previous research has found that phloretin has a dramatic inhibitory effect on water transport in avian species. With a spectrophotometric method, it was observed that avian erythrocyte hemolysis time was increased 6 to 10 fold by 1 mM phloretin. The data reported here obtained by spectrophotometric and video microscopic methods, show that this phloretin inhibition of water transport is pH sensitive. In the lower pH region, phloretin has no inhibitory effect. As pH increases, the inhibitory effect of phloretin becomes more and more prominent. At pH 9.00, 1 mM phloretin treatment increased the swelling time of turkey erythrocytes by 758%. The same pH effect was confirmed in duck osmotic hemolysis.

**Keywords:** pH effect, Phloretin, Digital microscopy, Erythrocytes

## 1. Introduction

Phloretin ( $C_{15}H_{14}O_5$ ), also called  $\beta$ -(4-Hydroxyphenyl)-2,4,6-trihydroxypropiophenone or 3-(4-Hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)-1-propanone, comes from a substance called phloridzin, which is found in the fresh root bark of the apple tree (Felter and Lloyd, 1898). Phloretin is well known in the study of membrane transport. It is a useful inhibitor of urea (Macey, 1970; Macey, 1984), urea analogues (Brahmand Wieth, 1977), glycerol (Abrami and Ripoche, 1995; Macey and Farmer, 1970; Macey et al, 1972) and glucose (Betz and Gilboe, 1975; Bowyer, 1957; Seyfang and Duszenko, 1991). Previous research reveals that while phloretin inhibits urea transport, it does not inhibit water transport in mammals (Reimer et al, 2003; Bowyer, 1957; Macey and Farmer, 1970). In this case, phloretin has been used as an effective inhibitor of water transport in turkey and duck red blood cells.

The effect of phloretin and pH on swelling time of avian erythrocytes has not been documented before. Here it is shown that increasing pH effects the inhibition of phloretin and in turn increases the swelling time of red blood cells in birds.

## 2. Methods

### 2.1. buffers

5 mM HEPES was used to buffer the water and isotonic NaCl solution for pHs 6.9-7.9, while 5 mM MES was used for pHs lower than 6.9 and 5 mM TAPS for the pHs higher than 7.9. HEPES, TAPS, and MES were purchased from

Sigma. The pH of all solutions was checked with a pH meter and adjusted to the proper range using hydrochloric acid or sodium hydroxide.

## **2.2. phloretin**

0.02743 g of phloretin (purchased from Sigma) was added to 1 mL of ethanol to make the concentration 100 mM. When testing on erythrocytes, phloretin was incubated in the suspension for 15 minutes prior to use.

## **2.3. spectrophotometric methods**

The hematocrit of the blood being used was found. This determined the concentration of the red blood cells in the blood. The blood was diluted at a ratio of 1:10 with isotonic saline (150 mM buffered saline) at the chosen pH and centrifuged for five minutes at 1200 rpm. This was the blood washing process. When finished, the supernatant was removed and the blood was resuspended 1:10 in saline. The blood washing process was repeated two more times and then the red blood cells were resuspended to three percent hematocrit. This was calculated using the numbers attained from the original hematocrit. The blood was then ready for experimentation.

When experiments were conducted with phloretin, a sample of the prepared blood was taken and placed in a separate tube. The desired molar concentration of phloretin was added to this blood and gently mixed. Then the phloretin was allowed to incubate in the blood for ten to fifteen minutes so that it reaches maximal binding to the cell membranes.

For experimentation, 125  $\mu$ L of blood was pipetted into a cuvette and placed in a Fisher Scientific spectrophotometer model 410 set at the wavelength of 510 nm. 2.35 mL of buffered water at the desired pH was squirted in quickly at an angle that left the blood and the water well mixed. The lid of the spectrophotometer was immediately closed and the recording of data commenced. The total volume in the cuvette was 2.475 mL. The spectrophotometer was connected to an Iworks/214 interface box and then a computer. Data were recorded in the software program Labscribe. The spectrophotometer was calibrated so that 100% transmittance registered as 100% hemolysis of the blood. In measuring the results, the time required for 75% of the blood to hemolyse was recorded. This method is based on work first published by Jacobs (Jacobs, 1952).

