Methods of Glucose Measurement and Carbohydrate Analysis

Lecture Goal(s): To introduce methods of glucose/carbohydrate analysis and discuss the underlying theory of each procedure/technique. Normal reference ranges will be given and the relationship between test and test result will be correlated with errors in carbohydrate metabolism.

Lecture Objectives: Upon completion of this class material each student will be able to do the following:

1. Cog/II Discuss the following methods of glucose/carbohydrate analysis. Describe the reaction, the analyte(s) specifically measured, problems and other noteworthy characteristics about each reaction with special emphasis placed on the newer enzymatic techniques.

   a. Folin-Wu
   b. Somogyi-Nelson
   c. Benedict's test
   d. neocuproine method
   e. Hoffman alkaline ferricyanide
   f. Hultman o-toluidine
   g. glucose oxidase
   h. hexokinase
   i. glucose dehydrogenase

2. Cog/II Note the most common specimen currently used for glucose analysis. Discuss the problems with the use of whole blood for these measurements.

3. Cog/II Explain specimen collection and storage requirements for glucose analysis.

4. Cog/I List normal reference values for the different types of glucose analyses performed and note panic values.

5. Cog/I Describe the proper conditions for a glucose tolerance test.

6. Cog/II Discuss the rationale and usefulness of collecting a urine sample along with each blood sample during an oral glucose tolerance test.

7. Cog/III Correlate results from an oral glucose tolerance test with health and disease. Explain the reason for increases and decreases in glucose levels in health and disease.

8. Cog/II Explain the use of the Wilkerson Point System for diagnosis of diabetes mellitus.

9. Cog/II Explain the usefulness of running an insulin level along with the glucose level during an oral glucose tolerance test.

10. Cog/II Discuss the use of the following tolerance tests and how each test is performed.

    a. IVGTT
    b. tolbutamide tolerance test
    c. epinephrine tolerance test
    d. lactose tolerance test

11. Cog/II Describe the glycation of proteins and the utility of measuring glycated proteins in monitoring diabetes mellitus.

12. Cog/III Compare and contrast the methods for measuring glycated hemoglobin.

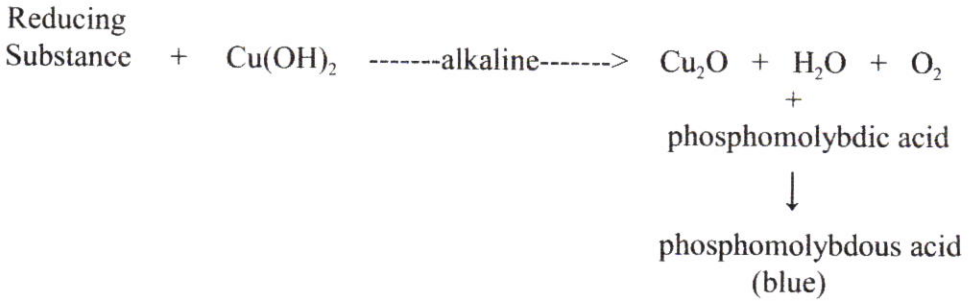
13. Cog/II Explain what is meant by microalbumin and relate the measurement of microalbumin in the urine to diabetes mellitus.

Methods of Glucose Measurement

Historical Methods

Many of the older methods for measuring glucose were based on its property of acting as a reducing agent in hot alkaline solution. In hot alkaline solutions, reducing substances, such as glucose, will reduce cupric (Cu^{+2}) to cuprous (Cu^{+1}) ions. Below a few of these methods are considered for historical purposes.

1. **Folin-Wu**: This is probably the oldest method of glucose analysis. This method is not limited, however, to just glucose. This method will detect any reducing substance. In this reduction method, the resulting cuprous ions primarily form cuprous oxide (Cu_2O). Cuprous oxide then reacts with phosphmolybdic acid (reduced form) to form phosphomolybdous acid (oxidized form) which produces a blue color in which the intensity of the color is directly proportional to the amount of reducing substance present in the sample.



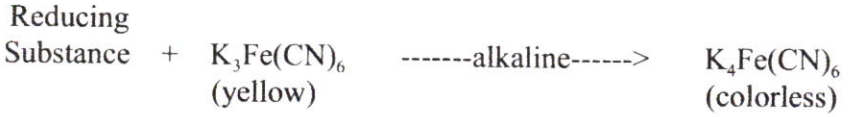
The specimen that was used for this procedure was whole blood. A protein free filtrate had to be prepared by the addition of tungstic acid in order to remove albumin which would interfere with the test. This reaction did not go to completion, therefore critical timing was very important. Also, temperature control and reagent concentration had to be monitored closely since the reaction did not go to completion. Other reducing substances present in the blood stream would interfere with this reaction. Some of these reducing substances include creatinine, uric acid, glutathione, and thionine. Finally, care had to be taken to reduce the exposure of cuprous oxide to air. Oxygen in the air would reoxidize cuprous oxide to cupric oxide. This was undesirable since the reaction was based on phosphomolybdic acid oxidizing cuprous oxide. Therefore, a special Folin-Wu tube was used for this analysis.

2. **Somogyi-Nelson**: This method is basically the same as the Folin-Wu method for glucose measurement with one difference. The protein free filtrate was prepared using a 3% barium hydroxide ($Ba(OH)_2$) and 3% zinc sulfate ($ZnSO_4$) mixture. This method of preparing the protein free filtrate has become a frequently used technique which is not limited to just glucose analysis, and, when used, the filtrate is known as a Somogyi-Nelson filtrate. This filtrate offers several advantages over the tungstic acid filtrate in the Folin-Wu technique. The two main advantages are: 1) in addition to removing protein, this method also precipitates all non-carbohydrate reducing substances thus making this analysis much more specific for carbohydrates; 2) the pH of the resulting filtrate is 7.4, physiologic pH. Before the development of enzymatic methods, this method was considered the most specific method for the determination of glucose in whole blood.

