

# **Sugar Substitutes: Packets of Poison?**

**Potential Mutagens in Artificial  
Sweeteners Found Harmless Using the  
Ames Test**

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## **Abstract**

Artificial sweeteners have long been rumored to possess negative side effects, including the risk of cancer. The sugar substitutes Splenda, Equal, and Sweet 'N Low were tested for mutagenicity using a slightly modified version of the Ames test. Our first trial showed 0.25M hydrazine sulfate to be mutagenic. We also tested ethidium bromide, but the results did not indicate mutagenicity as they should have.

## **Introduction**

Since the introduction of artificial sweeteners, mass media outlets have warned the public of potential cancer risks allegedly related to the use of these substances. Artificial sweeteners are found in a variety of goods available on today's market, including breath mints, cereals, chewing gum, cocoa mixes, gelatin desserts, instant teas and coffees, juice beverages, laxatives, multivitamins, milk drinks, shake mixes, soft drinks, tabletop sweeteners, topping mixes, wine coolers, and yogurt. With their promise of weight control, artificial sweeteners appeal to a majority of consumers; in 1987, the Calorie Control Council reported that approximately 40 million Americans used artificial sweeteners for dieting purposes.

Boasting zero calories and a flavor 300 times sweeter than sugar, saccharin became the first artificial sweetener to hit the market in 1879 (Platkin). Saccharin is the sweetener found in Sweet 'N Low and has been shown to cause bladder cancer in rats when fed to them in high doses (Diehl). Regarding saccharin, Michael Jacobson, executive director of the Center for Science in the Public Interest, states, "You don't need the artificial sweeteners that could possibly cause cancer – especially since there are others available that are perfectly safe" (Platkin). The question of whether or not saccharin is truly carcinogenic remains a mystery.

The artificial sweetener sold under the brand name Equal contains aspartame, which is also undergoing speculation as a carcinogen: twelve rats developed malignant brain tumors after receiving an aspartame-containing feed for two years (Diehl). However, hundreds of clinical studies around the world and investigations conducted by the Centers for Disease Control indicate there is no evidence of health risk from aspartame. Michael Jacobson says, "Aspartame appears to be safe, but should be retested since it is so widely consumed" (Platkin).

Sucralose, marketed as Splenda, is a chlorinated sucrose derivative. Splenda has been in existence since 1976, but only recently received approval by the U.S. Food & Drug Administration (FDA) in 1998 (Platkin). Research performed prior to the approval of Splenda revealed that sucralose causes shrunken thymus glands and enlarged liver and kidneys (Sucralose Toxicity). Due to the relative newness of this sweetener, more studies should be conducted pertaining to the mutagenicity of sucralose.

We used the Ames test to determine the mutagenicity of several artificial sweeteners. The Ames test is a worldwide standard for testing new compounds to determine if they are mutagenic. Developed by Bruce Ames and his colleagues during the 1970s at the University of California at Berkeley, the test originally utilized a mutant strain of histidine dependent *Salmonella typhimurium*. This bacterium carries a defective gene, leaving it unable to synthesize the amino acid histidine from ingredients present in its culture medium. However, this mutation can be reversed, allowing the gene to regain its function. This is known as a back mutation, and these revertants are then once again able to grow on a medium lacking histidine (Kimball). The Ames test involves placing a large number of the *S. typhimurium* bacteria into a petri dish containing a nutrient agar that lacks histidine. A small number of the bacteria will spontaneously revert to his<sup>+</sup>, even without the presence of a mutagen. Counting the number of these revertant

bacterial colonies provides a basis to check the validity of the laboratory procedures to come. Another separate but identical petri dish lacking histidine is then prepared, adding *Salmonella* bacteria in addition to mammalian enzymes required for metabolism (usually rat liver enzymes). Because *S. typhimurium* is a prokaryote and therefore does not provide an accurate model of the human body, adding such enzymes supplies a more realistic measure of the mutagenicity of a substance as applied to mammals. Revertant bacteria appear on the plate and flourish into visible colonies. The number of colonies is counted after the plates have been incubated at 37°C for 48 hours (Felton).