## **2.4. digital microscopic method**

The digital images of erythrocyte hemolysis were captured with a Motic National DC3-163 Digital Microscope with phase contrast lens interfaced to an Athlon XP 1400 megahertz computer. The software used was Motic Image Plus 2.0. The output size of the pictures was 640 x 480, which is the maximal resolution of the digital microscope.

A slide of erythrocytes suspended in isotonic saline solution was used to make sure the microscope was in focus. A hemocytometer was used to reduce the movement of the cells. The grids on the hemocytometer also helped to keep track of each cell.

During the preparation of the slide for data recording, the 40x objective lens was tuned the side. For each experiment, 0.20  $\mu$ L of the washed 3% blood suspension was placed in the center of the hemocytometer. Immediately following that, the auto capture sequence in Motic Plus was started. Since the objective lens was turned to the side, the first several images captured were the black background. This is to make sure the program can capture the earliest possible image of cells lysing.

8  $\mu$ L of hemolysing reagent was placed in the center of a clean cover slide. Quickly but very gently, the cover slide was turned upside-down, the hemolysing reagent facing down, and put right on top of the blood drop, to make sure the blood drop and hemolysing reagent had maximal contact and that they are evenly mixed throughout the slide. The mixing between the blood and hemolysing reagent marks the beginning of the hemolysis process. Very rapidly, the 40x objective lens was turned into position. Fine adjustments were made to obtain the sharpest image. A series of picture were taken every second. The auto capture sequence was stopped once all the cells on the hemocytometer had lysed.

## **2.5. measuring swelling time**

Two sets of picture sequences were obtained for 15 different pH values. The first picture in each set was considered the time zero. Five groups of cells, four of them at each corner, and one in the center of the picture, each composed of five erythrocytes, were followed to record the swelling time. A graphic view, Irfanview was used to view the sequences of all the pictures in order. Individual cells were followed from the beginning shape, which is elliptical, to when they first turned into a round shape. This time length was recorded as swelling time. The hemolysis time, which is the time from when the cells were elliptical to when they disappear, was also recorded.

### 3. Results

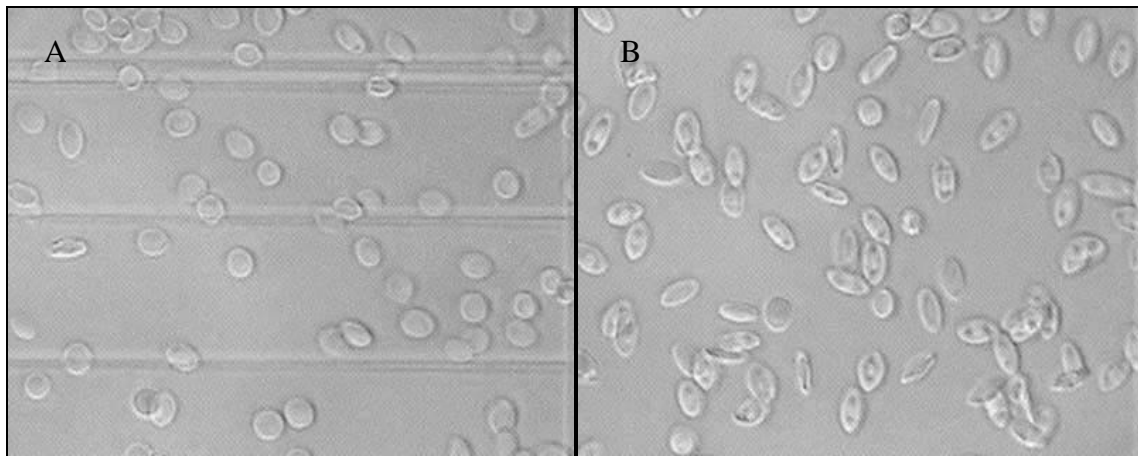


Figure 1- Phloretin Effect on the Swelling of Turkey Erythrocytes at pH 7.9. 1- A is taken 2 seconds after hemolysis started in non-phloretin treated cells. 1- B is taken 2 seconds after hemolysis started in 1 mM phloretin cell suspension. When the non-phloretin treated erythrocytes were already spherical, the cells treated with 1 mM phloretin still maintained an elliptical shape.

