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Prediction of fetal age

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PREDICTION OF FETAL AGE

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Introduction

Under certain circumstances it becomes necessary for a physician to determine the gestational age of a fetus whose maturity is in doubt. These circumstances are many and varied. Perhaps the fetus is affected with erythroblastosis fetalis as has been previously determined by means of maternal antibody titers; should intrauterine transfusion be performed or is the fetus mature enough to have a better chance of survival in the nursery environment? There occurs a time during such a gestation that the morbidity and/or mortality associated with intrauterine transfusion equals or exceeds that associated with prematurity. If the fetus is severely affected, it is at this time that delivery should be carried out. Other instances where knowledge of fetal age is extremely important include cases of premature labor, maternal hypertension, pre-eclampsia, antepartum hemorrhage, cesarean section, and hydramnios. In all of these circumstances and others not mentioned, there is a question of whether to effect immediate delivery by induction of labor or by Cesarean section or to postpone delivery for some time in order to allow the fetus to mature further. It is in this position that the physician places a premium upon the ability to determine the gestational age of the fetus.

But what if the fetus is delivered before term? What age the risks of prematurity and are they great enough

to warrant any hesitation when the question is whether or not to deliver immediately? Without listing all the causes for neonatal morbidity and/or mortality, several facts are clear. For the infant of less than 1500 grams birth weight, the overall mortality rate in the neonatal period exceeds 60%.[47] For the 1500-2000 gram group the mortality rate is 21% and for the 2000-2500 gram group it drops to 5%. If the birth weight exceeds 2500 grams, the mortality rate drops to 0.8%. There is also a greatly increased incidence of neonatal complications, associated with prematurity. The most common of these is respiratory distress syndrome which affects 13% of all prematures (By definition, prematurity means a birth weight of less than 2500 grams.), over 25% of those of less than 2000 grams birth weight, over 50% of those of less than 1500 grams at birth.[14] Overall mortality for infants so affected averages 50%. Also, to be considered is the fact that premature infants who survive to be discharged from the hospital have a mortality rate of approximately three times that of full-term infants during the first two years of life.[29] In addition, there has been noted an increased incidence of physical and mental handicaps later in life.[13]

Thus, it can easily be seen that when there are conditions which endanger the baby, the mother or both, two factors must be considered. First, the baby must be delivered as soon as possible in order to prevent further

harm either to the baby or to the mother; and second, the delivery must be postponed as long as possible in order to give the baby the best possible chance for survival. The optimal time for delivery in most of these cases would be a gestational age of 36 weeks which is equal to a birth weight of 2500 grams. Generally, the increased survival rate beyond this time is overshadowed by the increased risk of the disease condition present.

In order to best reconcile this question of to deliver or not to deliver, an accurate knowledge of gestational age is necessary. It is really not sufficient to merely estimate whether or not the fetus is "mature" (2500 grams or more) but rather it is imperative to be as accurate as possible. A reasonable degree of accuracy might be considered as plus or minus one week of gestation, a range or two weeks. This would allow the physician to realistically estimate chances of fetal survival in any case at any time during the gestation. Although this idea seems improbable at present, this degree of accuracy should be considered the future goal in the determination of fetal age.

What are the present methods of predicting fetal age? Are they accurate? The answer to these questions are that the present methods have reasonable accuracy when applied to large groups of cases but generally fail to be correct over two-thirds of the time when applied to an individual case.

Among the commonly used current methods are those of patient history (usually correlated with uterine fundal height) and X-ray techniques. Some of the newer and consequently less tested methods are those of amniotic fluid cytology, levels of amniotic fluid creatinine and alkaline phosphatase and spectrophotometric determination of the optical density difference at 450 millimicrons in amniotic fluid.

These methods will be presented listing both their advantages and disadvantages as well as the presentation of a small series of cases comparing certain of these methods.

Current Methods of Determining Fetal Age

Patient's History

Much may be determined concerning fetal age merely by consulting the mother. The application of Naegele's rule to the date of the beginning of the last menstrual period experienced by the patient is probably the most common means of estimating the gestational age of the fetus. Naegele's rule states that one may calculate the expected date of delivery by subtracting three months from the first day of the last menstrual period and adding 7 days. This yields a gestation of 280 days from the first day of the last menstrual period or 267 days from probable date of ovulation. In large series of pregnancies, this calculation is quite accurate. In a group of 77,300 live births in which the obstetrical

history was relatively certain, there was a mean duration of gestation of 280.8 days from first day of last menstrual period to delivery with a standard deviation of 12.88 days.[16]

However, certain problems do arise with this method, not the least of which concerns the patient's reliability. Even after overlooking this factor there are several other factors which may cast uncertainty upon the "first date of the last menstrual period." Bleeding during the time between conception and the time of positive or probable diagnosis of pregnancy may occur. According to Speert and Guttmacher,[46] over 20% of gravidas who eventually deliver viable infants may be expected to demonstrate macroscopic vaginal bleeding at some time during the first or second trimester. This bleeding was found to be three times more common in multigravidas than primigravidas. Also, to be contended with are the estimated date of ovulation (and consequently, conception) and anovulatory cycles. Collet, et al,[11] concluded that the incidence of anovulatory cycles was approximately 14% for all age groups, the 17-18 year old group showing a 30.8% incidence. Chances for an anovulatory cycle to precede a given instance of ovulation are about one in seven. This alone could cause an error in gestational age of about four weeks. Time of ovulation during the cycle is also important. Naegele's rule assumes ovulation to have taken place on approximately the thirteenth day of the menstrual cycle. However, in any given case, ovulation may occur at any time

between the fourth and thirty-ninth days of the cycle, 25% of women in all age groups falling outside of the tenth to eighteenth day range. This range may cause the expected date of delivery as calculated by Naegele's rule to be in error by as much as ten days on the early side to three weeks on the late side.