3. **Benedict's Test**: This is another variation of the same copper reduction technique which was adapted for semi-quantitative measurement of reducing sugars in urine. This method stopped with the production of a red-colored cuprous oxide compound. The intensity of the color was once again proportional to the relative amount of reducing substances present in the urine sample.

4. **Neocuproine Method**: This is another variation of the same copper reduction technique in which cuprous oxide is reacted with neocuproine (2,9-dimethyl-1,10-phenanthroline hydrochloride) to produce an orange-colored product where the intensity of the orange color was directly related to the concentration of reducing substance present. This end product was three times more intense than that produced with phosphomolybdous acid in the Folin-Wu method. Otherwise, the reaction was very much like the Folin-Wu technique and suffered many of the same limitations.

5. **Hoffman Alkaline Ferricyanide**: This method was also directed at detecting reducing substances in the blood stream. The different aspect about this method is that the oxidized form potassium ferricyanide (which is yellow) was reduced under hot alkaline conditions to a colorless potassium ferricyanide.



The drop in color intensity is proportional to the amount of reducing substance present in the sample. In order to measure this on a spectrophotometer, the reagent blank had to be set to a high absorbance, e.g. 2.000, and then the amount of decrease was plotted versus concentration. This technique was extensively used on the original AutoAnalyzer, but was difficult to run manually. Another important drawback to this technique is that the reagent, in particular cyanide, is poisonous.

6. **Hultman o-Toluidine**: This last historical method differs from the others in that this is a condensation reaction instead of a copper reduction reaction. In this reaction there is condensation of an aldose (hence more specific than previous methods) with an aromatic amine (o-toluidine) in hot acetic acid to form a glycosylamine. This glycosylamine readily undergoes dehydration to form a green-colored Schiff base. The intensity of this green color is directly proportional to the concentration of aldoses in the sample being analyzed.

Aldose + o-toluidine ----hot acetic acid----> glycosylamine ---- minus H_2O----> Schiff Base (green)

This method offers several advantages over the other historical tests for glucose. As a matter of fact, you still find this method being used from time to time. Some of the advantages include: 1) this was the first method in which a protein free filtrate was not required; 2) this could be run on serum; 3) this method is reported to be sensitive to 20 mg/dL on a 20 µL sample - this small sample size now permitted glucose measurement on fingersticks; 4) this method was considered to be very specific for glucose, since it is by far the most abundant aldose found in the blood stream.

Current Methods

Most of the currently used techniques involve enzymatic reactions which react specifically with glucose. The two major enzymes utilized to measure glucose levels include hexokinase and glucose oxidase.

As you review this section on the CD you will note that the hexokinase reaction system involves the same reaction seen as the first step of metabolism; the hexokinase reaction. The second step in this reaction sequence involves the first step of the pentose phosphate pathway. In this second step we see the reduction of NAD or NADP to NADH or NADPH. The coenzyme used depends on the source of the enzyme. If the glucose-6-phosphate dehydrogenase is isolated from yeast, then NADP is used as the coenzyme while NAD is the coenzyme if this enzyme is isolated from bacteria. As far as the spectrophotometric analysis goes, neither NAD nor NADP absorb light at 340 nm while both NADH and NADPH do absorb light at 340 nm. Spectrophotometrically speaking, we are not concerned with which coenzyme is present since they both absorb at the same wavelength. Note that the product of this reaction appears colorless to human eyes since absorption is at 340 nm.

The hexokinase reaction discussed may also be utilized as a reference method. This would, however, require the preparation of a Somogyi-Nelson protein free filtrate which makes this too time consuming to be utilized routinely in a clinical laboratory. An alternative to preparing the protein free filtrate is to prepare a specimen blank. Either one of these steps gives the hexokinase procedure the accuracy necessary to be considered a reference method. In practice, however, neither step is typically taken. Thus, hexokinase is typically used as a field/derived method.

The **glucose oxidase** procedure is the second enzymatic technique used to quantify glucose levels. Note that this is an enzymatic reaction that produces a colored product which can be read spectrophotometrically in the visible region.

The instrument utilized by this approach has an oxygen electrode placed in the reaction chamber. Notice that the first step of the glucose oxidase reaction involves the consumption of oxygen. The oxygen electrode measures the rate of oxygen consumption in the reaction mixture after the sample is added, thus, the second step of the reaction does not occur. With this system, a standard is first analyzed and the oxygen consumption is adjusted to that standard value. Then controls and patient samples are run. To consider this in simple terms, the instrument then sets up a ratio to determine the glucose level in the controls and patient samples. The ratio would look something like this.

$$\frac{\text{oxygen consumption for the standard}}{\text{concentration of the standard}} = \frac{\text{oxygen consumption for the unknown}}{\text{concentration of the unknown}}$$

In this equation, the unknown would be either the control or the patient sample. In this ratio, the only thing that would not be known would be the concentration of the unknown and this is what the instrument would calculate and display.

This approach was first made popular by Beckman Instruments.

A list of the current methodologies and their reactions are as follows:

Methods of glucose analysis - From the Methods of Analysis section on the CD included with the text.

(EP, End-point analysis mode; CSF, cerebrospinal fluid; GDH, glucose dehydrogenase; K, kinetic analysis mode)

Method 1: Benedict's; qualitative, semiquantitative

Principle of analysis:

$$Cu2+ + \text{Glucose} \xrightarrow[OH-]{\text{heat}} Cu2O\downarrow + CuOH\downarrow$$

(red) (yellow)

Comments: Basis of semiquantitative tests for total reducing sugars in urine; Used in combination with more specific glucose oxidase/peroxidase urine screen to differentiate glucosuria from other sugars in urine, especially in neonates

Method 2: Alkaline ferricyanide; quantitative

Principle of analysis:

$Fe(CN)^{3}_{-6}$ (ferricyanide) (yellow) $\xrightarrow{\text{heat, OH-}}$ $Fe(CN)^{4}_{-6}$ (ferricyanide) (colorless)

