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ABSTRACT

This publication is the sixth of a series of seven supplementary investigative materials for use in secondary science classes providing up-to-date research-related investigations. This unit is structured for grade levels 10 through 12. It is concerned with the crystallization of an enzyme, lysozyme, from egg white. The first part of this guide provides the teacher with: (1) materials needed; (2) suggestions to facilitate classroom use for the investigation; and (3) suggested readings. The second part provides students with background information and one major investigation - crystallization of lysozyme. The investigation consists of: (1) materials needed for a four-student team; (2) procedures; (3) questions for thought; (4) extending the investigation; and (5) suggested readings. (HM)

science study aids

6

lysozyme:
the cooperative enzyme

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TO THE TEACHER

This Science Study Aid (SSA) is concerned with the crystall.zation of an enzyme, lysozyme, from hen egg white. The SSA provides students with information on the history and nature of the enzyme as well as the procedure for insolating it from egg white. There is one major Investigation and eight supplementary Investigations for the student. In this "To the Teacher" section, suggestions to facilitate classroom use are provided for each of the Investigations.

Lysozyme: The Cooperative Enzyme is structured for grade levels 10 through 12. It is designed to stimulate creative thinking and to act as a focal point for class or group discussion. This SSA is an appropriate supplement to general science courses. Also, it is readily adaptable to biology and chemistry classes.

INVESTIGATION 1

Part A

Lysozyme is crystallized from a seeded egg white solution.

Steps 2 and 3 require the use of a blender (kitchen or laboratory type) equipped with a low speed. During the homogenization of the egg white (Step 2) and the mixing of the salt (Step 3), air must not mix with or be whipped into the egg white solution. To keep air out, place a glass beaker or jar about one inch under the surface of the egg white while it is being blended. Maintain a clearance of a few centimeters from the inside of the blender. This will prevent a vortex from forming which would introduce air into the egg white.

In Step 3, students are asked to check the pH of the egg white solution with indicator

MATERIALS LIST

For your convenience, the materials needed to perform all the Investigations in this Science Study Aid are listed below. The following list gives the quantities needed for each two-student team:

STUDENT MATERIALS

(Appropriate size household glasses, baby food jars, etc., can be used instead of laboratory beakers.)

1 250 ml beaker
2 150 ml beakers
1 50 ml beaker
6 chicken eggs
1 glass rod
1 balance
sodium bicarbonate (baking soda)*
distilled white vinegar*
pH indicator paper (one 1-inch long strip)

TEACHER MATERIALS

1 gm isoelectric lysozyme
1 blender
1 centrifuge (several pairs of lady's stockings or pantyhose may be substituted -- see page 4, this section)
NaCl - sodium chloride

OPTIONAL

5 gm Micrococcus lysodeikticus
59.2 ml 1N NaOH (sodium hydroxide)
13.6 gm KH_2PO_4 (monobasic potassium phosphate)
sodium bromide *
hydrobromic acid *
sodium nitrate *
hydronitric acid *

* Quantity needed depends on volume of egg white.

paper (pHydrion or Fishers Alkacid). Most commercially purchased eggs will have a pH of 9.5, the most suitable for the crystallization of lysozyme. If your solution is not in the range of 9.0 - 9.5, adjust it accordingly with dilute sodium hydroxide or vinegar. This solution is then seeded with a minute amount of isoelectric lysozyme crystal. The lysozyme crystallized from the egg white in Investigation 1 may, in turn, be used to seed the egg white preparation in further Investigations. If you use your own seed crystals in further Investigations, recrystallize them 2 to 3 times following the procedure given in Part B, Steps 1-4. The more lysozyme is recrystallized, the purer it becomes.

Isoelectric lysozyme may be obtained from:

Worthington Biochemical Corporation
Freehold, New Jersey 07728

Order: Lysozyme, Code LY

Dust or any other small particles in the seeded egg white will prevent growth of larger lysozyme crystals by favoring the formation of tiny crystals. This cleanliness is especially important in the recrystallization of lysozyme.

Part B

Steps 1 and 3 involve the use of a centrifuge. If your laboratory is not so equipped, the crystallized lysozyme can be separated from the egg white with a section of ladies' hose. This technique is less reliable and will not produce as much lysozyme. We have found the toe to be most satisfactory. Stretch the clean, dry hose over a glass jar or beaker, allowing part of it to sag inward, forming a small cup. Next, pour the egg white into the hose cup and allow it to filter through. The lysozyme crystals, which are bound up with the egg white chalazae and mucin, will collect on the top of the panty hose. This material, to be used in Step 2, can be removed from the panty hose with a small knife. The same hose can be used in Step 3.

The largest crystals will form in an uncontaminated solution of the dissolved lysozyme at room temperature.