For safety reasons, rather than utilizing *S. typhimurium*, our version of the Ames test used a strain of *Escherichia coli* that cannot metabolize arabinose. The mutant gene that prevents the *E. coli* from producing arabinose renders cells unable to grow unless they are placed in a medium containing food other than arabinose. This variation of the Ames test determines substances that are able to change from ara- to ara+, suddenly gaining the ability to break down arabinose. Because the incorporation of rat liver enzymes complicates our experiment, we did not use them for our tests. Instead, we cut circular wells in the center of our agar plates and filled these holes with each of our test substances. Our number of resulting back mutations were then counted and recorded.

The Ames test does not directly indicate the potential of a substance to cause cancer, but there is a relatively high correlation between mutagen strength and carcinogen strength in rodent studies (Ames Test). While the U.S. Food and Drug Administration (FDA) continues to insist there is no relationship between artificial sweeteners and the occurrence of cancer in humans, many argue otherwise. Our experiment provided further understanding regarding ingredients of artificial sweeteners as potential mutagens.

## **Materials & Methods**

The study was conducted on three artificial sweeteners: Sweet'N Low, Splenda, and Equal. We used the world standard Ames test, with a specific *E. coli*, Rel 606, to test the potential mutagenic affect of these sweeteners. Our *E. coli* strain was provided by Richard Lenski of the microbiology department at Michigan State University.

The agar used for our plates was a DM agar that was slightly modified from Richard Lenski's personal website. Using his ingredients and measurements as a template, we came up with our version of DM agar. Our agar was adjusted to the correct pH of 7.0 and completely sterilized by the autoclave or through filter sterilization depending on what would precipitate when autoclaved. If the solution would precipitate when autoclaved, that part of the solution had to be filter sterilized separately and added to the autoclaved part later. After completely sterilizing the agar a sterile vitamin solution was added to the total solution. Now we were able to pure approximately 100 plates which were used by both groups in section one.

To have Rel 606 available for cell suspensions we inoculated some from the main plate that was provided to us. Having colonies accessible we could make cell suspensions in sterile saline. We based the turbidity of these suspensions to a McFarland standard. The McFarland standard was made by adding 5 $\mu$ l of BeCl<sub>2</sub> to a test tube containing 995 $\mu$ l of H<sub>2</sub>SO<sub>4</sub>.

Using fresh DM plates we had to punch holes in the center of the plate. These holes are where we will eventually add our potential mutagens. To produce these holes we had to flame the top of a micropipette and while it was still hot push down into the center of the plate. We used a sterile micropipette tip to remove the piece of agar remaining in the hole. This technique was used for all plates.

The first technique used for spreading of the bacteria was with a Q-tip® dipped into the cell suspension then spread onto the plate. Using this method we did not obtain very thick lawns, therefore, we decided to utilize the “hockey stick” method. We spread 75µl of cell suspension on the plates and using the hockey sticks to disperse the cells we were able to grow better lawns that were more evenly distributed on the agar medium compared to the swabbing method. If this method is used the cells have to be spread before you punch holes in the agar medium. After the cells were added we added approximately 50µl of our positive control, 0.25M hydrazine sulfate, and all three artificial sweeteners. For the negative control we left the holes empty. These plates were then placed agar side down in the 37°C incubator until sufficient growth was seen. There was no set incubation time.