Comments: Rarely used, of historical interest; 1 mg of creatinine = 1 mg of glucose; 0.5 mg of uric acid = 1 mg of glucose; very poor specificity

Method 3: o-Toluidine; quantitative, EP

Principle of analysis: Increased absorbance at 630 nm

Comments: Serum or urine; rarely used in automated analysis; o-toluidine is a suspected carcinogen; other sugars, especially mannose and galactose, give positive interferences; turbidity can cause a positive bias

Enzymatic Methods

Method 4: Hexokinase (HK); quantitative, spectrophotometric K or EP

Principle of analysis:

$$\text{Glucose} + \text{ATP} \xleftrightarrow{\text{HK}} \text{Glucose 6-phosphate} + \text{ADP}$$

$$\text{Glucose 6-phosphate} + \text{NADP+} \xleftrightarrow{\text{G6PD}} \text{6-Phosphogluconate} + \text{NADPH} + \text{H+}$$

Increased absorbance at 340 nm related to glucose concentration

Comments: Serum, CSF, urine; automated; most commonly used method; has been proposed as basis of reference method; very good accuracy and precision

Method 5: Glucose oxidase coupled reaction ("Trinder")

a. Quantitative, using various types of dyes as final O2 acceptor; K or EP
b. Quantitative or semiquantitative in dipstick screen, visual or reflectance photometry

Principle of analysis:

$$\text{Glucose} + \text{O2} \xleftrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + \text{H2O2}$$

$$\text{H2O2} + \text{Reduced dye} \xleftrightarrow[\text{peroxidase}]{\text{Horseradish}} \text{Oxidized dye (colored)} + \text{H2O}$$

Peroxidase indicator reaction — Increased absorbance related to glucose concentration

Comments:

a. Serum, urine, CSF; easily and usually adapted to automated analysis; second indicator reaction susceptible to false-positive interferences from a variety of compounds; good accuracy and precision
b. Used in all dipstick screens; serum, urine

Method 6: Glucose dehydrogenase; quantitative, EP, K

Principle of analysis:

$$\text{Glucose} + \text{NAD}^+ \xrightarrow{\text{GDH}} \text{D-Gluconolactone} + \text{NADH} + \text{H}^+$$

Increased absorbance at 340 nm is related to glucose concentration

Comments: Rare, serum, CSF; can be adapted to automated instruments

Method 7: Glucose oxidase (GO) oxygen consumption; quantitative, polarographic measurement using O2 electrode; K

Principle of analysis:

$$\text{Glucose} + \text{O2} \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + \text{H2O2}$$

H2O2 consumed in side reactions
O2 consumption measured polarographically by oxygen electrode

Comments: Serum, CSF; semiautomated and fully automated systems; correlates best with the proposed reference method; very good accuracy and precision

Rules for Selecting the Correct Number of Decimal Places to Report

It is very important that the clinical laboratory be consistent in reporting out the same number of decimal places for a given laboratory test. If different technologists report test results with different numbers of decimal places, physicians and other health care professionals may begin to lose confidence in the laboratory. Thus, there are a couple of simple rules to follow. These rules reach a happy medium between analytical accuracy and pathological significance. Even though our glucose analysis may be accurate to two decimal places, there is absolutely no pathological significance between a glucose of 101.22 and 101.78. Therefore, there is no need to report results to this level of accuracy. These rules are based on the normal/reference ranges for the analyte being considered and are as follows.

1. If the normal/reference range is greater than 10, you report **no** decimal places. For example, the normal/reference range for fasting glucose is 75 mg/dL - 105 mg/dL.
2. If the normal/reference range is less than 10, you report **one** decimal place. For example, the normal/reference range for total protein is 6.0 g/dL - 8.0 g/dL. Note that you **do** include the zero to locate the decimal place even when there are no tenths.
3. If the normal/reference range crosses 10, you report **one** decimal place. For example, the normal/reference range for serum calcium is 9.0 mg/dL - 11.0 mg/dL.

There are only a few exceptions to these general rules and these will be noted as they are

considered.

Specimens for Glucose Analysis

The most frequently used specimen is serum/plasma. Another sample that was used in the initial glucose methods and is being used more and more today is whole blood. Whole blood is common in home glucose monitoring and in point-of-care testing. The concern with this is whole blood is a two compartment system which makes interpretation of laboratory results more difficult. As an example, whole blood values for glucose (and other analytes) varies with the hematocrit. As hematocrit decreases, the aqueous content of blood increases (erythrocytes contain 73 mg/dL water while plasma contains 93 mg/dL). Thus, as hematocrit decreases, measured glucose levels increase. For example, consider a glucose level measured on whole blood is 100 mg/dL when the hematocrit is 45%. If the hematocrit decreased to 20%, the whole blood glucose level would increase to 104 mg/dL. Also, if the hematocrit was to increase to 60%, the whole blood glucose would decrease to 91 mg/dL.

Other problems with the use of whole blood are as follows.

1. Whole blood must be mixed thoroughly, but gently, before sampling. This is inconvenient for many automated systems and is sometimes overlooked in point-of-care testing.
2. Whole blood from capillary punctures must flow freely and not be forced. If pressure is applied to collect the sample, whole blood is diluted with tissue juices leading to inaccurate results.
3. Analytes such as glucose are more stable when serum/plasma is separated from the cells.
4. Serum/plasma is easier to store than whole blood.
5. The specificity of most testing procedures is improved in serum/plasma when cellular components of whole blood are removed.

Thus, whole blood is typically not the sample of choice for most clinical chemistry analyses. There are some assays where whole blood is required in clinical chemistry and these will be considered as they arise.

The specimen of choice for glucose determinations is venous blood. In cases where infants are involved, or, when there is a difficult stick on an adult, a capillary specimen can be used. When capillary or arterial samples are used, a note should be placed in the comments section of the patient report indicating the type of sample used since normal/reference ranges may vary somewhat.