The time of quickening as reported by the patient has also been used to assist in determining fetal age. In a reliable patient, the first faint fetal movements are usually felt between the eighteenth and twentieth weeks of gestation.[17] However, they are frequently spurious and are definitely unreliable in the great majority of patient unless also detected by the physician and interpreted in the light of other findings.

The use of fundal height to predict fetal age is also mentioned here only to point out that its accuracy is not good and subject to many variables. It too should be interpreted in the light of all other findings.

X-ray Findings

Probably the most commonly used method of determining fetal age at the present time is that of X-ray. X-rays are taken of the maternal lower abdomen to include the uterus and fetus. They are then examined to determine the presence or absence of certain features which develop at reasonably predictable times during gestation. Currently the most popular

of these features is that of the distal femoral epiphysis with the proximal tibial epiphysis supplying "corroborating" evidence.

The distal femoral epiphysis develops at approximately the 34th week of gestation, the normal range being the 32nd to 36th weeks inclusively, the proximal tibial epiphysis appearing approximately 2 weeks later.[37] In infants born at various gestations it is present (in X-rays taken after delivery) in 5% of infants born during the eighth fetal month, 33% of those born during the ninth month, 86% during the tenth month, and 95% of those born at full term.[1] In these same infants, the proximal tibial epiphysis was found in 6% in the ninth month, 40% in the tenth month, and 85% of the full-term infants. Thus, the presence of the distal femoral epiphysis indicates a gestational age of approximately 35 to 36 weeks. The presence of both the distal femoral and proximal tibial epiphyses indicates a gestational age of over 36 weeks. Also to be noted is the fact that females develop these growth centers slightly earlier than males[1] and Negroes develop them earlier than Caucasian infants.

However, the distal femoral ossification center may also develop in fetuses at earlier ages than 35 weeks. In a group of 42 infants weighing less than 2000 grams at birth, the distal femoral epiphysis was found in one of 11 White males, three of six White females, two of 11 Negro

males and seven of 14 Negro females.[9] Considering that the fetus weighs 2000 grams at approximately 33 1/2 weeks of gestation, this means that among these obviously premature infants, 14% of the males, 50% of the females, 36% of the Negroes and 23% of the White infants would have been incorrectly diagnosed as mature by X-ray technique. Another study of 100 cases[10] presents its results as follows: In 26 cases judged premature both by X-ray and history, 62% were premature by birth weight; in 12 cases judged premature by X-ray but mature by history, 67% were premature; in 15 cases judged premature by X-ray but mature by history, 40% were premature and in 47 cases judged mature by both methods, 6% were premature. In other words, when X-ray techniques judged prematurity, they were incorrect 37% of the time and when judged mature, they were incorrect 15% of the time. It must be pointed out that in this last group there was a preponderance of cases in which history overwhelmingly pointed toward maturity which might tend to bias the results in favor of the X-ray diagnosis of maturity.

In the above-mentioned studies the X-rays have been taken after delivery. Under normal circumstances it is not possible to do this because we are interested in the fetal age so that we can decide whether or not to deliver the infant. Thus, the maternal soft tissues and skeleton must be added to the X-rays thereby complicating

the reading of the films. One study[35] showed that if the distal femoral epiphysis was considered present only if it exceeded 4 x 3 mm. in size. In this study, only 78% of all full-term infants demonstrated this size growth center. One could expect to consistently identify any epiphysis present if it were this size; but if smaller, the diagnosis could only be considered speculative.

Further doubt as to the reliability of epiphyseal development as a means of predicting fetal age was cast by Scott and Usher[44] who reported that epiphyseal development is late if the fetus is moderately underweight or otherwise underdeveloped for a known gestational age. In their series, 37% of "malnourished" infants (25% or more underweight for known term gestation) failed to radiologically demonstrate a distal femoral epiphysis. Even when present, the growth center averaged 35% less in diameter than that found in infants of normal birth weights. The proximal tibial epiphysis was absent in 84% of these infants.

Thus, it may be seen that the X-ray diagnosis of fetal age is accurate in about two-thirds of the cases. It may be in error in either direction by equal amounts, that is, it may predict maturity when the fetus is immature or it may predict immaturity when the fetus is mature. If both the distal femoral and the proximal tibial epiphyses are present, one can fairly safely say that the fetus has attained the gestational age of 36 weeks.

Newer Methods of Determining Fetal Age

Amniotic Fluid Cytology

It was first noted by Kittrich[24] that certain cells present in amniotic fluid exhibit differential staining characteristics that are of assistance in estimating fetal age. He obtained vaginal vault aspirates from women who were near term and who had a question of whether or not the membranes had ruptured. The aspirates were stained with Nile Blue sulfate and examined for the presence or absence of cells staining orange or red. If the membranes had previously ruptured, the cells were usually seen but were always absent if the membranes were intact. At this time Kittrich noted that "reliability of the method seems to vary with the quantity of fat-containing epithelium shed from the skin of the fetus with the liquor--it increases with term."

Further investigation of this method was performed by Brosens and Gordon in a series of articles.[6,7,19] In these series specimens were obtained by amniocentesis or by aspiration of the bag of forewaters at time of delivery. Slides were made from the uncentrifuged amniotic fluid and stained with 0.1% Nile Blue sulfate. Four types of cells are found on slides made in this manner:[48,22] (1) blue-staining anucleate cells probably from the fetal epithelium, (2) blue-staining nucleate cells from the amnion,

(3) unstained anucleate cells which are identical to those found in slides prepared from vulvar scrapings, and (4) orange-staining anucleate cells. These orange-staining cells may stain diffusely orange or contain vacuoles which appear orange against a rather poorly colored cytoplasm. These cells are found in the amniotic fluid generally around the thirty-fifth week of gestation and tend to increase in number as term nears. Their origin has been traced to the fetal sebaceous glands. In frozen-section specimens of stillborns, cells staining orange with Nile Blue sulfate were found only in the active sebaceous glands. No cells were present if the section contained no sebaceous glands.