The time required for lysozyme to crystallize out of solution in Parts A & B can range from 3 to 7 days each time. Schedule your class accordingly.

EXTENDING THE INVESTIGATION

When your students have successfully completed each part of Investigation 1, you can direct their attention to the Investigations in this section. Each of the Investigations follows from Investigation 1, Parts A and B.

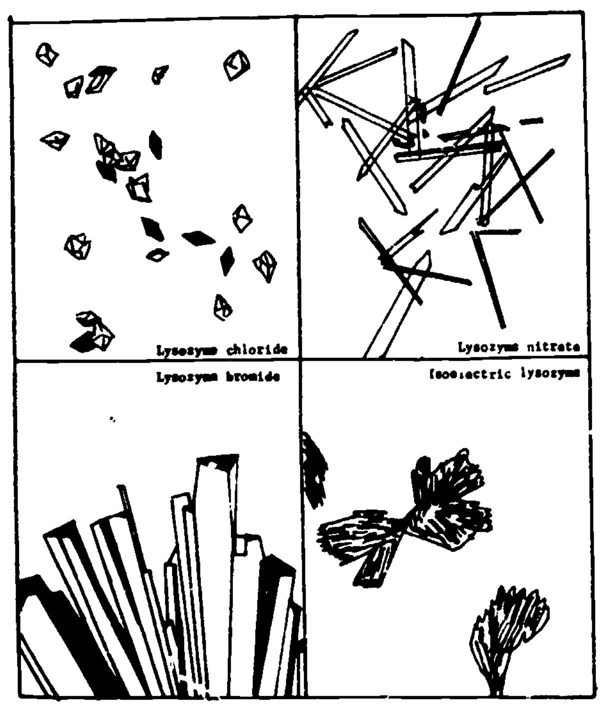

FIG. 1

Part B

The first Investigation suggests using different inorganic salts to crystallize various salts of lysozyme from egg white. The procedures for these crystallizations are the same as those in Investigation 1 with one exception: in Step 4, Part B, substitute 5% of the selected salt for sodium bicarbonate. It is also recommended that the acid of the salts (1 part acid to 10 parts water) be used rather than vinegar (Part B, Step 2). Use hydrobromic acid with sodium bromide and hydronitric acid with sodium nitrate. (See Fig. 1 for sketches of the different cyrstalline forms.)

The third Investigation requires the use of the bacterium Micrococcus lysodeikticus. This may be purchased from the Worthington Biochemical Corporation, Freehold, New Jersey 07728.

The solution in which the bacterium is suspended should be buffered. Prepare the buffer according to the following directions:

1. In a one-litre beaker, add 13.6 gm of KH_2PO_4 (monobasic potassium phosphate) to 59.2 ml of 1N NaOH.
2. Add a small amount of water to completely dissolve the KH_2PO_4 solution.
3. Add water to bring the solution up to about 1 litre.

Pour this buffer solution into individual test tubes. Add enough bacteria to lightly cloud the solution. The introduction of crystalline lysozyme will lyse the bacterial cell walls, causing the solution to clear. The rate and degree of lysing depends on several factors including the amount of lysozyme, the concentration of both bacteria and enzyme, and the temperature of the buffer.

SUGGESTED READING

Isolation of Lysozyme from Egg White, Alderton, G., Ward, W. H., and Fevold, H. L., Journal of Biological Chemistry, Vol. 157, No. 1, p. 43 (January 1945). This article is the first of several scientific descriptions of the procedures and results of Mr. Alderton's method for the crystallization of lysozyme from egg white.

Direct Crystallization of Lysozyme from Egg White and Some Crystalline Salts of Lysozyme, Alderton, G., and Fevold, H. L., Journal of Biological Chemistry, Vol. 164, No. 1, p. 1 (July 1946). An excellent article describing the direct crystallization of lysozyme with several excellent photographs of different salts of the enzyme.

TO THE STUDENT

When a bicycle mechanic repairs a ten-speed derailleur or when an agricultural scientist investigates the growth pattern of Mexican wheat, several needs are shared in common by both specialists. For example, each needs knowledge of the subject with which he is working. Can you think of other common requirements? How about tools? Both the mechanic and the scientist need special tools. The mechanic requires spanner wrenches, taps, dies, rivet extractors, etc. The scientist needs such equipment as a scanning electron microscope, Pyrex glassware, and pH meters. These tools are man-made, that is, they are designed and built by man. But not all the tools a scientist uses are man-made. Enzymes are an example.

Enzymes are organic substances that are formed from protein molecules. They are found in the protoplasm of cells. They are involved in many biological reactions, from helping with the growth of plants to assisting with digestion of food in our intestinal tracts. When isolated and used in the laboratory, enzymes enable scientists to influence many chemical reactions. For example, the rate at which dates mature or the degree of heat which bacterial spores resist can be influenced by enzymes. Lysozyme is one of the many enzymes used as research tools by scientists.