It is important to separate serum/plasma from the cells as soon as possible. At room temperature, glucose in a whole blood sample is metabolized at a rate of 7 mg/dL/hr. At refrigerator temperatures (4°C), the rate of glucose metabolism is 2 mg/dL/hr. Therefore, serum/plasma should be separated from the cells within one-half hour. If serum/plasma will remain in contact with the cells for an hour or longer, a preservative such as sodium fluoride (NaF) should be used.

Laboratory Tests Used to Evaluate Glucose Metabolism

A **fasting blood glucose** (also known as a fasting blood sugar, FBS) is an analysis of glucose following a specified period of time (e.g., 8 hrs., 10 hrs.) during which there is no caloric intake.

Please note that throughout clinical chemistry normal/reference ranges will be given. The ranges that are given are those for which I am most familiar. These may vary somewhat from your textbook. It is not expected that you know all normal/reference ranges to the exact number, but you should have a good idea what the approximate range is. Recall past discussions regarding the development of normal/reference ranges and how each clinical facility should establish their own ranges. Because of this you are not expected to know each exact range; an approximate range will be acceptable.

The normal range for a fasting blood glucose that will be used here is 70 mg/dL - 105 mg/dL. An elevated fasting blood glucose of greater than 126 mg/dL **on more than one occasion**, is diagnostic for diabetes mellitus.

In the fasting state, arterial and capillary blood samples for glucose are approximately 5 mg/dL higher than venous samples. Thus, once again, it is important to note on the patient report the type of sample used when it varies from the norm.

It is important to note that other diseases, such as the endocrine diseases previously considered may also lead to an elevated blood glucose level, especially after fasting. Thus, the physician must match the laboratory data they receive with the patient symptoms. If the elevated fasting glucose and diabetes mellitus does not match with the symptoms presented by the patient, then additional laboratory testing may be needed to help pinpoint the problem.

A **2-hour postprandial (2-hrpp) glucose** (Refer to page 596, "Postprandial Plasma Glucose" in Kaplan) is an analysis of glucose in a blood sample collected two hours after a meal or glucose load. The purpose of this test is to assess a patient's ability to metabolize glucose. A normal 2-hrpp is 120 mg/dL or less. A 2-hrpp glucose level of 200 mg/dL or greater **on more than one occasion** is diagnostic of diabetes mellitus.

There is a wide gray area between 120 mg/dL and 200 mg/dL. An elevation of the 2-hrpp glucose in this area may act as an early indicator of some problem with glucose metabolism. In general, there are two factors that may lead to an elevation of the 2-hrpp glucose. These include diabetes mellitus and age.

A **random blood glucose** is an analysis of glucose in a blood sample collected with no restrictions as to caloric intake or timing since the last caloric intake. We know that a fasting blood glucose reference range is 70 mg/dL - 105 mg/dL. We also know that blood glucose levels typically reach a maximum of 130 mg/dL - 160 mg/dL following a meal. In addition, blood glucose levels should never go above 200 mg/dL. Even though we know all of these specifics, a random blood glucose is said to have **no** normal/reference range. In the clinical laboratory, we simply do not know when the last caloric intake occurred or how much was ingested.

A **CSF glucose** is an analysis of glucose in spinal fluid. A CSF glucose is typically ordered in conjunction with a CSF protein. The significance of this combination of tests on CSF will be discussed in the proteins section. The normal/reference range for CSF glucose is 60% to 70% of the plasma glucose level which typically equates to 40 mg/dL - 70 mg/dL. Keep in mind that CSF glucose levels **must** always be compared with concurrently measured serum/plasma glucose for adequate clinical interpretation.

In addition to normal/reference ranges, clinical laboratories also have **panic values**. Each laboratory will have its own set of rules on how to proceed when panic values are obtained. Such steps involve notifying the physician directly, etc. The following are panic values related to glucose for which you are responsible.

1. Serum/plasma glucose of greater than 600 mg/dL
2. Serum/plasma glucose of less than 40 mg/dL
3. CSF glucose of greater than 150 mg/dL

Another measure of a patient's ability to metabolize glucose is a **glucose tolerance test (GTT)**. This test is typically performed by administering a glucose challenge orally. Therefore, this is also typically referred to as an oral glucose tolerance test (OGTT) in order to distinguish this test from the less commonly used intravenous glucose tolerance test (IVGTT) described below. The OGTT is described in Kaplan on page 1596. Note that an OGTT is rarely necessary for the diagnosis of diabetes mellitus and is not recommended for routine clinical use. Kaplan goes on to list the situations where the OGTT may be indicated.

For the results of an OGTT to be meaningful, there must be several conditions met. It is important that the patient consume an adequate diet in CHO. For three days prior to the GTT, a diet of at least 150 grams of CHO per day is required. An additional two days may be required prior to the test if the patient had not been consuming a diet sufficient in CHO prior to this time. The presence of anorexia would invalidate the test. The test should **only** be performed on ambulatory patients and not on hospitalized, acutely ill, or inactive patients since glucose tolerance will be impaired in these situations. Also, a decision will be made between the patient and their physician as to whether or not the patient should remain on certain medications. Diuretics, oral contraceptives, as well as other medications are known to interfere with this test. Prior to the day of the test, the patient should ingest a normal evening meal and then have no caloric intake until after the test. This restriction includes coffee! Note, however, that reasonable amounts of water may be ingested during the fasting period. During the GTT the patient should remain at rest and refrain from smoking.