For a moment let us examine the differential stain used in these studies. Nile Blue sulfate is a commerical name for a differential fat stain which is a mixture of an oxazine sulfate (true Nile Blue) and the oxazone (Nile Red). Neutral lipids tend to be stained varying shades of red to orange while other substances are stained various shades of blue. The most commonly used concentration is a 0.1% aqueous solution. Other differential fat stains have not as yet been reported as being helpful in the diagnosis of fetal age by amniotic fluid cytology. Pinacyanole chloride (Vernitest) as described by Averette[3] has been used for this purpose but this is not truly a differential fat stain. With this test it is difficult to consistently determine the difference

between the fetal and maternal cells and contamination is a frequent problem. Van Leeuwen, et al,[48] have described amniotic fluid cytology using the staining method of Papanicolaou but this method fails to produce a good differentiation between the lipid-containing and non-lipid-containing cells. However, their description of "acidophilic-staining nuclear ghosts" are probably the same cells as those described as "orange-staining" by Brosens and Gordon.

In 1966 amniotic fluid cytology was presented as a method expressly for the determination of fetal maturity. Brosens and Gordon[19] correlated weeks of gestation with percentage of "orange-staining cells" as follows: Less than 1%--less than 34 weeks' gestation; 1 to 10%--34 to 38 weeks'; 10 to 50%--38 to 40 weeks'; over 50%--over 40 weeks' gestation.

Several recent articles have been published further evaluating the method. Anderson and Griffiths[2] found no cells of the sebaceous type prior to 36 weeks' gestation in 62 patients. They further stated that "zero counts do not necessarily indicate immaturity." In their method they utilized a Newbauer counting chamber and reported their findings in cells per cubic millimeter. Their correlation of counts in excess of 1000 orange-staining cells per cubic millimeter with a minimum of 40 weeks of

gestation would roughly equal 50% by Brosen's and Gordon's method. Counts in the range of 200 to 500 cells per cubic millimeter correlate with gestation of 37 to 40 weeks.

Amniotic Fluid Alkaline Phosphatase

It was first noted by Beck and Clark[4] that the maternal levels of plasma alkaline phosphatase were significantly elevated over normal nonpregnant levels, the increase being greater during the latter stages of pregnancy. The increase was shown to occur in two stages, a moderate increase beginning during the mid-portion of the second trimester, the increase becoming greater during the third trimester. The increase reaches a peak just prior to or at the time of delivery. Postpartum there is an abrupt decline in the maternal plasma alkaline phosphatase until normal nonpregnant levels are reached approximately six weeks after delivery. The magnitude of the overall increase tends to vary with the individual but ranges are usually considered to be 200 to 300% of normal. The increase tends to be more or less constant for a given individual in succeeding pregnancies.

Although certain of the serum or plasma enzymes which increase in the maternal circulation show a corresponding increase in the cord blood (e.g., glutamic oxalacetic transaminase and lactic dehydrogenase), alkaline

phosphatase is not elevated in the cord blood.[18] Simultaneous values in the maternal plasma and the amniotic fluid reveal a significant increase in the amniotic fluid levels but not to the same extent as the maternal levels.[25] Maternal values in this study were found to be in the range of 5.5 to 19.1 Shinowara-Jones-Reinhart units with an average of 11.9. Simultaneous amniotic fluid levels (at time of delivery) were found to be 2.5 to 12.5 with an average of 5.6.

This degree of elevation of maternal levels applies only to single pregnancies, a somewhat larger increase occurs with twin pregnancies.[34] The increase found with twin pregnancies averages about 32% higher than single pregnancies. Also, to be noted is that the increase is greater when the fetus is male than when it is female.[4] This increase in favor of the males is statistically significant during the tenth lunar month and averages 28% greater than levels found with female fetuses.

The source of the elevated maternal alkaline phosphatase is not the fetal circulation because, as was previously mentioned, fetal (cord blood) levels of alkaline phosphatase are not elevated at birth. A portion of the increase is probably indirectly due to the fetal growth, however; that portion being due to an increased mobilization of calcium from the maternal skeleton which transfers to the fetus. This portion of the alkaline phosphatase

increase beings at approximately the fourteenth week of gestation, the same time at which the fetal skeleton begins to calcify. However, the majority of the alkaline phosphatase rise has been traced to one of the isoenzymes of alkaline phosphatase which is produced by the placenta. This fraction has been characterized electrophoretically[43] but is more easily identified by its unusual ability to withstand heat denaturation.[36] "Placental alkaline phosphatase" retains virtually 100% of its activity following heating to 56° C. for 30 minutes. "Regular alkaline phosphatase" (originating from bone, liver, etc.) loses over 90% of its activity under the same conditions. The source in the placenta of this isoenzyme has been shown to be the syncytial border of the placental villi.[12] In this region it remains separated from the fetal circulation by the mesenchyme of the villi. The exact method by which it appears in the maternal circulation and in the amniotic fluid is not certain.

The continued increase in maternal alkaline phosphatase during the latter stages of pregnancy is paralleled by the growth of the placenta. As might be expected, in cases where the placenta is abnormal in size or function (e.g., toxemia) the maternal serum level of heat-stable alkaline phosphatase is low.[33] The changes may be even more striking in cases of severe fetal distress or fetal death.

In cases in which the fetus and placenta are considered to be normal, the alkaline phosphatase levels should rise in such a manner as to make possible a correlation between alkaline phosphatase level and fetal age. However, normal cases show a wide range of values for a relatively certain gestational age. Perhaps a portion of the heat-stable fraction is inactivated by the liver or excreted in some manner. If so, possibly amniotic fluid levels of alkaline phosphatase or the heat-stable placental isoenzyme would give a more accurate picture of the placental and fetal growth.