### **Result Figures**

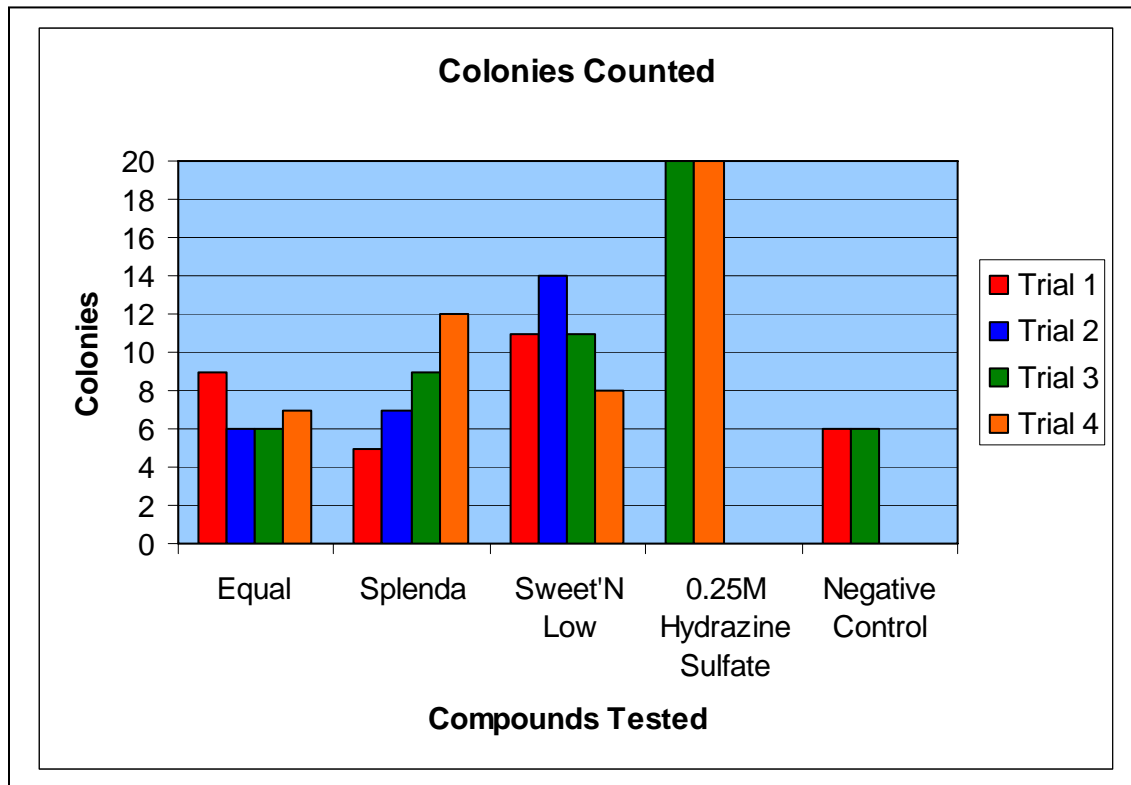


Figure 1: The number of colonies counted on each plate during each trial ran. The hydrazine sulfate had more colonies than twenty which is seen on the graph. The actual number of colonies growing on these plates were uncountable. See figure 2 below. Ethidium bromide is not seen here because we did not use it to interpret our results.



Figure 2: Positive control which was 0.25M hydrazine sulfate. This is exactly what is supposed to be seen as a positive control. The colonies growing on the outer edge of the plate are most likely spontaneous mutations not effected by the hydrazine sulfate

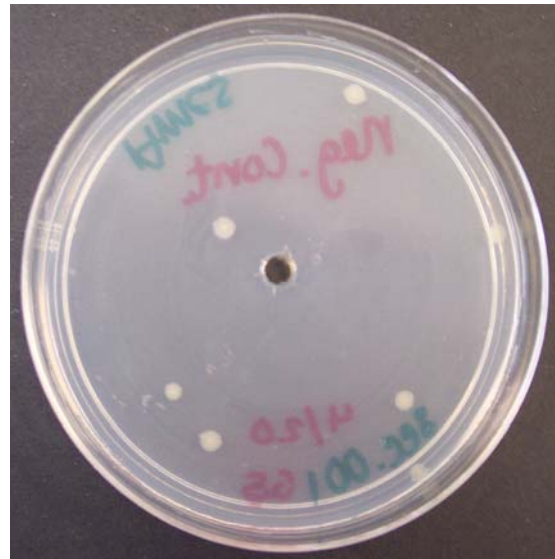


Figure 4: Negative control which resembles spontaneous back mutations that are happening naturally with no mutagen present. This plate contains six mutations.

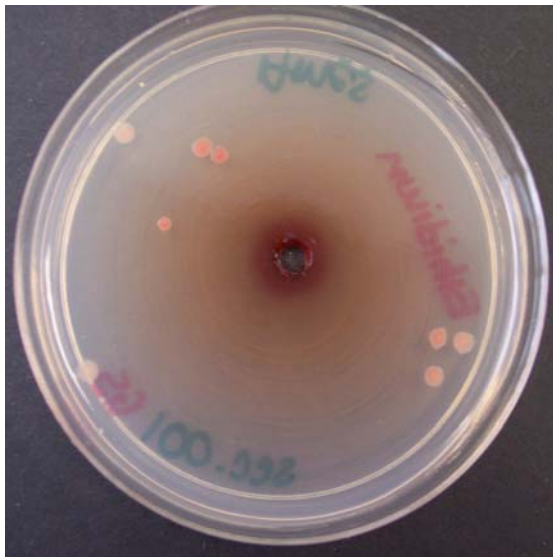


Figure 3: Ethidium bromide which turned out to be a false positive control. You can also see that this plate also contains eight spontaneous back mutations that corresponds to our negative control.