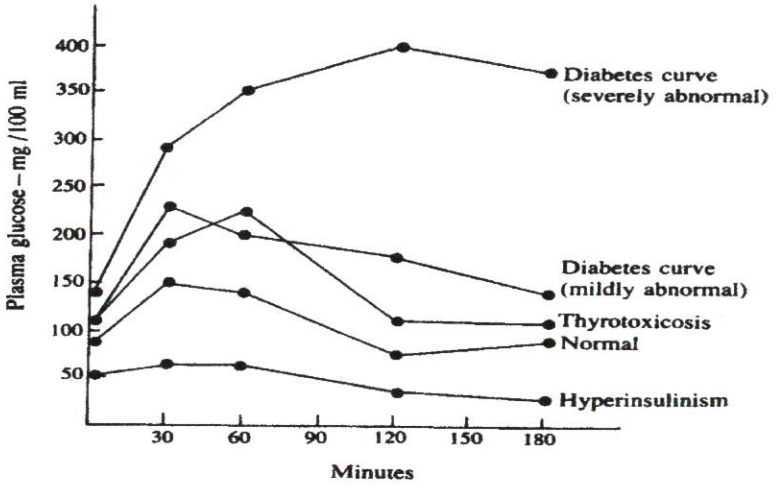
The OGTT should begin between 7:00 a.m. and 9:00 a.m. after 30 minutes of rest. The reason for this is based on the diurnal variation in glucose metabolism throughout the day. This diurnal variation results from the diurnal fluctuation of cortisol secretion. You will recall that cortisol is one of the hormones that influences CHO metabolism. Cortisol is at its highest level of secretion between 7:00 a.m. and 9:00 a.m. Also, keeping in mind the diurnal variation just described, the normal/reference ranges for the OGTT are established for this time of the day.

The OGTT procedure begins with the collection of a fasting blood glucose. A fasting blood glucose should have already been ordered on the patient before the OGTT is scheduled. Thus, if the patient had a high fasting glucose such that administration of a glucose challenge may put the

patient in a life-threatening situation, the OGTT would never be scheduled. For this reason, the fasting blood glucose is frequently not analyzed by the laboratory until the test is completed. (NOTE: even though the sample is not analyzed until the test is complete, the serum/plasma must still be separated from the cells.) After the fasting blood glucose sample is collected, a synthetic product called "Glucola" is administered. This product consists of a hydrolyzable saccharide of corn syrup and carbonated water with flavoring added. Seven ounces of Glucola are equivalent to 75 g of glucose, which is the typical glucose challenge. Two situations where the level of glucose challenge differ include testing for gestational diabetes mellitus (discussed below) and in pediatric patients. In pediatric patients it is recommended that a dose of 40 grams of glucose per square meter of body surface area be administered. The Glucola should be ingested within a five-minute period.

Next, blood samples are collected at 30 minutes, 1 hour, 2 hours, and 3 hours following the administration of Glucola. Nausea is a somewhat frequent problem with Glucola. If a patient becomes nauseated, the timing of collections should still occur if possible. The purpose for the 30-minute collection is primarily to confirm adequate gastric absorption if the patient becomes nauseated. The 30-minute collection is of little diagnostic value otherwise.

If we were to prepare a plot of the glucose levels versus the time of collection after Glucola administration, we can find some very characteristic patterns of health and disease. Consider the graph below.



In a normal (i.e., non-diabetic) patient we would see glucose levels peaking somewhere between the 30-minute and 1-hour time. By two hours, the blood glucose level should be close to the fasting level or slightly below the fasting level if insulin overshot. This slightly decreased 2-hour blood glucose level is somewhat commonly seen, and, in the normal individual, is corrected by the 3-hour sample due to the counter-regulatory action of glucagon. In a situation of diabetes mellitus, we see a higher and more prolonged increase in blood glucose levels due to problems with insulin production or action. Also notice that other disease states may produce characteristic patterns on this plot. For example, hyperinsulinism may produce an almost flat line pattern. Some laboratories will have their laboratory information system (LIS) electronically evaluate the OGTT data and print an appropriate statement in the comments section on the patient's chart. The LIS programs that carry out this function typically utilize a point system to

evaluate patient data, an example of which is the Wilkerson Point System. This point system assigns points if a pre-defined glucose level is exceeded. For example, if the fasting glucose level is greater than 126 mg/dL, 1 point is assigned. If the 1-hour glucose level is greater than 195 mg/dL, ½ point is assigned. If the 2-hour glucose level is greater than 120 mg/dL, ½ point is assigned. If the 3-hour glucose level is greater than 126 mg/dL, 1 point is assigned. Following electronic evaluation of each point, if there is a total of two or more points, this is considered diagnostic for diabetes mellitus and an appropriate comment is placed on the patient's chart.

In the past a urine sample was collected after each blood sample and urine glucose levels were analyzed. The use of urine samples in addition to blood samples is now considered unnecessary by most in the field. It is known that the threshold for glucose in the kidney is 160 mg/dL to 180 mg/dL, and if the blood glucose levels exceed this limit, glucose will spill over into the urine. Thus, we can predict with great accuracy when glucose is going to appear in the urine by considering the blood glucose levels making the need for collection of multiple urine samples during the OGTT procedure unnecessary.

The measurement of both glucose and insulin levels on OGTT samples may be useful in distinguishing the type of diabetes mellitus present. First, in non-diabetic individuals, plasma insulin levels peak approximately one hour after a glucose load and return to fasting levels within two to three hours. As you consider the following points please note that type 1 and type 2 diabetes mellitus will be compared and contrasted in great detail below.

1. Type 1 diabetics respond to a glucose load with little or no insulin increase above fasting levels.
2. Type 2 diabetics respond to a glucose load with an abnormally late and often excessive increase in insulin levels.
3. Type 1 diabetics often have low fasting insulin levels.
4. Type 2 diabetics often have variable fasting insulin levels.

Insulin levels **alone** are generally not considered to be clinically useful for diagnosis of diabetes mellitus. The greatest utility of insulin levels alone is in the diagnosis of insulinoma (described below). (Refer to Fig. 32-2, page 599, "Criteria for Evaluation")

Other tolerance tests may also be utilized to evaluate various aspects of CHO metabolism, five of which are considered here.

Intravenous Glucose Tolerance Test (IVGTT) - Poor absorption of orally administered glucose may result in a flat glucose tolerance curve. Some patients are unable to tolerate a large CHO load orally which may lead to vomiting. Other patients may have altered gastric physiology resulting from procedures such as gastric resection. In these patients, an IVGTT may be performed to eliminate factors related to rate of absorption. Plasma insulin assays may also be requested in conjunction to the test.