Amniotic Fluid Creatinine

It has been noted that certain nitrogenous constituents of the amniotic fluid are found to be elevated with respect to the concentrations normally found in the maternal serum or plasma.[31,8] Among these constituents is creatinine, the concentration of which appears to vary according to length of gestation.

The difference in creatinine concentration between the maternal serum and the amniotic fluid was first described by Makepeace, et al, in 1931.[31] While analyzing amniotic fluid specimens for various other substances, four samples were checked for creatinine concentration. Three of these samples were from term pregnancies, one was obtained by

amniocentesis at approximately four months of gestation. By the method of Folin and Wu, the following results were obtained for the term specimens: 1.67 mg.% (serum) vs. 2.30 mg.% (amniotic fluid), 1.54 vs. 2.61 and 1.43 vs. 2.00. The results from the four month gestations were 1.39 vs. 1.43. No conclusions pertaining to the cause of this elevation were drawn at this time.

What about the maternal creatinine concentration? Does it also rise normally during pregnancy as does the amniotic fluid creatinine? Does the amniotic fluid creatinine rise with increasing gestation? And, at what point during pregnancy does the rise begin? An extensive study of renal function during pregnancy was carried out by Sims and Krantz in 1958.[45] They found that the true plasma creatinine by the Hare method (to be described later) was lower in pregnancy than in a group of nonpregnant controls. The values were 0.67, 0.14 mg.% for the nonpregnant controls vs. 0.46, 0.13 mg.% for the pregnant subjects with very little overlap between groups. There is no statistically significant change in plasma creatinine levels during pregnancy however there is a prompt return to normal nonpregnant levels within twelve weeks postpartum. These changes are the result of an increased renal function during pregnancy, namely, renal plasma flow increases approximately 25%, glomerular filtration rate increases approximately 50% throughout pregnancy and the filtration fraction increases

approximately 40% by term and decreases irregularly to normal over several months postpartum as opposed to the others who promptly return to normal.

It appears then that maternal creatinine is somewhat lower than normal during pregnancy due to increased renal clearance function. Why then does the amniotic fluid show an increase in creatinine late in gestation? According to studies of creatinine transport between the fetus and the maternal circulation, creatinine appears in the amniotic fluid due to excretion by the fetal kidneys.[28] These kidneys begin to function at approximately twelve weeks of gestation but probably do not excrete significant amounts of urine until well into the third trimester. The creatinine then passes into the maternal circulation by means of free diffusion through the chorioamnion. There is no known transport mechanism for this process.

The attempt to correlate amniotic fluid creatinine with fetal age was made by Pitkin and Zwirek in 1967.[38] Obtaining amniotic fluid by amniocentesis, they analyzed it for creatinine concentration only. There was no detectable correlation between the creatinine concentration and maternal age, parity, or maternal complications of pregnancy such as anemia, chronic hypertension, pre-eclampsia, diabetes mellitus, urinary tract infection or Rh sensitization. It was noted that the creatinine undergoes a gradual

increase until approximately the 34th week at which time it begins a more abrupt rise. The relationship between a pregnancy of 37 or more weeks and amniotic fluid creatinine level of 2 mg.% or more was determined to be highly significant by statistical analysis ($p < 0.05$). Also, obtained were maternal serum creatinine levels selected at random. No variations from the previous observations that maternal creatinine levels are slightly lower than nonpregnant levels were found.

Spectrophotometric Analysis of Amniotic Fluid

The newest of the methods of predicting fetal age to be discussed here involves the use of spectrophotometry. For several years spectrophotometric analysis of the amniotic fluid has been used as the prime means of predicting the severity of fetal involvement in erythroblastosis fetalis. 5,26,30,41,42.

The use of spectrophotometry for this purpose is based on the observations that certain pigments present in the amniotic fluid in cases of erythroblastosis fetalis produce characteristic peaks of absorbance or transmittance when they are analyzed on a spectrophotometer.[5] The peak which most closely correlates with the degree of affliction present is the bilirubin peak which occurs at 450 to 455 millimicrons. There are several other peaks present in the range frm 350 to 700 millimicrons over which the analysis is performed

and they must be noted so as to not cause false values to be calculated. The most common of these peaks are: meso-bilirubin--425 millimicrons, unidentified heme pigments--410 to 415 millimicrons, oxyhemoglobin--415 and 540 and 575 millimicrons, methemalbumin--630 millimicrons and meconium--400 to 405 millimicrons.[26] The meconium peak is usually present if there is some degree of fetal distress, the oxyhemoglobin peaks are present if there is some degree of contamination of the specimen with blood during the amniocentesis. The spectrophotometer upon which the analysis is performed may be of any available type, however, the continuous-recording type is used, the wave lengths to be recorded should be the following (all in millimicrons): 350, 365, 380, 400, 410, 415, 420, 430, 440, 450, 460, 470, 485, 500, 515, 530, 540, 555, 570, 585, 600, 620, 640, 460, and 700.[26] These points will provide a rough form upon which the entire curve may be hand drawn. If the curve is plotted on semilogarithmic paper on a continuous recording spectrophotometer at a constant speed, a linear plot is obtained. A curve is plotted along the so-called base line, that which would be expected if there were no bilirubin present in the amniotic fluid. The distance between this base line and the 450 millimicron peak is converted into its corresponding optical density (O.D.) difference. This value is then plotted on a chart which gives the range of severity of fetal affliction. These

ranges correlate well with expected fetal survival rates.[26,42]

It has been observed that if serial determinations of this O.D. difference are performed and the plotted on a chart with weeks of gestation as the abscissa, definite trends can be noted. In general, if the succeeding values tend to decrease, the fetus has an excellent chance of survival. However, if succeeding values increase, the disease is progressing and therapy (intrauterine transfusion or delivery) should be instituted.