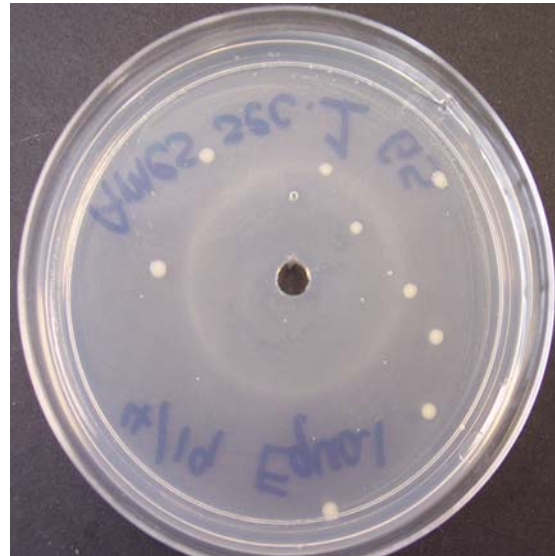


Figure 5: Equal which is seen to contain only spontaneous mutations and no mutations caused by the Equal itself.

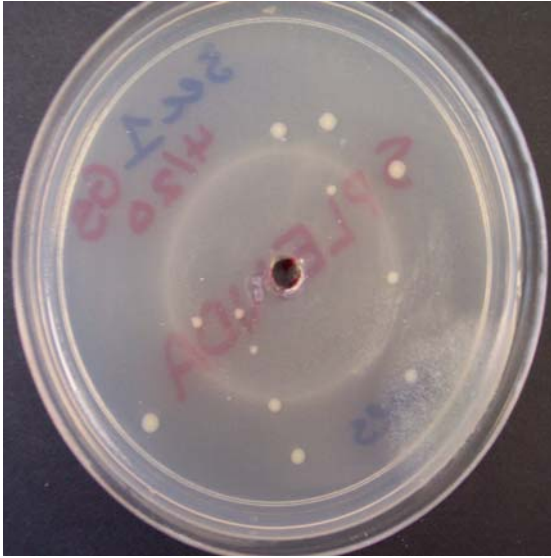


Figure 6: Splenda which contains only spontaneous back mutations and no mutations caused by the Splenda itself.

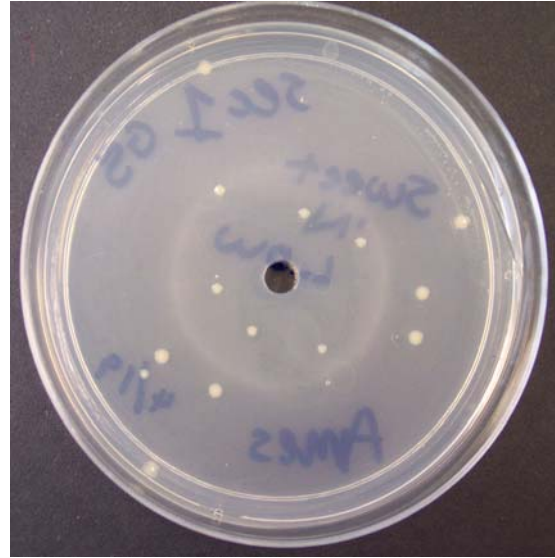


Figure 7: Sweet'N Low which contains only spontaneous back mutations and no mutations caused by the Sweet'N Low itself.

