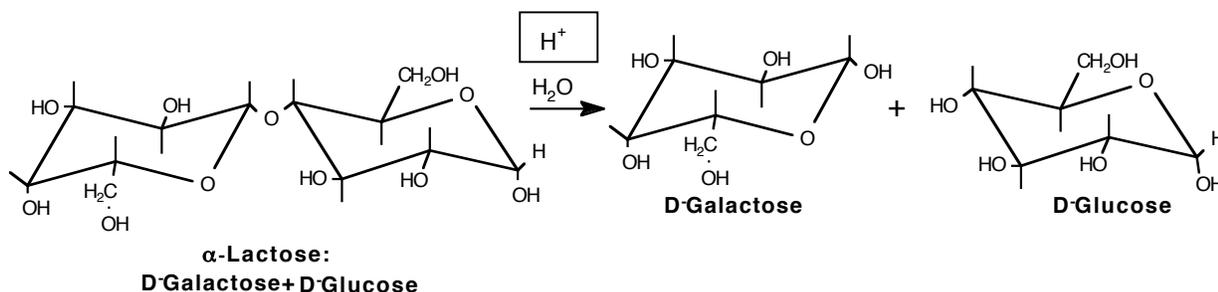


# Isolation of Casein, Lactose, and Albumin from Milk

Adapted by R. Minard (Penn State Univ.) from *Introduction to Organic Laboratory Techniques: A Microscale Approach*, Pavia, Lampman, Kriz & Engel, Saunders, 1990. Revised 3/20/2000

## PreLab Exercise:

Draw a mechanism for the acid-catalyzed hydrolysis of the acetal bond in lactose; see reaction below. Hint: your textbook may be of some help; first identify the functional group that is undergoing a reaction and consider the catalyzed used.



## Introduction: The Chemistry of Milk

Milk is a food of exceptional interest. Not only is milk an excellent food for the very young, but humans have also adapted milk, specifically cow's milk, as a food substance for persons of all ages. Many specialized milk products like cheese, yogurt, butter, and ice cream are staples of our diet.

Milk is probably the most nutritionally-complete food that can be found in nature. This property is important for milk, since it is the only food young mammals consume in the nutritionally significant weeks following birth. Whole milk contains vitamins (principally thiamine, riboflavin, pantothenic acid, and vitamins A, D, and K), minerals (calcium, potassium, sodium, phosphorus, and trace metals), proteins (which include all the essential amino acids), carbohydrates (chiefly lactose), and lipids (fats). The only important elements in which milk is seriously deficient are iron and Vitamin C. Infants are usually born with a storage supply of iron large enough to meet their needs for several weeks. Vitamin C is easily secured through an orange juice supplement. The average composition of the milk of each of several mammals is summarized in the accompanying table.

Average Percentage Composition of Milk from Various Mammals

	Cow	Human	Goat	Sheep	Horse
Water	87.1	87.4	87.0	82.6	90.6
Protein	3.4	1.4	3.3	5.5	2.0
Fats	3.9	4.0	4.2	6.5	1.1
Carbohydrates	4.9	7.0	4.8	4.5	5.9
Minerals	0.7	0.2	0.7	0.9	0.4

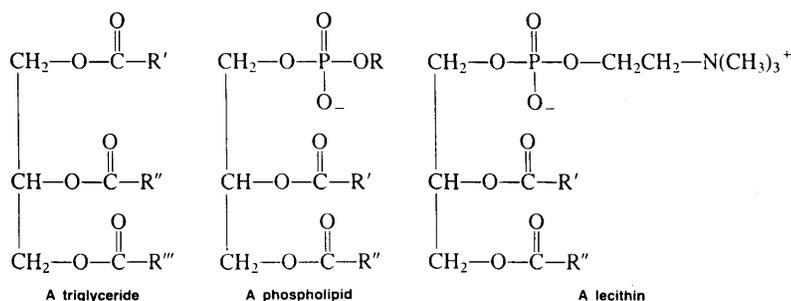
## FATS

Whole milk is an oil-water type of emulsion, containing about 4% fat dispersed as very small (5-10 microns in diameter) globules. The globules are so small that a drop of milk contains about a million of them. Because the fat in milk is so finely dispersed, it is digested more easily than fat from any other source. The fat emulsion is stabilized to some extent by complex phospholipids and proteins that are adsorbed on the surfaces of the globules. The fat globules, which are lighter than water, coalesce on standing and eventually rise to the surface of the milk, forming a layer of **cream**. Since vitamins A and