It is this decrease of the optical density peak at 450 millimicrons which has been reported as an aid in determination of fetal maturity. Mandelbaum, et al[32] have reported that readings of 0.01 or greater (O.D. difference at 450 millimicrons) are seen infetuses of 35 weeks' gestation of less while readings of 0.00 are seen only if the fetus is 36 weeks of gestation or more. They noted that successive values should be obtained and that the decrease to readings of 0.00 may occur very rapidly (less than one week). In infants delivered (spontaneously or Cesarean section) after the readings were .00, birth weights all exceeded 5 pounds, 85% of them exceeded 6 pounds. In the group whose readings were greater than .00, all weighed less than 5 1/2 pounds except for one infant who died within several days of respiratory distress syndrome.

Speculation on the reason for this decrease in

the 450 millimicron optical density peak has failed to yield a cause. It is possible that the fetal hepatic function increases at this time so that the bilirubin pigments are more efficiently metabolized by the fetus. If this occurred, less bilirubin would be available for excretion by the fetus and less would appear in the cord blood to be transferred across the placental barrier into the maternal circulation where it is metabolized by the maternal liver. However, at this time, this possibility has not been thoroughly investigated.

Comparison of Newer Methods of Predicting Fetal Age

Materials

A total of 16 amniotic fluid specimens were obtained from a total of 13 patients. Each specimen was analyzed for creatinine, alkaline phosphatase (10 specimens were also analyzed for heat-stable alkaline phosphatase), spectrophotometric analysis to determine the optical density difference from normal at 450 millimicrons and cytologic examination to determine the percentage of fat-containing cells present.

Ten specimens were obtained by amniocentesis between the 29th and 38th weeks of gestation. Amniocentesis was performed using the method of Queenan and Adams[40] with minor modifications. All patients were Rh negative and had been previously sensitized as was determined by antibody titer.

Three specimens were obtained at time of Cesarean section by aspiration of the amniotic sac just prior to incision of the uterus. An 18-gauge needle and 20 cc. syringe was used. In two other cases aspiration failed because of an anterior placenta which caused the specimens to be grossly contaminated with blood. In another instance no fluid was obtained because of oligohydramnios although no fetal abnormalities were detected at the time of delivery.

The remaining three specimens were obtained at time of vaginal delivery. A 20-gauge needle and 20 cc. syringe was used to aspirate the presenting bag of forewaters as soon as the cervix was dilated sufficiently to permit the procedure to be performed without danger to the fetus. It was noted that this was the least satisfactory of the methods of obtaining amniotic fluid. There was an excess amount of vernix caseosa in the fluid which was rather difficult to remove even by prolonged centrifugation. Also as will be noted later, labor itself induces a mild degree of fetal distress and the amniotic fluid may contain meconium at the time of aspiration. This adds greatly to the problem of spectrophotometry and negates any alkaline phosphatase results.

Immediately after obtaining the specimens they were placed in brown bottles to protect them from the light. They were centrifuged within 30 minutes at speeds of 2000 to 2500 rpm for a minimum of 15 minutes. If the procedures were not to be carried out immediately, the specimens were

then frozen until the following morning. Prior to analysis all specimens were subjected to vacuum filtration through a Millipore filter to assure complete removal of all particulate matter.

Methods

1. Amniotic fluid cytology.

Prior to centrifugation of the amniotic fluid specimens, approximately 0.5 milliliter was removed from the specimen and saved for cytologic examination. If the examination was not to occur until the following morning, the sample was refrigerated overnight.

Slides for cytologic examination were made as follows: Two drops (approximately 0.05 milliliter) of uncentrifuged amniotic fluid were placed in the center of a clean glass microscope slide. Three drops of 0.1% aqueous Nile Blue sulfate stain was then added to the slide. The stain and fluid were thoroughly mixed on the slide by means of a small glass stirring rod. A coverslip was then placed over the mixture. The slide was heated gently for a period of two minutes by warming over open gas flame. At no time was the temperature sufficient to cause the specimen to boil. After cooling, the slide was examined under the low power objective on a microscope. Two such slides were examined for each amniotic fluid specimen. A count of 200 cells was made on each slide and the average of orange-staining cells

was determined. For a cell to be counted as "orange-staining" it was not necessary for the entire cell to stain orange, but rather if there were orange or red vacuoles present in the cytoplasm the cell was considered positive. Red blood cells and white blood cells when present were not considered in the total 200 cell count. When clumps of cells were present, the number of cells in the clumps was estimated as well as possible. In a number of the specimens additional slides were prepared but were not heated. It was ascertained that if the slides were allowed to stand for 1/2 hour, no heating was necessary. Shorter periods of time were not tested nor were any slides read immediately after preparation and prior to heating.

2. Creatinine

Creatinine levels were determined following the centrifugation and filtration process. The samples were tested by the Jaffe picric acid method[21] which has been adapted for use with the Technicon Auto-Analyzer. This method is based on the development of a yellow to red color which is produced by the interreaction of creatinine and picric acid in an alkaline solution. This reaction also measures certain other "chromogens" in addition to creatinine.[15] Some of these are acetone, pyruvic acid, ascorbic acid, proteins, barbiturates, phenolsulfonphthalein, and sodium sulfobromophthalein. Their interference with the true levels of creatinine is dependent upon the method

of deproteinization of the specimen and the time allowed for color development. Standardization of the time involved such as is controlled on the Auto-Analyzer prevents most of the interference caused by these other "chromogens." True creatinine levels may only be obtained if the specimen is first treated with Lloyd's reagent of fuller's earth. The creatinine is adsorbed onto these substances and is then removed by elution. The Jaffe reaction is then carried out on the Lloyd's reagent or fuller's earth. Values obtained by this method are slightly lower than those utilizing the Jaffe method. As studied by Hare,[45] true creatinine varies as a percent of the total chromogens but is in the range of 80 to 90% of the values obtained by the Jaffe method.