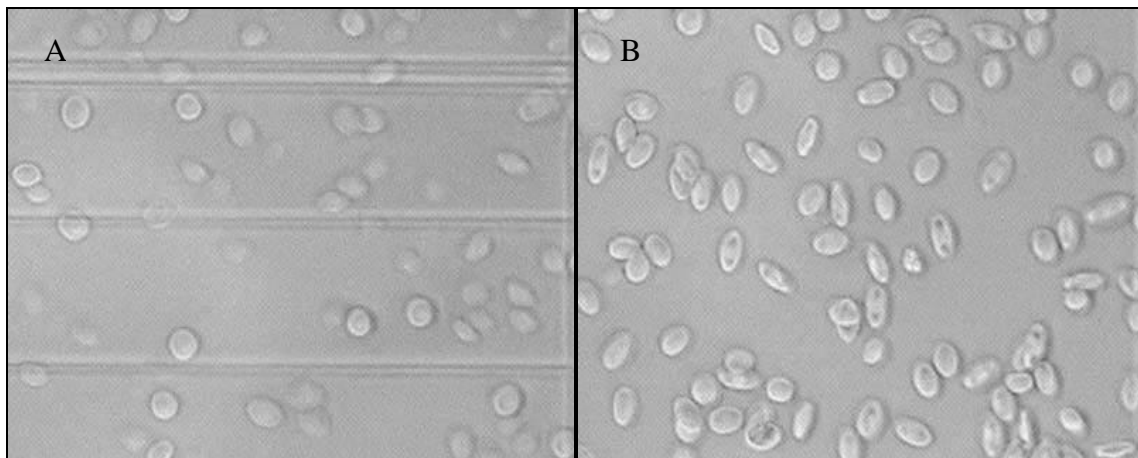


Figure 2 – A and B are turkey erythrocytes without phloretin and with 1 mM phloretin in pH 7.9 respectively 5 seconds after hemolysis process started. Most of the non-phloretin treated cells have already lysed, while the cells in 1 mM phloretin were still elliptical, but rounder than when at 2 seconds (figure 1- B).

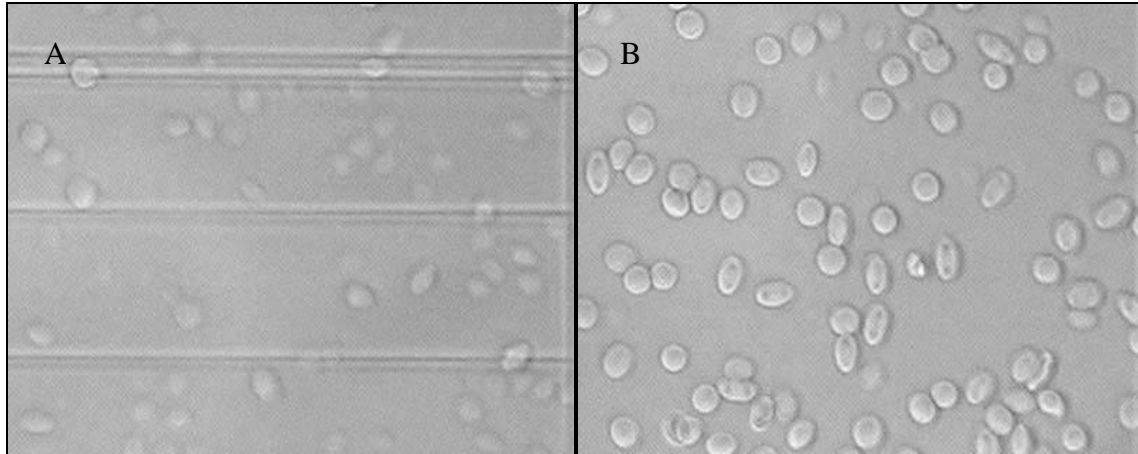


Figure 3 - A and B are turkey erythrocytes without phloretin and with 1 mM phloretin in pH 7.9 respectively 10 seconds after hemolysis process started. Notice that all the cells without phloretin have turned into ghost cells and cells in 1 mM phloretin were still elliptical or spherical.