D are fat-soluble vitamins, they are carried to the surface with the cream. Commercially, the cream is often removed by centrifugation and skimming and is either diluted to form coffee cream ("half and half"), sold as **whipping cream**, converted to **butter**, or converted to ice cream. The milk that remains is called skimmed **milk**. Skimmed milk, except for lacking the fats and vitamins A and D, has approximately the same composition as whole milk. If milk is **homogenized**, its fatty content will not separate. Milk is homogenized by forcing it through a small hole. This breaks up the fat globules and reduces their size to about 1 to 2 microns in diameter.

The fats in milk are primarily triglycerides; about two thirds of all the fatty acids in milk are saturated, and about one third are unsaturated. Milk is unusual in that about 12% of the fatty acids are short-chain fatty acids (C<sub>2</sub>-C<sub>10</sub>) like butyric, caproic, and caprylic acids.

Additional lipids (fats and oils) in milk include small amounts of cholesterol, phospholipids, and lecithins (phospholipids conjugated with choline). The structures of phospholipids and lecithins are shown. The phospholipids help to stabilize the whole milk emulsion; the phosphate groups help to achieve partial water solubility for the fat globules. All the fat can be removed from milk by extraction with petroleum ether or a similar organic solvent.



## PROTEINS

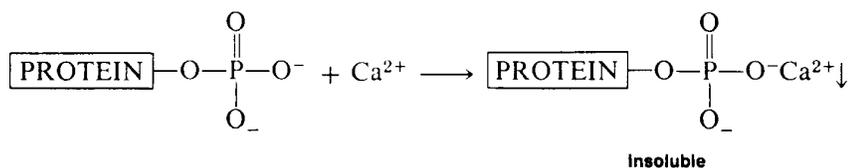
Proteins may be classified broadly in two general categories: fibrous and globular. Globular proteins are those that tend to fold back on themselves into compact units that approach nearly spheroidal shapes. These types of proteins do not form intermolecular interactions between protein units (H bonds and so on) as fibrous proteins do, and they are more easily solubilized as colloidal suspensions. There are three kinds of proteins in milk: caseins, lactalbumins, and lactoglobulins. All are globular.

**Casein** is a phosphoprotein, which has phosphate groups are attached to some of the amino acid side-chains. These are attached mainly to the hydroxyl groups of the serine and threonine moieties. Actually, casein is a mixture of at least three similar proteins, which differ primarily in molecular weight and amount of phosphorus they contain (number of phosphate groups).

CASEIN	MW	PHOSPHATE GROUPS/MOLECULE
$\alpha$	27,300	~9
$\beta$	24,100	~4-5
$\kappa$	~8,000	~1.5

Casein exists in milk as the calcium salt, calcium caseinate. This salt has a complex structure. It is composed of  $\alpha$ ,  $\beta$ , and  $\kappa$  caseins which form a **micelle**, or a solubilized unit. Neither the  $\alpha$  nor the  $\beta$  casein is soluble in milk, singly or in combination. If  $\kappa$  casein is added to either one, or to a combination of the two, however, the result is a casein complex that is soluble owing to the formation of the micelle.

A structure proposed for the casein micelle is shown on the following page. The  $\kappa$  casein is thought to stabilize the micelle. Since both  $\alpha$  and  $\beta$  casein are phosphoproteins, they are precipitated by calcium ions.



The  $\kappa$  casein protein, however, has fewer phosphate groups and a high content of carbohydrate bound to it. It is also thought to have all its serine and threonine residues (which have hydroxyl groups), as well as its bound carbohydrates, on only one side of its outer surfaces. This portion of its outer surface is easily solubilized in water since these polar groups are present. The other portion of its surface binds well to the water-insoluble  $\alpha$  and  $\beta$  caseins and solubilizes them by forming a protective colloid or micelle around them. Since the entire outer surface of the micelle can be solubilized in water, the unit is solubilized as a whole, thus bringing the  $\alpha$  and  $\beta$  caseins, as well as  $\kappa$  casein, into solution.