3. Alkaline phosphatase and heat-stable alkaline phosphatase.

Total and heat-stable alkaline phosphatase was determined using the Powell and Smith[39] modification of the Kind and King[23] method. In this procedure the substrate used is phenyl disodium phosphate which is hydrolyzed to phenol by the action of the alkaline phosphatase. The phenol is then reacted with 4-amino-antipyrene and oxidized with alkaline potassium ferricyanide which yields a red colored product. This is measured colorimetrically and is calculated in King-Armstrong units. This entire process is

run on a Technicon Auto-Analyzer. For the heat-stable alkaline phosphatase the amniotic fluid was first heated to 56° C. for 30 minutes and then treated the same as the unheated specimens which were used for the total alkaline phosphatase determinations.

4. Spectrophotometric analysis.

Specimens were subjected to spectrophotometric analysis over the 350 to 700 millimicron wavelength range. The instrument used was a Coleman Hitachi Model 124 double-beam ultraviolet-visual ratio-recording spectrophotometer. A water blank was used to determine 100% transmittance. Readings were printed on semilogarithmic paper on the continuous recording readout portion of the spectrophotometer. The baseline was determined by drawing a straight line from the base readings on either side of the 450 millimicron peak. The optical density difference between this baseline and the peak was then calculated and recorded.

Discussion

In order to estimate the fetal weight at time of examination for specimens not obtained within one day of delivery, Lubchanco's growth curve[27] was applied to the birth weight of the infant and the weight for a given gestation prior to this time was calculated. An example would be if an infant were born with a birth weight of 3400 grams. This correlates with 40 weeks' gestation on the growth curve.

If a specimen of amniotic fluid was obtained 4 weeks prior to this time, we note on the curve that the weight at 36 weeks would be expected to be 2475 grams. This would then be assumed to be the fetal weight at the time of examination. Although this may cause a minor degree of error in certain cases, this approximation is accurate enough for most purposes and would be expected to be more accurate than the use of Naegele's rule in calculating gestational age.

Using these weights, graphs correlating weight at time of examination with the following parameters were prepared: (1) amniotic fluid cytology, (2) creatinine, (3) alkaline phosphatase (including heat-stable alkaline phosphatase wherever applicable), and (4) optical density difference at 450 millimicrons. The graphs are seen on the following pages.

It should be noted that a preponderance of the samples (9) were obtained for the purpose of ascertaining severity of fetal involvement with erythroblastosis fetalis. In each of these cases the maternal Rh antibody titer was positive to a titer of 1:8 or greater. The prime purpose of attempting to determine fetal age of these amniotic fluid samples was to conduct a pilot program to determine the feasibility of a larger study to follow. However, we can make certain observations from the results obtained.

The cytologic diagnosis of fetal age as previously discussed involves a relatively simple distinction between

positive cells (those staining orange or red with Nile Blue sulfate) and negative cells (those staining various shades of blue). This distinction can easily be made under the scanning lens of a microscope after the observer has a certain amount of experience with the method. There tends to be an extremely rapid rise in percent of positive cells after the 36th week of gestation or 2500 grams gestational weight as has been previously noted by others.[7,19,2] Prior to this time the percentage generally stays below 3%. To calculate the cytology by means of a counting chamber and reporting the number of positive cells per cubic millimeter as has been advocated by Anderson and Griffiths[2] seems to offer no particular advantages over merely performing a differential count on a wet mount as was done here.

As may be noted in Table II, creatinine levels in the amniotic fluid form almost a straight-line function when plotted against gestational weight. The method used to determine these values was the Jaffe method[21] which was previously discussed in detail. Since the study was started, Thiede has advocated the exclusive use of the Hare method of determining creatinine in amniotic fluid. How much difference there actually is between the methods awaits further investigation. If fetal kidney function is the prime producer of the amniotic fluid creatinine, then the Hare method[20] would appear to be the best method of determining

the level of activity present because the presence of 0.2 mg.% of "false" creatinine in a given sample could cause an error in calculation of 300 grams of fetal weight or approximately 10 days of gestation according to the results determined here. Of the methods tested here, creatinine produced the most reliable results. It was not affected by the presence of bilirubin or meconium in the amniotic fluid nor by the maternal factors such as diabetes mellitus.

Alkaline phosphatase levels in the amniotic fluid have not to my knowledge been reported as a test to determine fetal age. It was found that values tend to increase with gestational age and weight but that the "scatter" of values for a given weight is large. The correlation of heat-stable alkaline phosphatase as a percentage of total alkaline phosphatase activity for a given gestation produced no meaningful results. A completely random character dominated these statistics. Several of the alkaline phosphatase readings were inordinately high for the fetal weight and it was noted that in each of these three specimens there was a definite spectrophotometric peak at 400 to 405 millimicrons indicating the presence of meconium in the amniotic fluid. One of the specimens was grossly stained with meconium. The alkaline phosphatase level in this specimen was "over 38 King-Armstrong units," no dilution was performed to further determine the exact level. In another specimen whose optical

density curve indicated the presence of moderate amounts of meconium but was mildly meconium stained produced a level of 220 King-Armstrong units which was determined by diluting the fluid.

Spectrophotometric analysis of the amniotic fluid specimens for fetal age was hampered to some extent by the prime reason for obtaining the majority of the specimens, that of determining severity of fetal involvement with erythroblastosis fetalis. As has been previously discussed, this produces a peak at 450 millimicrons in proportion to the degree of fetal involvement. The prediction of fetal age by optical density difference at this same wavelength is based upon the decrease in this peak to almost zero during the latter portions of gestation. For this reason only four of the samples demonstrated this strikingly low level, all of these having a gestational weight of at least 2800 grams. Two of the specimens were found to have meconium present to such a degree that they completely overshadowed the 450 millimicron peak and therefore made the optical density difference at this reading usefulness.

Conclusions

Realizing the shortcomings of a small series of cases, several conclusions may be drawn from the results presented here.

Of the methods presented, only amniotic fluid creatinine fails to be affected by maternal diabetes mellitus, hydramnios, erythroblastosis fetalis, meconium staining of the amniotic fluid or placental insufficiency. The standard deviation from the straight-line values in Table II was less than 0.2 milligrams percent for all values.