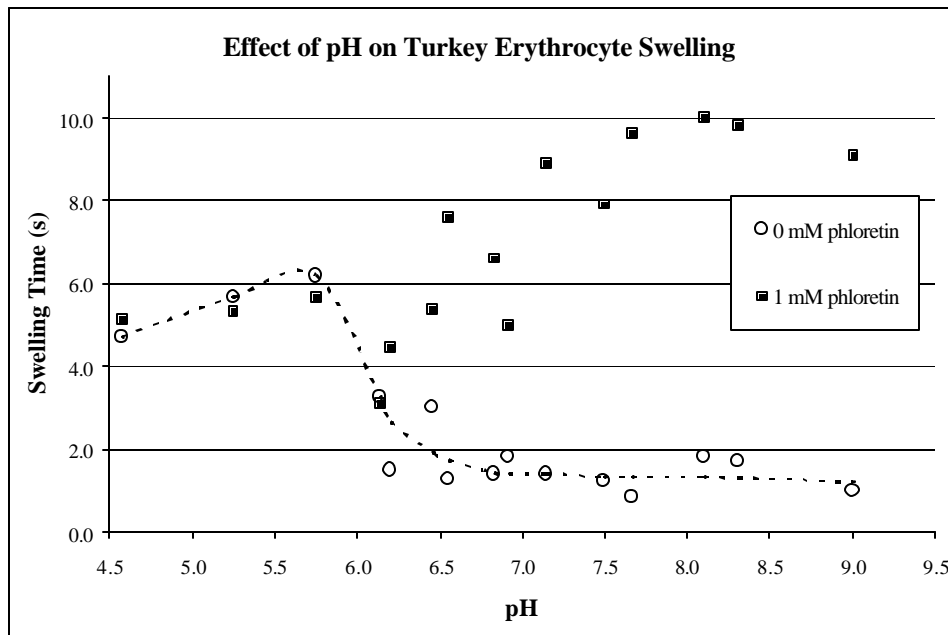


Figure. 4 - Phloretin Inhibitory Effect in Different pHs of Turkey Erythrocytes. The Swelling time is the time it takes the cells to swell from elliptical to round. Note that starting from about pH 6.2 phloretin begins to show powerful inhibitory effect.

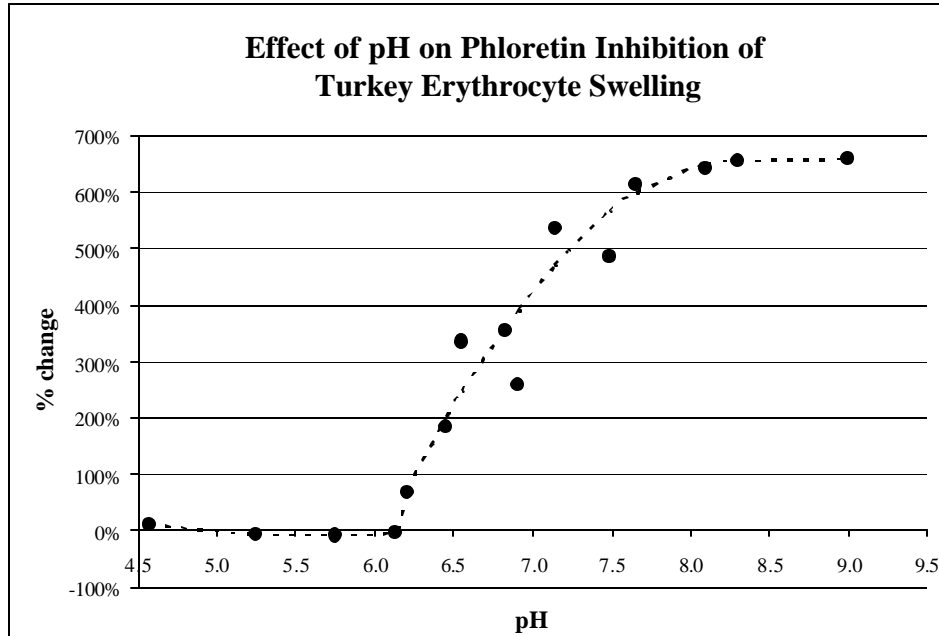


Figure 5 - Phloretin Effect in Different pHs. Phloretin has a concentration of 1 mM in the cell suspension. "% change" is a measure of the phloretin effect on osmotic hemolysis. It was calculated with the formula,

$$\% \text{ change} = \frac{\text{phloretin swelling time} - \text{control swelling time}}{\text{control swelling time}} \times 100\% .$$

This data were collected by digital video microscopy.