The dose of glucose is 0.5 g/kg of body weight, given as a 25 g/dL solution. The dose is administered IV within 2 - 4 minutes and blood is collected every 10 minutes for one hour. If insulin assays are performed, a specimen is also obtained five minutes after the start of the injection. Blood glucose levels decrease in an exponential manner and the rate of glucose disappearance can be calculated. It has been found that in at least 50% of patients who have

undergone both IVGTT and OGTT, test results do not correlate. This has led to questions being raised regarding the usefulness of the IVGTT.

Since there is some question as to the utility of the IVGTT, you are not responsible for the calculation. For those who are interested, the calculation is as follows.

Glucose disappearance is calculated from the formula $K = 70/t_{½}$, where K is the rate of disappearance of blood glucose expressed as percent per minute of the 10-minute level and $t_{½}$ is the number of minutes required for the blood glucose to fall to one-half of the 10-minute level. To determine $t_{½}$, glucose values are plotted on the log scale of semilog paper versus time on the abscissa. A line drawn through the points is extrapolated to a glucose concentration equal to half the 10-minute level. The time interval between 10 minutes and the extrapolated point is equal to $t_{½}$. In normal individuals, K usually exceeds 1.5%; values below 1.0% are considered diagnostic of diabetes mellitus.

In the formula $K = 70/t_{½}$, the value 70 is derived from the logarithmic nature of the decrease in glucose concentration with time. The concentration of glucose at 10 minutes will be twice that of the value obtained from the plot at $t_{½}$. Using natural logarithms, the rate of decrease in glucose concentration expressed as percent per minute (K) is given by:

$K = 100(\ln 2 - \ln 1)/t_{½} = 69.3/t_{½}$ which is rounded to $K = 70/t_{½}$ for ease of calculation.

Insulin Tolerance Test - This test is sometimes used to evaluate patients with resistance to administered insulin or with certain endocrine disorders. The patient is placed on a diet containing at least 300 g of CHO daily for two or three days before the test. With the patient in the fasting state, blood is taken for a baseline glucose level, after which insulin is injected IV by the medical staff in an amount corresponding to 0.1 unit/kg of body weight. Blood specimens are then collected for glucose determination at 20, 30, 45, 60, 90 and 120 minutes after the insulin is administered. During this test the patient is to be monitored closely by the medical staff, and, if signs of a hypoglycemic reaction appear, glucose should be administered immediately and the test stopped.

Normally the blood glucose decreases to about 50% of the fasting level within 30 minutes and then returns to normal fasting limits by 90 to 120 minutes. There are two types of abnormal response. The insulin-resistant type of response shows only slight or delayed decrease in blood glucose, and this is seen with adrenal cortical hyperfunction (Cushing's syndrome), in acromegaly, and in some cases of diabetes mellitus.

In the second type of response, blood glucose levels fall normally, but the subsequent rise is delayed or does not occur at all. This situation occurs with hypofunction of the anterior pituitary or the adrenal cortex (Addison's disease), and in hyperinsulinism. In cases of suspected pituitary or adrenal insufficiency, it is recommended that half the usual dose of insulin be given and that the patient be watched carefully for signs of hypoglycemia. With severe insulin intolerance, an excessive hypoglycemic response with detrimental results (e.g., convulsions) can occur. Glucose solutions or fruit juice should normally be given to patients at the end of insulin tolerance tests.

Tolbutamide Tolerance Test - Tolbutamide, 1-butyl-3-(p-tolylsulfonyl)urea (trade name Orinase), is a compound that stimulates the normal pancreas to produce insulin. Following IV injection, the normal response is similar to that observed with the insulin tolerance test; the blood glucose decreases about 50% of the fasting level by 30 minutes, then returns to normal. If the blood glucose level at 20 minutes is between 80% and 84% of the fasting level, the patient is said to have a 50% probability of having diabetes mellitus. In more severe cases, the response will be even less since the pancreas is unable to secrete adequate insulin. The test is also claimed to be valuable in evaluating hypoglycemic states caused by insulinomas. In this condition, injection of tolbutamide results in a marked decrease in blood glucose to values in the range of 20 - 30 mg/dL and persistent hypoglycemia up to three hours. If the insulin response to tolbutamide is measured, diagnostic data can usually be obtained by 60 minutes typically reducing the risk of severe hypoglycemia. As with the insulin tolerance test, patients must be watched carefully for hypoglycemic reactions, and, the test terminated if the symptoms appear by the IV administration of glucose.

Epinephrine Tolerance Test - This test is used to evaluate one form of glycogen storage disease - Type I, von Gierke's Disease. von Gierke's Disease is a condition in which there is a deficiency or absence of the enzyme glucose-6-phosphatase in the liver. This enzyme is the catalyst for the final step in the formation of blood glucose from hepatic glycogen. Individuals with von Gierke's Disease have low blood glucose, increased liver glycogen but decreased **availability** of liver glycogen as shown by the less than normal or no increase in blood glucose following administration of epinephrine. In a normal person, after IM injection of 1 mL of a 1/1000 (1 g/L) solution of epinephrine hydrochloride, the blood glucose increases 35 - 45 mg/dL in 40 - 60 minutes and returns to the fasting level by 2 hours. Blood specimens are taken at 30, 45, 60, 90, and 120 minutes after injection.

Lactose Tolerance Test - A lactose tolerance test can be used to evaluate a deficiency of small bowel mucosal lactase. This has been found to be a rather common condition in healthy adults. Such deficiency may be associated with intolerance to lactose manifested by diarrhea and other symptoms following the ingestion of milk. The diarrhea will usually disappear if lactose is eliminated from the patient's diet. The diagnosis is often apparent from the clinical history.