Among the other methods, cytologic examination of the amniotic fluid was the most accurate. The increasing values in this method tend to be logarithmic, the steepest increase occurring after 36 weeks' gestation or 2500 grams fetal weight.

Spectrophotometric analysis is virtually useless in predicting fetal age in cases of erythroblastosis fetalis, a group of patients comprising a large percentage of those in whom fetal age is in question.

Alkaline phosphatase levels in the amniotic fluid were found to rise significantly with gestational weight but tend to vary considerably from case to case. Values tend to be spuriously high in cases where there is meconium staining of the amniotic fluid and abnormally low when there is placental malfunction.[30] Calculation of heat-stable alkaline phosphatase activity as a percentage of total alkaline phosphatase activity produced no meaningful results.

Summary

Several methods of determining the fetal age and

weight have been presented with the purpose of determining the feasibility of a larger study comparing the reliability of several methods upon the same specimen. Each of the methods presented is relatively simple, quick to perform, available in most hospital laboratories, and considerably cheaper than presently used X-ray methods. The methods presented, with the exception of X-ray and patient history, all require the use of amniocentesis to obtain amniotic fluid. While amniocentesis is not without risks, it has an extremely low incidence of morbidity. Of more importance is the risk of danger to the pregnancy if fetal age is not determined so that the proper therapy may be instituted.

Of the methods compared, creatinine levels in the amniotic fluid demonstrated the greatest degree of accuracy and reliability. The presently used methods of X-ray and patient history are accurate in only two-thirds of the cases and variation in a given range of fetal age is great.

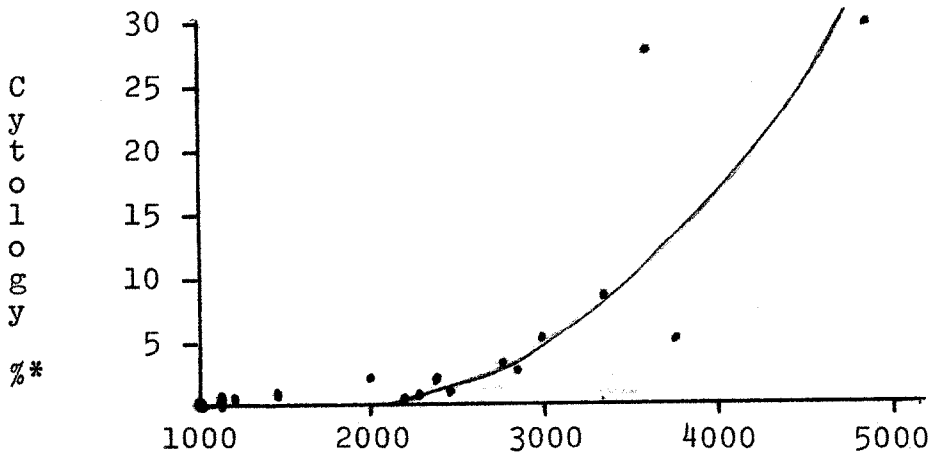
It is hope that these studies will be enlarged upon and the value of using several methods to determine fetal age will be borne out.

TABULATION OF RESULTS

Case No.	Gestational Weight (gm)	Cytology (%)	Creatinine (mg.%)	Alk. Phos. K-A u.	Heat Stable (% of total)	OD Diff.
1.	2300	0.2	1.2	1.6		.084
2.	2500	0.6	1.6	2.8		.049
3.	1250	0.1	1.1	1.0		.055
4.	2000	2	1.6	3.0		.066
5.	1000	0.1	1.0	2.0		.055
6.	1500	0.5	1.2	2.0		.092
7.	3825	5	2.2	4.5	50	.009
8.	2215	0.1	0.9	2.2	73	.219
9.	3375	10	1.8	4.0	50	.009
10.	2800	4	21.	212	32	.000
11.	4800	30	2.9	1.4	79	.019
12.	2350	1	1.9	over 38	over 90	.369
13.	2950	6	2.0	220	5	.120
14.	2900	3.5	1.8	4	75	.056
15.	3675	28	1.4	62	8	.070
16.	1150	0.1	0.9	1	100	.060

TABLE I

Gestational Weight (grams)



* Percentage orange-staining (fat-containing) cells

TABLE II

Gestational Weight (grams)