In 1922, Alexander Fleming, the English scientist who discovered penicillin, took a sample of nasal mucus from a patient who had a cold. He placed a small drop of this nasal mucus on a culture of bacteria with which he was experimenting. The next day he observed that the bacteria which had not come in contact with the mucus was thriving, but that the bacteria which had come in contact had dissolved. Fleming later associated the lysing (dissolving) of the bacteria with an enzyme in the nasal mucus. He appropriately named it lysozyme. The ensyme subsequently was discovered in many tissues and secretions of the human body as well as in many plants. Its highest concentration was found in chicken egg white.

Today, the detection of high levels of lysozyme in blood and urine aids in diagnosing the presence of tuberculosis, certain types of leukemia and cancer, and severe infections which cause white blood cell destruction.

In research laboratories such as those at ARS' Western Regional Research Center, lysozyme also is used to investigate the structure and properties of bacteria. ARS researchers use the enzyme to dissolve cell walls in susceptible bacteria, thus exposing the cell body or cytoplasm. This enables them to investigate the characteristics of the bacteria and their resistance to heat, for example. Other agents cannot lyse the cell wall without also destroying the cytoplasm.

Because of the wide application of lysozyme as a research and pharmacological tool, there is a significant need for the purified form. Isolation of enzymes generally is a lengthy and costly operation. Their availability in pure form often depends on complicated techniques requiring sophisticated equipment. In 1944, Gordon Alderton, an ARS scientist at WRRC, made a significant discovery. He found that high yields of lysozyme could be recovered from hen egg white using a simple and inexpensive procedure. His method has made large quantities of this valuable research tool available to scientists throughout the world.

In this SSA, you and your classmates will crystallize lysozyme from egg white using the process developed by Mr. Alderton. After isolating the enzyme, you will be able to use your new research tool and knowledge to design and carry out additional investigations.

INVESTIGATION I

Crystallization of Lysozyme

In this Investigation, you will crystallize lysozyme directly from egg white. The eggs can be purchased from your local supermarket. In order for the crystals to form, the egg white usually must be seeded with a small quantity of isoelectric lysozyme. ("Isoelectric" refers to the free base of the lysozyme molecule. The term means that the molecular structure of the lysozyme crystal which will form is not determined by the salts present in the egg white solution but rather by its own molecular structure.) The results of your investigation also will be isoelectric crystals. You may use them to seed egg white for further investigations of your own design.

MATERIALS PER STUDENT TEAM

Parts A and B

1 250 ml beaker
1 glass rod
1 balance
pH indicator paper

Part A

2 150 ml beakers
6 chicken eggs
NaCl *
Isoelectric lysozyme

Part B

1 10X hand lens
1 12 cm sq. section of wax paper
sodium bicarbonate (baking soda) *
distilled white vinegar *

* Quantity depends on volume of egg white

PRELIMINARY PROCEDURE

1. Before you begin the activities in this Investigation, carefully read through the procedures.
2. It is important that all your equipment is clean -- dust-free and uncontaminated.

PROCEDURE

Part A

1. Carefully separate the egg whites from their yolks. Allow no portion of the yolk to mix with the egg white. Put the white in one 150 ml beaker and the yolk in the other.
2. Pour the entire class's egg whites into the blender. Homogenize the egg whites at a very low speed. BE SURE that no air enters the mixture during the blending (your teacher will tell you how). Air will precipitate egg proteins and prevent recovery of the crystalline lysozyme.
3. Add 5% NaCl (1 gm NaCl to every 20 ml solution) to the homogenized egg white. Blend at a low speed, again insuring that no air enters the mixture. After this final blending, check the solution's pH. it should be about 9.5. If it is not, adjust accordingly with vinegar or sodium hydroxide.
4. Now pour equal amounts of the egg white solution into each team's 250 ml beaker.
5. Gently stir a small amount of crystallized isoelectric lysozyme, equal to the amount which could fit on the point of a ball-point pen, into the mixture of each of the beakers. Two to five gentle stirrings with a CLEAN glass rod will be sufficient.
6. Place the seeded egg white mixture in a refrigerator or in crushed ice (where the temperature is about 4° C.). Stir solutions 2 to 3 times a day rapidly. As the lysozyme crystallizes (3 to 5 days), it will become bound up with the egg white's chalazae and mucin, giving it the appearance of thick snowflakes when viewed under a magnifying lens.

QUESTIONS FOR THOUGHT

1. You supply lysozyme to hospitals and research laboratories. What ideal conditions would you want to establish for the first crystallization of the enzyme?
2. Why do you think it is necessary for all lab equipment to be dust-free when growing crystals?

EXTENDING THE INVESTIGATION

1. Does agitation effect how quickly lysozyme precipitates out of solution? Design your own investigation and find out.
2. Determine the effect of higher

temperatures on the crystallization of lysozyme.