Calcium caseinate has its isoelectric (neutrality) point at pH 4.6. Therefore, it is insoluble in solutions of pH less than 4.6. The pH of milk is about 6.6; therefore casein has a negative charge at this pH and is solubilized as a salt. If acid is added to milk, the negative charges on the outer surface of the micelle are neutralized (the phosphate groups are protonated) and the neutral protein precipitates:



The calcium ions remain in solution. When milk sours, lactic acid is produced by bacterial action (see below), and the consequent lowering of the pH causes the same **clotting** reaction. The isolation of casein from milk will be carried out in this experiment.

The casein in milk can also be clotted by the action of an enzyme called **rennin**. Rennin is found in the fourth stomach of young calves. However, both the nature of the clot and the mechanism of clotting differ when rennin is used. The clot formed using **rennin, calcium paracaseinate**, contains calcium.



Rennin is a hydrolytic enzyme (peptidase) and acts specifically to cleave peptide bonds between phenylalanine and methionine residues. It attacks the  $\kappa$  casein, breaking the peptide chain so as to release a small segment of it. This destroys the water-solubilizing surface of the  $\kappa$  casein, which protects the inner  $\alpha$  and  $\beta$  caseins and causes the entire micelle to precipitate as calcium paracaseinate. Milk can be decalcified by treatment with oxalate ion, which forms an insoluble calcium salt. If the calcium ions are removed from milk, a clot will not be formed when the milk is treated with rennin.

The clot, or curd, formed by the action of rennin is sold commercially as cottage cheese. The liquid remaining is called the whey. The curd can also be used in producing various types of cheese. It is washed, pressed to remove any excess whey, and chopped. After this treatment, it is melted, hardened and ground. The ground curd is then salted, pressed into molds, and set aside to age.

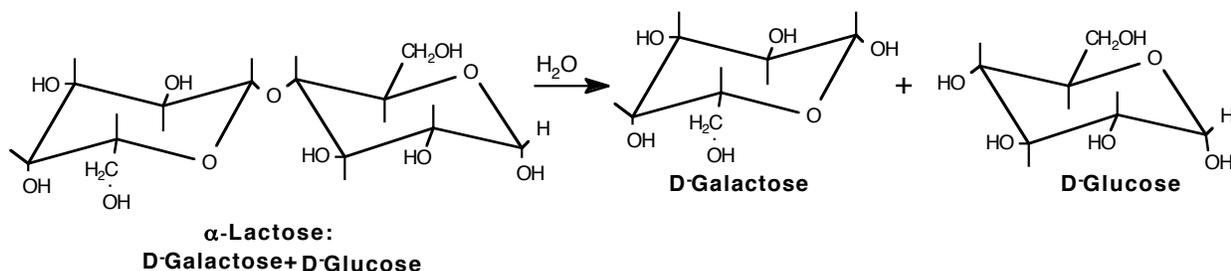
**Albumins** are globular proteins that are soluble in water and in dilute salt solutions. They are, however, denatured and coagulated by heat. The second most abundant protein types in milk are the lactalbumins. Once the caseins have been removed, and the solution has been made acidic, the lactalbumins can be isolated by heating the mixture to precipitate them. The typical albumin has a molecular weight of about 41,000.

A third type of protein in milk is the lactoglobulins. They are present in smaller amounts than the albumins and generally denature and precipitate under the same conditions as the albumins. The lactoglobulins carry the immunological properties of milk. They protect the young mammal until its own immune system has developed.

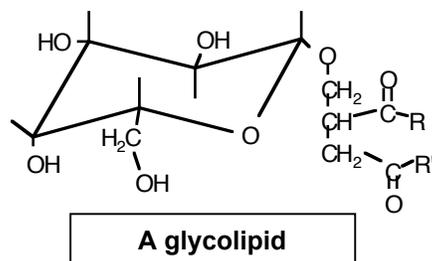
## CARBOHYDRATES

When the fats and the proteins have been removed from milk, the carbohydrates remain, as they are soluble in aqueous solution. The main carbohydrate in milk is **lactose**.