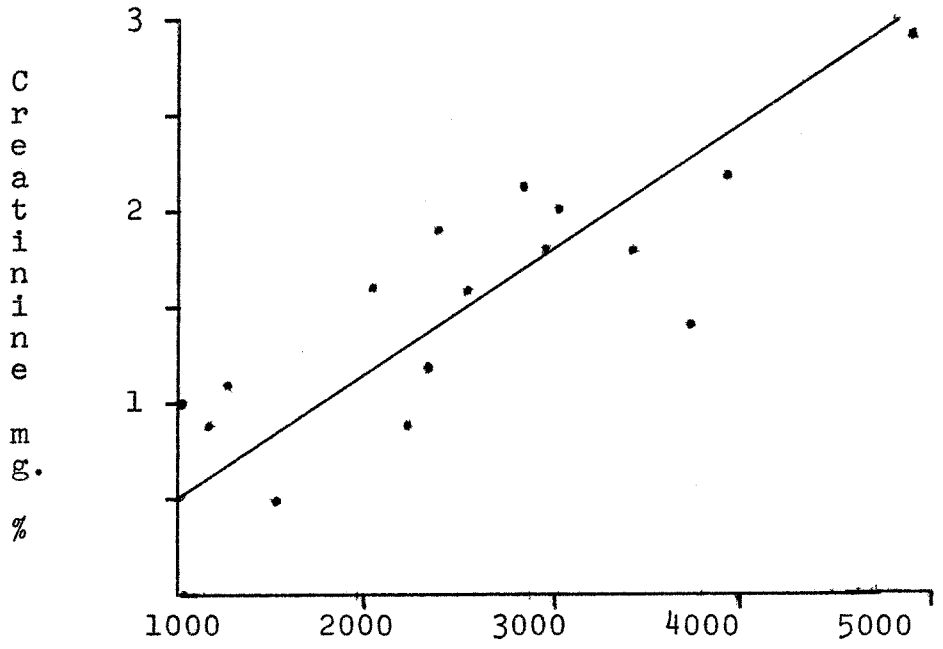
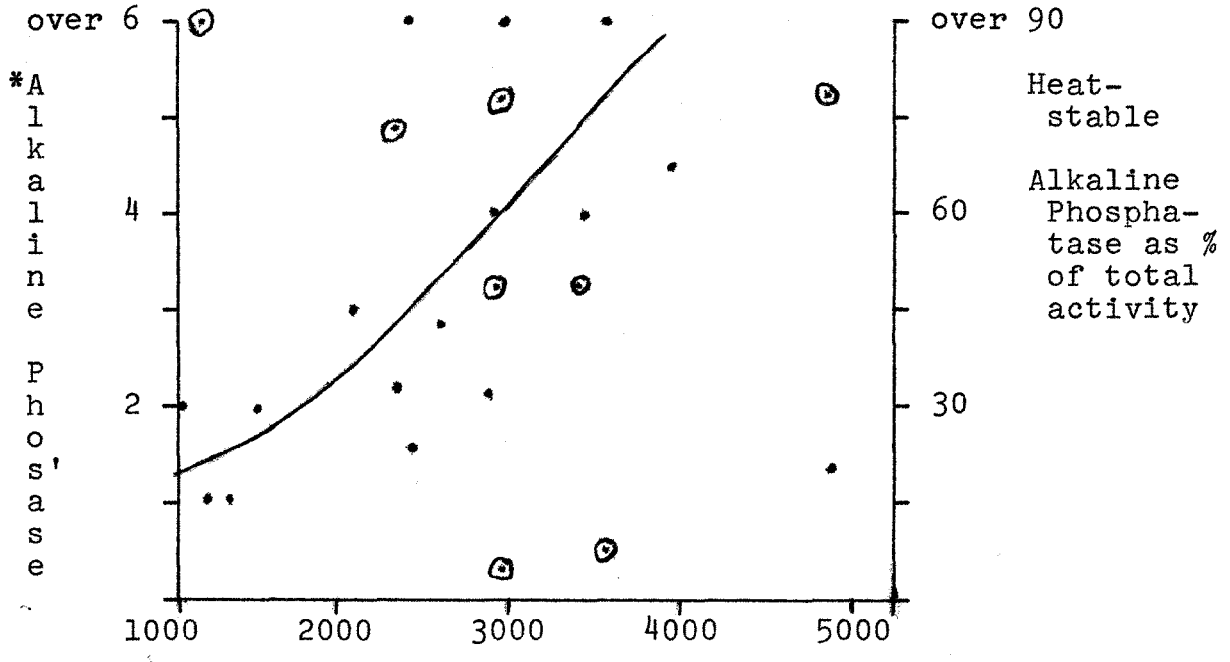


TABLE III

Gestational Weight (grams)



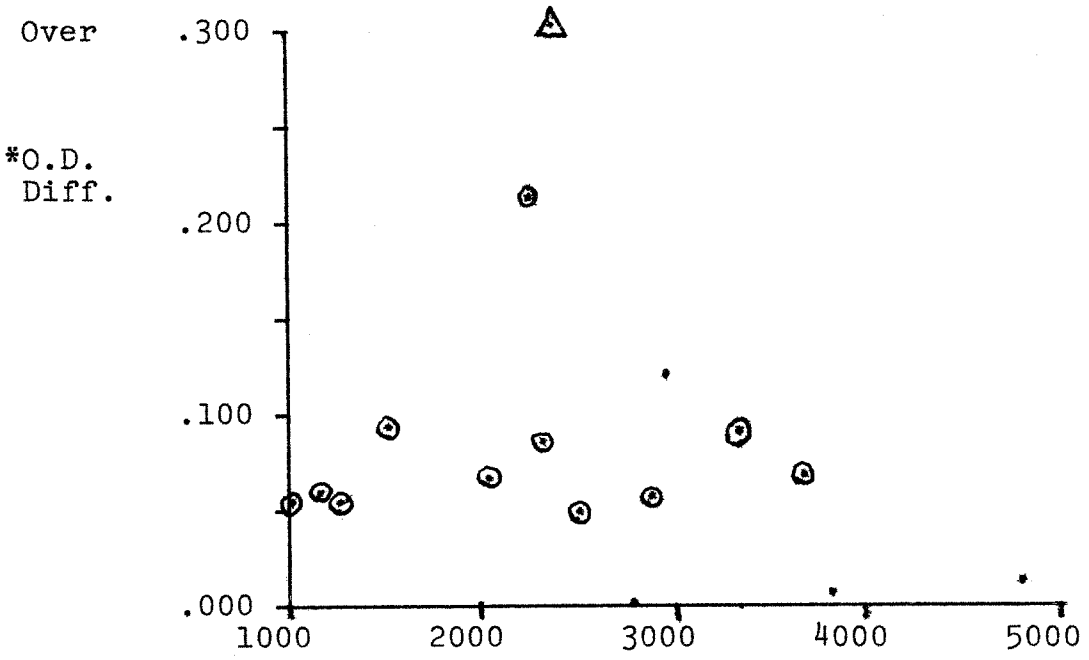
* Alkaline Phosphatase (King-Armstrong units)

· = Alkaline Phosphatase

0 = Heat-stable Alkaline Phosphatase as % of total activity

TABLE IV

Gestational Weight (grams)



*O.D. Difference at 540 millimicrons

△ = Meconium-stained amniotic fluid

⊙ = Rh-negative sensitized patients

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