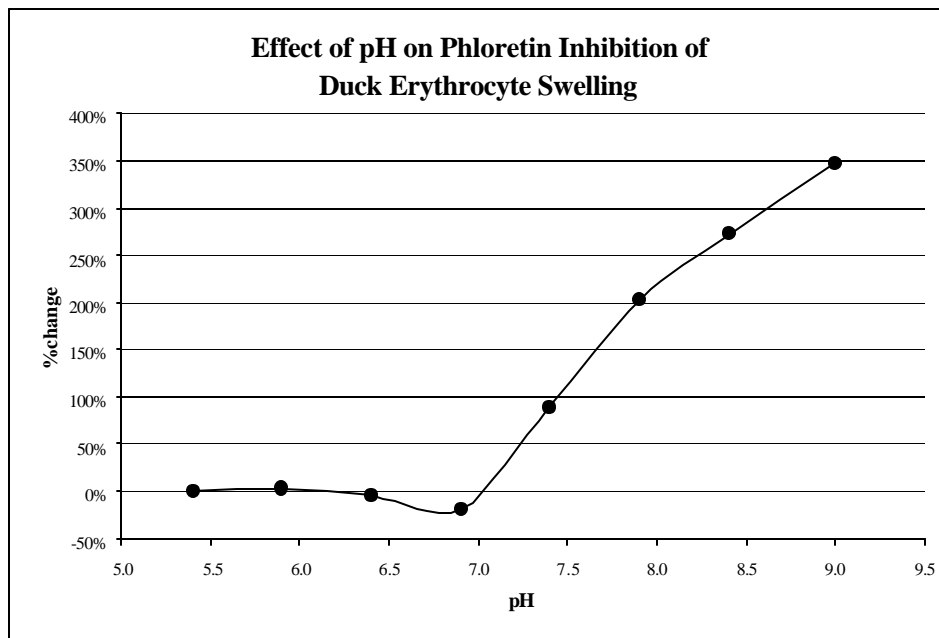


Figure 6 – Effect of pH on Phloretin Inhibition of Duck Erythrocyte Swelling. It was expressed as the percentage of difference in phloretin swelling time over the control swelling time. Data were collected with spectrophotometric method. It also shows that the inhibitory effect of phloretin is pH sensitive.

## 4. Discussion

Figures 1, 2 and 3 prove that phloretin has an inhibitory effect on the swelling time of turkey erythrocytes. This conclusion agrees with other documented literature (Reimer et al, 2003; Miller and Manalis). Figure 4 shows the difference in swelling time of phloretinated and non-phloretinated blood in a range of differing pHs. It can be observed that phloretin does not inhibit at the lower pHs (pH 4.9-6.0); it follows the same general trend as the control (buffered water). However, after pH 6.0, inhibition of phloretin is visible.

In Figures 5 and 6, the pH effect of phloretin inhibition in turkey and duck are displayed. It is shown that they exhibit the same trend. At lower pH, phloretin does not display any inhibitory effect on the swelling time. The inhibitory effect becomes stronger as more basic hemolysing reagents are reached. The graphs above show that phloretin has a pH threshold at 6.2 in turkey measured by digital microscopy and 6.9 in duck measured by the spectrophotometer.

Notice that the shapes of the curves in Figure 5 and Figure 6 are not quite the same. The similar general trend obtained by spectrophotometric method confirmed the data collected by the microscopic method. In comparison, the digital microscopic method is more accurate. This method directly measures the swelling time of each individual cell, as compared to spectrophotometric method which measures the time period of swelling and stress time combined for a population of cells. Swelling time and stress time are the two components of hemolysis. Swelling time is the most accurate measurement of the speed of water transport into the cell. Stress time is the time after the elliptical avian erythrocyte turned spherical until the cell lyses (Jay and Rowlands, 1975). Since the digital microscope method can measure swelling time by itself, it is the better of the two methods.

## 5. Conclusion

The natural product phloretin has been found to have inhibitory effect on water transport in avian erythrocytes (Reimer et al, 2003; Miller and Manalis). A further investigation of pH effect on phloretin inhibition was conducted. It was found that phloretin inhibitory effect is pH dependant. In turkey erythrocytes, phloretin had no effect in pH smaller than 6.2, but as pH went up to the more basic side, 1 mM phloretin showed more and more powerful inhibition of water transport. It lengthened the swelling time of turkey erythrocytes in pH 8.9 almost seven fold. Phloretin also has similar sensitivity to pH in duck erythrocytes, which proposes the hypothesis that phloretin has this pH sensitivity in all avian species, though more erythrocyte samples from different avian species need to be investigated to support this hypothesis.

## 6. References

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