To perform the test, a suspension of 50 g of lactose in about 200 mL of lemon-flavored water is prepared. For children, a suitable dose is 2 g of lactose per kilogram of body weight not to exceed 50 g. A fasting blood specimen is collected. Then the patient consumes the lactose suspension over a five minute period. Blood samples are collected at 15, 30, 45, 60, and 90 minutes after the lactose is consumed. The blood samples are analyzed for **glucose** by methods that are specific for glucose, primarily the hexokinase or glucose oxidase methods.
An increase of 30 mg/dL over the fasting glucose level is considered normal. A rise of 20 - 30 mg/dL is inconclusive. An increase of less than 20 mg/dL is evidence for a deficiency of intestinal lactase. Any abnormal test must be followed by a control in which 25 g each of glucose and galactose are given to ensure that the individual has normal tolerance to the two monosaccharides that make up lactose.

Another laboratory evaluation of CHO metabolism is to consider **glycated proteins**.

Reading: Kaplan Chapter 32, page 598 "Glycated Hemoglobin and Plasma Albumin".

A good brief introduction to glycated proteins is at the beginning of this section in Kaplan. Even though measurement of glycated proteins should not be used in the diagnosis of diabetes mellitus, this measurement serves as an excellent approach to monitoring long term glucose levels in those with diabetes mellitus.

There are two glycated proteins of interest. These include **glycated hemoglobin** and **glycated albumin**. Both of these proteins are glycated with glucose by a non-enzymatic reaction. As a side note, this is the second biochemical reaction we have considered that does not require an enzyme. Recall that the other non-enzymatic reaction that has previously been considered is the conversion of acetoacetate to acetone. The point is that most biochemical reactions do require enzymes, with only a few exceptions.

Glycated hemoglobin will be considered first. To understand the usefulness of this test you must first recall two facts: hemoglobin is found in the erythrocyte and the erythrocyte does not require insulin for glucose entry. The amount of glucose that enters the erythrocyte is directly proportional to the amount of glucose in the blood stream. When glucose enters the erythrocyte, it will react chemically with the N-terminal valine on the beta chain of hemoglobin forming an aldimine (formally known as a Schiff base). This reaction occurs rapidly and is reversible. As extracellular levels of glucose increase, intracellular levels of glucose will also increase. With this increase in intracellular glucose there will be an increased formation of the aldimine. Since this is reversible, as extracellular and intracellular levels of glucose decrease, the aldimine will reverse. Thus, the aldimine is said to be labile. As a result of the reversible nature of this reaction, transient increases in blood glucose will not result in permanent glycation of hemoglobin. On the other hand, if there is a prolonged increase in blood glucose, such as with diabetes mellitus, there will be a prolonged existence of the aldimine. In this case, the aldimine will undergo a slow reaction known as an Amadori Rearrangement forming a permanently glycated hemoglobin. This glycated hemoglobin will remain in the cell until the cell is removed from the circulation. The presence of glucose on hemoglobin does not affect the function of hemoglobin. Therefore, the level of glycated hemoglobin reflects the long term average of blood glucose for approximately the last six to eight weeks. As noted earlier, this serves as a good measure of glucose control which has great utility in monitoring diabetics. This is especially true when compared with a single serum/plasma glucose which reveals the blood glucose level at the time the specimen is collected. Glycated hemoglobin levels are measured using a hemolysate of whole blood . This whole blood sample can be collected at any time during the day and the patient does not have to be fasting. The normal/reference range for glycated hemoglobin is 4% - 8%. Those with uncontrolled diabetes mellitus may have a glycated hemoglobin as high as 25%. Once the patient has been controlled, the glycated hemoglobin level typically falls back to around 9%, or a little less. Note that this return to a level close to the upper normal/reference level would not be evident for three to five weeks during which time the erythrocytes exposed to the high levels of blood glucose are removed from the blood stream. Glycated hemoglobin levels should be performed twice a year in those patients who are meeting treatment goals and four times a year (i.e., quarterly) in those patients who are not meeting glycemic goals.

As methods of analysis for glycated hemoglobin are considered below, please pay close attention to the analyte being measured and the measurement technique. Variations in technique will result in different names for the analyte since different substances are being measured and may lead to confusion. As described below, the method of choice is affinity chromatography, and, when this technique is used, we measure **glycated hemoglobin**. On the other hand, when **some** types of ion exchange chromatography are used, the analyte measured is **Hb A_{1c}**. These are obviously different analytes and they have different normal/reference values. Even with this confusion it is still possible to use all of the techniques described below, and, if performed correctly, laboratory data can be obtained which can be used to monitor diabetics. Preference for one test over another is a matter of practical laboratory operations, equipment available, and concern for certain interferences. The trouble with this confusion is the use of one name, either Hb A_{1c} or glycated hemoglobin, as a general term with no regard to the method being used. Thus, careful attention is required as these methods are considered.

Perhaps the best technique to measure glycated hemoglobin involves chromatography. There have been several types of chromatography utilized for this purpose. The first method was developed by **Trivelli**. This method used cation exchange chromatography to separate hemoglobin into four fractions on the basis of charge: Hb A_o (normal adult hemoglobin), Hb A_{1a}, Hb A_{1b}, Hb A_{1c}. Hb A_{1a} and Hb A_{1b} were not completely resolved, and their combined concentration came to 2.5%. Hb A_{1c} represented about 5.5% and Hb A_o the remainder. Although the Trivelli method was cumbersome for routine use, it did establish the clinical value of the glycated hemoglobin assay. It dramatically illustrated that there are significant differences in Hb A_{1a+b} and Hb A_{1c} levels in normal and diabetic people.

Most experts at this time felt that **Hb A_{1c}** was a single species formed when glucose attached to the N-terminal valine in the beta chain of hemoglobin. This slight modification of hemoglobin was sufficient to change the net charge on the hemoglobin molecule making it possible to separate Hb A_{1c} from other hemoglobins by ion exchange chromatography.