Lactose, a disaccharide, is the only carbohydrate that mammals synthesize. It is synthesized in the mammary glands. Hydrolyzed, it yields one molecule of D-glucose and one of D-galactose.



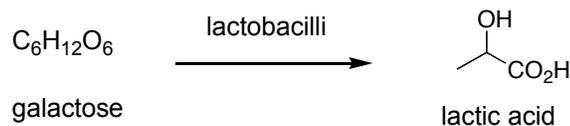
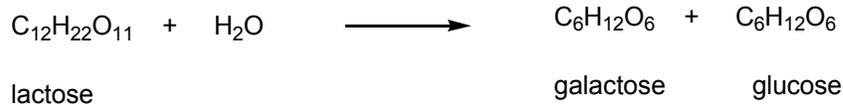
In this process, one molecule of glucose is converted to galactose and joined to another of glucose. The galactose is apparently needed by the developing infant to build brain and nervous tissue. Brain cells contain glycolipids as a part of their structure. A glycolipid is a triglyceride in which one of the fatty acid groups has been replaced by a sugar, in this case galactose. Galactose is more stable (to metabolic oxidation) than glucose and affords a better material for forming structural units in cells.



Although almost all human infants can digest lactose, some adults lose this ability on reaching maturity, since milk is no longer an important part of their diet. An enzyme called lactase is necessary to digest lactose. Lactase is secreted by the cells of the small intestine, and it cleaves lactose into its two component sugars, which are easily digested. Persons lacking the enzyme lactase do not digest lactose properly. As it is poorly absorbed by the small intestine, it remains in the digestive tract, where its osmotic potential causes an influx of water. This results in cramps and diarrhea for the affected

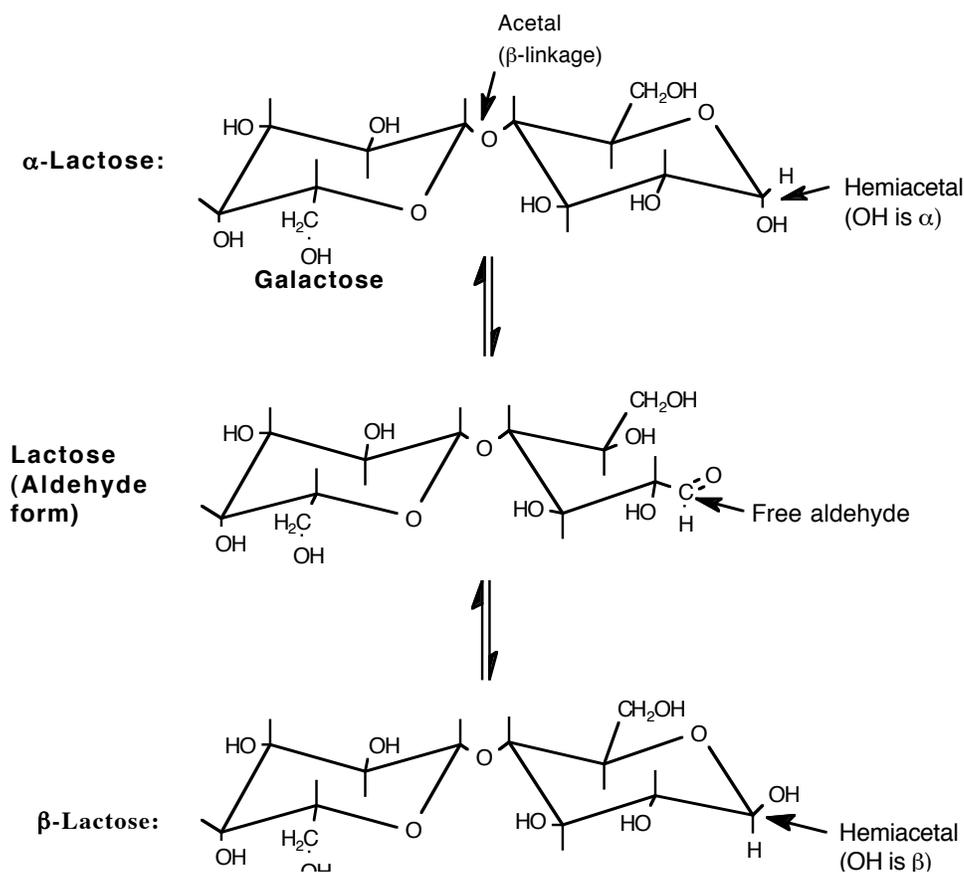
individual. Persons with a lactase deficiency cannot tolerate more than one glass of milk a day. The deficiency is most common among African-Americans and older Caucasians.