## Results

Using an *E. coli* strain that was defective in the arabinose operon and DNA repair, we tested for the mutagenicity of three artificial sweeteners. We ran four trials, the first and second with ethidium bromide as our positive control and the third and fourth trials with hydrazine sulfate as our positive control. Ethidium bromide (figure 3) did not give a correct positive control possibly for reasons that are stated in our discussion. Therefore all of our results were compared to the hydrazine sulfate (figure 2) which gave a perfect positive control. By looking at these two figures you will be able to notice why ethidium bromide was not considered as a positive control in this experiment. Our negatives for each trial contained Rel 606 solution but nothing was pipetted into the hole. We used this negative control technique to understand the natural spontaneous mutation rate. From our two negative plates we obtained good results and each plate had six naturally occurring mutations (figure 1). We decided not to run anymore negative controls because we obtained corresponding results from these to plates. On one of our

negative plates we did see contamination possible from an airborne particle, however, this colony was not counted for obvious reasons in our data in figure 1.

For the first and second trials we swabbed the solution of Rel 606 and sterile saline on the agar using the McFarland standard as a guide for turbidity. For the third trial we pipetted 50  $\mu$ l of Rel 606 solution that was more turbid than the solution from trials one and two and spread it using the hockey stick technique. In the fourth trial, 75  $\mu$ l of the more turbid solution was spread using the hockey stick method. We used these different methods because we were having trouble obtaining a full lawn throughout the plate. However, the plates from trials one and two which mostly likely had the least number of cells plated did turn out fine when compared with trials three and four. These plates did grow slower than the plates from trials three and four and were placed in the incubator longer. The plates from trials three and four had more cells plated to obtain a thicker more evenly spread lawn. We did this to make sure that we were not getting uneven coverage on the plates, which would give bad results if we did not address this problem.

After we allowed all plates time to grow we found that the number of cells plated did not have an effect on our results. All colonies growing on our artificial plates were seen to only be spontaneous mutations occurring naturally. Using figure 1 as a reference we could see that the number of colonies counted did statistically agree with the number of colonies counted on our negative controls. We did not use a statistical test to show this because we could decide for ourselves that none was needed to interpret our results. Therefore in conclusion we said that Sweet’N Low, Splenda, and Equal are not mutagenic compounds.

## Discussion

The purpose of our experiment was to determine if artificial sweeteners mutated *E. coli* that was unable to metabolize arabinose into being able to metabolize arabinose. Any mutations that we did see had to be classified as either back mutations (the sign of a mutagen), or spontaneous mutations (mutations that occur naturally). Results showed hydrazine sulfate caused mutations in *E. coli* cells after it was absorbed into the agar. Ethidium bromide showed some mutations, but they were classified as spontaneous mutations and thus disregarded. All of our artificial sweeteners also showed spontaneous mutations and were decided not to be mutagenic.

We see from figure 1 that all four trials yielded a consistent number of mutations, even though trials one and two were plated eight days before trials three and four. We determined that the reason the results from trials three and four were received faster than those from one and two was because of the turbidity of our McFarland standard. In trials one and two our Rel 606 cell suspension matched that of a McFarland standard containing 5 $\mu$ l of BeCl<sub>2</sub> and 995 $\mu$ l of H<sub>2</sub>SO<sub>4</sub>. This solution proved not to be turbid enough so we added more BeCl<sub>2</sub> for trials three and four and matched our Rel 606 solution to this. Also for trials one and two we swabbed the Rel 606 on rather than pipetting it on and you can never be sure with the swab how many cells you are actually plating. Based on our results it can be assumed that the more cells you place on the plate to be tested, the faster your results are going to come back. This was shown to be the case after we increased the turbidity of the cell suspension. With fewer cells it might take longer to utilize the small amount of glucose provided in the agar to start a lawn before any mutations become necessary.

We knew from Dr. Urbance that ethidium bromide was a known mutagen. The fact that it did not give us results that showed it to be mutagenic was confusing. It can only be imagined that it is so mutagenic, that it killed all the *E. coli* that came into contact with it. Spontaneous mutations were found but only in the area around the outside where the concentration of ethidium bromide must have been too low to kill off any *E. coli*. The concentration in the ring was too strong to allow any back mutations to live.

We know that since the amount of colonies on the artificial sweeteners plates were so similar to those on the negative control plate that they are not mutagenic. It was unnecessary to run a statistical test seeing that the difference in the numbers of mutations between our positive control and negative control were so great and the artificial sweeteners results are almost identical to those of the negative control. We can conclude from these similarities that the artificial sweeteners are not mutagenic.

**Works Cited**

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