Next in the evaluation of glycated hemoglobin came the **Fast Hb Test System**. This method used an ion exchange resin similar to that of Trivelli to separate the total Hb A_1 fraction (Hb A_{1a} + Hb A_{1b} + Hb A_{1c}) from other hemoglobins (Hb A_o). This simplified procedure extended availability of glycated hemoglobin to any clinical laboratory. It was felt at the time of the development of this test that total Hb A_1 reflected long-term blood glucose control and there was no need to separately measure Hb A_{1c} or the combined fraction Hb A_{1a} plus Hb A_{1b}.

Ion exchange chromatography columns consist of a stationary phase typically referred to as a resin. The ion exchange resin in the Trivelli, and similar, columns separate hemoglobin variants on the basis of their charge. Thus, by the Trivelli column, Hb A_{1c} could be separated from Hb A_{1a} and Hb A_{1b} since the charge on Hb A_{1c} has been altered by the addition of the glucose to the N-terminal valine.

The Fast Hb Test ion exchange resin is adjusted so that it has the same charge as the Hb A_1 fraction by passing the appropriate buffer(s) through the resin. Then, as the solution containing the hemoglobin passes through the resin, the Hb A_1 does not bind to the column and passes through the column first. Thus the name - Fast Hb Test. Recall that it was felt at the time of the development of this test that total Hb A_1 reflected long-term blood glucose control. Hb A_o has a slight positive charge and sticks to the column and does not interfere with the measurement of Hb A_1. A notable concern with this technique which is discussed below is that any hemoglobin variant with a charge similar to Hb A_1 (like Hb F) would also come through first and be measured as Hb A_1.

Returning to the confusion with terminology, some who measured Hb A_1 by the Fast Hb Test would refer to it as Hb A_{1c}. To add to this confusion, other techniques were also developed. These included:

1. High pressure liquid chromatography methods which could measure either Hb A_1 or Hb A_{1c}. (more confusion)
2. Electrophoretic separation by isoelectric focusing for measuring Hb A_{1c}.
3. Colorimetric procedure using thiobarbituric acid (TBA) for measuring Hb A_1.
4. Immunoassays for measuring Hb A_{1c}.

After this confusing welter of methods entered the scene a new complication arose. The original studies of glycated hemoglobin indicated that the glucose-hemoglobin interaction was an irreversible interaction and the level of glycated hemoglobin was not affected by recent diet. Subsequent studies indicated, as described above, that there is an intermediate that consisted of a reversible interaction between glucose and hemoglobin whose concentration depends on current blood glucose levels. This intermediate, which is known as the **labile fraction**, could be elevated for dietary reasons and cause a diabetic's results to be falsely elevated.

Ion exchange chromatography methods are unable to distinguish between the labile and non-labile forms of glycated hemoglobin. In addition, ion exchange techniques suffer from variations due to temperature which have to be corrected or controlled. Also, hemoglobin variants with a charge similar to the species being measured could completely confuse the test results. Thus, ion exchange methods suffer from a lot of limitations.

Finally **affinity chromatography** arrives. While the development of an additional method with a specific affinity for glucose may seem, at a first glance, to have complicated the problem, it has, in fact, provided a clearer picture. Affinity chromatography uses disposable minicolumns filled with a phenylboronic acid resin. Two of the hydroxy groups on the boron atom are spaced perfectly for interaction with the cis-hydroxys of the glucose that has been covalently bound to hemoglobin. As a result, any hemoglobin that has been glycated will be retained by the resin by a reversible linkage. Non-glycated hemoglobins will pass directly through the resin. Once all of the non-glycated hemoglobins have passed through the minicolumn, sorbitol is passed through the column to displace the glycated hemoglobins. The glycated hemoglobins are collected as they come off of the minicolumn and are measured spectrophotometrically.

A major advantage of the affinity chromatography method is there is no interference from non-glycated hemoglobins and negligible interference from the labile fractions. Thus, what is measured by this technique is true glycated hemoglobin. It has been found that Hb A_{1c} is not a pure compound. Only about 75% is glycated and the remainder consist of hemoglobin with something other than glucose attached to the N-terminal of the beta chain.

Another major advantage of the affinity chromatography method is it will detect all glycated hemoglobins. It has been found that there are more glycated hemoglobins than Hb A_{1c}. These are formed when glucose attaches to sites other than the end terminal valine on the beta chain. Affinity chromatography measures these as well, providing a more accurate measure of true glycated hemoglobin.

Affinity chromatography is not bothered by hemoglobin variants. It allows measurement of glycated Hb F, or S, or A, or E, etc. - whatever is glycated.

A final advantage of affinity chromatography is that it is unaffected by variations in temperature.

From all of this it is easy to see the advantages of affinity chromatography. These include:

1. It measures true glycated hemoglobin - not just Hb A_{1c} or Hb A_1.
2. Hemoglobin variants don't complicate the assay.
3. The labile fraction does not significantly interfere.
4. There is little temperature variation.

The second glycated protein is **glycated albumin**. Glycated albumin is known as **fructosamine**. The term fructosamine is a misnomer because the reaction leading to its formation is unrelated to fructose. Instead, this reaction occurs between serum proteins (primarily albumin) and glucose. The difference between glycated hemoglobin and fructosamine has to do with the half life of these two glycated proteins. The half life of glycated hemoglobin is approximately three to five weeks and the half life of fructosamine is two to three weeks. Thus, fructosamine would serve as an indicator of short term glucose control. The clinical utility of fructosamine has been overshadowed by glycated hemoglobin

In addition to monitoring glycemic control in association with diabetes mellitus, physicians are also concerned about the chronic problems associated with this disease. (These chronic problems will be considered in the next section.) One chronic problem likely to occur is nephropathy. It has been found that **urinary albumin excretion** may serve as the earliest indicator of this chronic complication of diabetes mellitus. Please read the first paragraph in the section entitled "UrinaryProtein" in Kaplan on page 599. Note in the reading that the term **microalbuminuria** is used to denote the presence of this albumin excretion. This term is misleading in that it suggests that there is a small form of albumin being excreted. This is **not** the case. The use of the term microalbuminuria is attempting to indicate that the excretion of albumin is greater than normal but less than previously detected levels.