When milk is allowed to stand at room temperature, it sours. Many bacteria are present in milk, particularly lactobacilli. These bacteria act on the lactose in milk to produce the sour lactic acid. These microorganisms actually hydrolyze lactose and produce lactic acid only from the galactose portion of the lactose. Since the production of the lactic acid also lowers the pH of the milk, the milk clots when it sours:



Many "cultured" milk products are manufactured by allowing milk to sour before it is processed. For instance, milk or cream is usually allowed to sour somewhat by lactic acid bacteria before it is churned to make butter. The fluid left after the milk is churned is sour and is called buttermilk. Other cultured milk products include sour cream, yogurt, and certain types of cheese.

Lactose is an example of a disaccharide. It is made up of two sugar molecules: galactose and glucose. In the following structures, the galactose portion is on the left and glucose is on the right. Galactose is bonded through an acetal linkage to glucose.



Notice that the glucose portion can exist in one of two isomeric hemiacetal structures:  $\alpha$ -lactose and  $\beta$ -lactose. Glucose can also exist in a free aldehyde form. This aldehyde form (open form) is an intermediate in the equilibration (interconversion) of  $\alpha$ - and  $\beta$ -lactose. Very little of this free aldehyde form exists in the equilibrium mixture. The isomeric  $\alpha$ - and  $\beta$ -lactose are diastereomers because they differ in the configuration at one carbon atom, called the anomeric carbon atom.

The sugar  $\alpha$ -lactose is easily obtainable by crystallization from a water-ethanol mixture at room temperature. On the other hand,  $\beta$ -lactose must be obtained by a more difficult process, which involves crystallization from a concentrated solution of lactose at temperatures about  $93.5^{\circ}\text{C}$ . In the present experiment,  $\alpha$ -lactose is isolated by the simpler experimental procedure indicated above.

$\alpha$ -Lactose undergoes numerous interesting reactions. First,  $\alpha$ -lactose interconverts, via the free aldehyde form, to a large extent, to the  $\beta$ -isomer in aqueous solution. This causes a change in the rotation of polarized light from  $+92.60^{\circ}$  to  $+52.30^{\circ}$  with increasing time. The process that causes the change in optical rotation with time is called **mutarotation**.

### **Polarimetry**

In this experiment, you will study the mutarotation of lactose by polarimetry. The disaccharide  $\alpha$ -lactose, made up of galactose and glucose, can be isolated from milk. As you can see in the structures shown above, the glucose unit can exist in one of two isomeric hemiacetal structures,  $\alpha$ - and  $\beta$ -lactose. These isomers are diastereomers because they differ in configuration at one carbon atom. The glucose part can also exist in a free aldehyde form. This aldehyde form (open form in the equation above) is an intermediate in the equilibration of  $\alpha$ - and  $\beta$ -lactose. Very little of this free aldehyde form exists in the equilibrium mixture.

$\alpha$ -Lactose has a specific rotation at  $20^{\circ}\text{C}$  of  $+92.60$ . However, when it is placed in water, the optical rotation decreases until it reaches an equilibrium value of  $+52.30$ .  $\beta$ -Lactose has a specific rotation of  $+34^{\circ}$ . The optical rotation of  $\beta$ -lactose *increases* in water until it reaches the same equilibrium value obtained for  $\alpha$ -lactose. At the equilibrium point, both the  $\alpha$  and  $\beta$  isomers are present. However, since the equilibrium rotation is closer in value to the initial rotation of  $\beta$ -lactose, the mixture must contain more of this isomer. The process, which results in a change in optical rotation over time to approach an equilibrium value, is called **mutarotation**.

Lactose can be removed from whey by adding ethanol. Lactose is insoluble in ethanol, and when the ethanol is mixed with the aqueous solution, the lactose is forced to crystallize. This is the process used in this experiment to isolate lactose from milk.