3. What is the effect of the NaCl concentration? What happens when you vary the concentration of sodium chloride (Step 3 in Investigation 1) to 1%? to 8%?
4. Prepare an investigation to determine if crystallization can be induced spontaneously in egg white without seeding with isoelectric lysozyme. Can crystallization be induced by seeding the prepared egg white with any of the other salts of lysozyme?

Part B

1. When no more crystals seem to be appearing (3 to 5 days), separate them from the egg white by centrifuging. If there is no centrifuge, your teacher will describe an alternate method.
2. Take the solid, white material from Step 6 and add it to 20 ml of a distilled white vinegar-water solution (1 part vinegar to 10 parts water). Stir until the crystals dissolve, leaving only the mucin and chalazae.
3. Separate the dissolved lysozyme from the chalazae and mucin by centrifuging or according to your teacher's instructions.
4. Take the filtrate (liquid portion) from Step 3 and slowly add 5% dry sodium bicarbonate while stirring gently. This will adjust the pH to 8. Check with indicator paper. Make sure the pH is 8. Now cover with wax paper. Place half of the class's solution at room temperature and half at 4° C. At which temperature do you predict the crystals will appear more rapidly? At which will they be larger? Write all predictions in your notebook.
5. Once the crystals form (3 to 5 days), observe them with a 10X hand lens. Sketch the crystal shapes and configurations in your notebook.

QUESTIONS FOR THOUGHT

1. You prepare lysozyme crystals for optical and crystallographic examination. The larger the crystal, the more convenient it is for these studies. What conditions are best suited for the growth of large crystals?

EXTENDING THE INVESTIGATION

1. ARS scientists have succeeded in crystallizing lysozyme using several different salts -- for example, sodium bromide (lysozyme bromide), sodium chloride (lysozyme chloride), and sodium nitrate (lysozyme nitrate). In its isoelectric form, the molecular structure of lysozyme itself determines its crystalline structure. When salts are used, they alter the structure of crystalline lysozyme according to their own molecular properties. By substituting 5% of the selected salt for the 5% sodium bicarbonate used in Step 4, Part B, observe the crystalline shapes of the different salts of lysozyme.
2. In Investigation 1, you crystallized isoelectric lysozyme from a solution with a pH of 8. Other salts of lysozyme are crystallized in solutions with a pH range of 4 to 5. Design an investigation to determine the effect of different pH's on the structure of the different crystalline forms of lysozyme.
3. Lysozyme is an extremely useful scientific tool because of its lysing effect on certain bacteria. Prepare suspensions of <u>Micrococcus lysodeikticus</u> according to your teacher's directions. Determine what concentrations of isoelectric lysozyme are most effective in lysing the bacteria. The more lysozyme is recrystallized (Steps 2-4, Part B), the purer it becomes. Is the second recrystallization of lysozyme as effective as the third in lysing <u>Micrococcus lysodeikticus</u>?

4. A knowledge of the optical and crystallographic properties of crystalline proteins such as lysozyme is desirable because it helps us understand their complex structure and the manner in which they function. One of the first measurements a crystallographer takes is the refractive index of the crystal. Design an investigation which will enable you to determine the refractive index of lysozyme chloride. See Practical Refractometry and Handbook of Chemical Microscopy in "Suggested Reading."

SUGGESTED READING

Flemings Lysozyme, Acker, Robert F., and Hartsell, S.E., Scientific American, p. 132 (June 1960). A well-written article describing the lysing effect of lysozyme on Micrococcus lysodeikticus. An excellent series of photographs accompanies the article.

Practical Refractometry, Allen, R. M., R. P. Cargille Laboratories, Inc., 33 Village Park Road, Cedar Grove, N. J., 1962, 2nd ed. An excellent lab manual for determining the refractive indices of different materials.

Handbook of Chemical Microscopy, Chamot, E. M., and Mason, C. W., Vol. 1, 3rd ed., John Wiley & Sons, Inc., New York, N. Y., 1958. Chapter 11, "Determination of Refractive Indices of Liquids and Solids," is an excellent source of information. On page 331 is a list of common liquid standards for refractive index determinations by immersion methods.

The Three-Dimensional Structure of an Enzyme Molecule, Phillips, David C., Scientific American, p. 78, November 1966. The first accurate description of the arrangement of atoms in an enzyme molecule. The enzyme is lysozyme. The article also explains how lysozyme lyses bacterial cell walls. Excellent reading.

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are a series of supplementary investigative materials for use in secondary science classes, grades 7 - 12. The materials are based on federal and private research programs. They are written by secondary science teachers working with scientists at research facilities throughout the country. Before being published, they are tested in the laboratory and in classrooms of cooperating teachers.

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