### **Isolation of a Protein, Casein, and a Sugar, Lactose, from Milk**

In this experiment, you will separate several of the chemical substances found in milk. First, you will isolate a phosphorus-containing protein, **casein**. The remaining milk mixture will then be used as a source of a sugar,  **$\alpha$ -lactose**. After you isolate the milk sugar, you will make several chemical tests on this material. Fats, which are present in whole milk, are not isolated in this experiment because powdered nonfat milk is used.

First, the casein is precipitated by warming the powdered milk and adding dilute acetic acid. It is

important that the heating not be excessive or the acid too strong, because these conditions also hydrolyze lactose into its components, glucose and galactose. After the casein has been removed, the excess acetic acid is neutralized with calcium carbonate, and the solution is heated to its boiling point to precipitate the initially soluble protein, albumin. The liquid containing the lactose is poured away from the albumin. Alcohol is added to the solution, and any remaining protein is removed by centrifugation.  $\alpha$ -lactose crystallizes on cooling.

### Cautions

The isolation of lactose requires the use of a bench-top centrifuge. Be sure to balance the centrifuge with two tubes of equal weight when operating, or damage can occur to the centrifuge and the centrifuge tube may shatter.

### Isolation and Purification of Casein

Make a dilute solution (approx. 10%) of acetic acid by adding 1 mL glacial (100%) acetic acid to 10 mL distilled water in 10-mL-Erlenmeyer flask. Mix thoroughly and set aside.

Place 4.0 g of powdered nonfat milk and 10 mL of water into a 50- or 100-mL beaker. Heat on a sand bath to about 40°C (top of sand bath at about 50°C). Monitor the temperature of the milk solution with a thermometer.

When the mixture has reached 40°C, add the dilute acetic acid dropwise to the warm milk. *Do not add all of the dilute acetic acid at one time!* Maintain the solution at about 40°C and **after every 5 drops**, stir the solution gently using a small spatula. Using the spatula, push the precipitated casein onto the side of the beaker so that most of the liquid drains from the solid (you will have to hold it on the side of the beaker to drain the liquid).

Then transfer the congealed casein to a 20-mL vial in small portions. The casein will stick together and be hard to transfer if you use large pieces. If any liquid separated from the casein in the vial, use a Pasteur pipet to transfer the liquid back into the beaker.

Slowly continue the dropwise addition of the 1 mL of dilute acetic acid solution to the beaker to complete the casein precipitation. Remove as much casein as possible from the beaker and transfer it to the vial. Avoid adding an excess of acetic acid to the milk solution, as this will cause the lactose in the milk to hydrolyze to glucose and galactose.

When most of the casein has been removed from the milk solution, add 0.2 g of calcium carbonate to the milk in the beaker. Stir this mixture for a few minutes and save it for use in the isolation of lactose below. (The mixture should be used as soon as possible during the current lab period.)

Transfer the casein from the 20 mL vial to a Hirsch suction filter funnel. Draw a vacuum on the casein for about 5 min to remove as much liquid as possible, pressing the casein with a spatula during this time. (The liquid contains the albumins and lactose--so a great loss of liquid at this point will result in decreased yields of these other two components.)

Transfer the casein to a 7- to 10-cm piece of filter paper, fold this over onto the casein, and press gently to absorb any remaining liquid. Place the solid on a **tared** watch glass, let air dry until the next lab period, and weigh. Casein is used to make white glue, so it is important that you don't leave it on the filter paper or it will become glued to it! Calculate the weight percent of casein isolated from the powdered milk.

## Isolation of the Sugar, Lactose, and Albumin Proteins from Milk

After the isolation of casein, the milk mixture contains the sugar (lactose) and the protein (albumin). Heat the milk mixture to about 75°C for about 5 min. on a sand bath. Heating results in a nearly complete denaturation and precipitation of the albumins from the solution.

Decant the liquid in the beaker away from the solid into a clean plastic 10-mL centrifuge tube (borrowed from the rack next to the Beckman centrifuge located at the end of lab 215). You may need to hold the solid with a spatula when transferring the liquid. Press the solid albumins with a spatula to remove as much liquid as possible and pour the liquid into the centrifuge tube. Save the albumins in the original beaker. *You should now have about 7 mL of liquid. If you have less than 2 mL, you need to add an additional 1 mL to the albumin residue, heat to about 75°C, and decant, combining the two liquid portions. This procedure will minimize the loss of lactose that may have crystallized along with the albumin precipitate.*

When the liquid has cooled to about room temperature, place it in the centrifuge. Make sure the centrifuge is balanced by placing another plastic centrifuge tube filled with water to the same level as your lactose tube, at a position 180° opposite the lactose tube. Centrifuge for 5 min according to the instructions posted on the wall above the centrifuge.

Following centrifugation, decant the liquid away from the solid into a 50- or 100-mL beaker. Add 15 mL of 95% ethanol to the beaker. Solids will precipitate. Heat this solution on a sand bath to about 60°C to dissolve some of the solid. Pour equal amounts of the hot solution into two 10-mL plastic centrifuge tubes (obtained from the rack next to the Beckman centrifuge in Room 215) and centrifuge this solution as soon as possible before it cools appreciably. Centrifuge for 2 min. It is important to centrifuge the solution while it is still warm to prevent premature crystallization of the lactose. A considerable quantity of solid material is deposited on the bottom of the centrifuge tubes.

Remove the warm, supernatant liquid from the tube using a Pasteur pipet, and transfer the liquid to a 25- or 50-mL Erlenmeyer flask. (You can discard the solid remaining in the centrifuge tube.) Stopper the flask and allow the lactose to crystallize for at least two days. Granular crystals will form during this time.

Collect the lactose by vacuum filtration on a Hirsch funnel. Use about 3 mL of 95% ethanol to aid the transfer and to wash the product.  $\alpha$ -Lactose crystallizes with one molecule of water of hydration per molecule of lactose and therefore its formula is  $C_{12}H_{22}O_{11} \cdot H_2O$ . Weigh the product after it is thoroughly dry. Calculate the weight percent of the  $\alpha$ -Lactose isolated from the powdered milk. Measure the optical activity of  $\alpha$ -lactose by weighing and dissolving all of your sample in 2 mL of distilled water and analyzing the solution by polarimetry (see Chapter 11 of the Lab Guide). Try to obtain a reading as soon as the polarimeter readings stabilize to the nearest tenth of a degree ( $\pm 0.1^\circ\text{C}$ ). Over a period of time, one can observe a gradual reduction in the specific rotation.

Important: If the polarimeter continues to scan in the positive direction, reverses, and scans to the negative values, and back again, then your sample is probably too cloudy. (Sometimes particles of albumin will precipitate while the lactose crystals are growing.) If this is the case, you can remove the sample solution from the cell and rinse the cell with methanol. Centrifuge the solution and return it to the cell. If centrifuging does not remove the suspended particles (that is, the suspended particles are really fine), fold a piece of filter paper in a cone shape and gravity filter the solution, and return it to the cell.

Allow the albumins to dry for 2-3 days in the original beaker in which they were precipitated. Break up the solid and weigh it. Calculate the weight percent of albumins isolated from the powdered milk.

### **Cleaning Up:**

Clean the plastic centrifuge tubes with soap and water, rinse with distilled water, **NOT ACETONE**, and place upside down in the rack by the Beckman centrifuge. All the filtrates from the above isolations are easily biodegraded and can therefore be flushed down the drain.

### **Final Report:**

Use the data for cow's milk given in the table titled *Average Percentage Composition of Milk from Various Mammals* to discuss your percent yields from dry fat-free milk and how these compare to what is found in whole milk or might be calculated for whole milk using your experimental data in conjunction with the table data.

### **Postlab Questions**

1.  $\beta$ -Lactose is present to a larger extent in the aqueous solution when the solution is at equilibrium. Why is this to be expected?
2. Draw the structure of  $\alpha$ - and  $\beta$ -lactose and the free aldehyde and refer to the drawings in your answer.
3. Why do you expect a gradual reduction in the optical rotation and why does it reach a point where it no longer continues to decrease?
4. In the precipitation of casein, why is it important that the temperature be maintained at 40°C?