gested as a lipid- specific fluorochroming substance in the diagnosis of rupture of the membrane ([4]). Nile blue sulphate, however, has the advantage that is free of danger for the user.

SUMMARY

Results obtained with fluorescence microscopy in the diagnosis of rupture of the membranes and in assessment of foetal maturity Nile blue sulphate are presented.

Foetal lipid cells, stained orange when viewed in ordinary light, display a deep yellow-green fluorescence and the method thus enables an immediate double evaluation to be made from the same slide.

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Assessment of foetal maturity

by

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In many cases of pregnancy at risk, the foetus may have better chances in a nursery than in the uterus. The risk of death as a result of prematurity must obviously be avoided. The concept of maturity or « the state of being fully developed » ([1]), which had been instinctively associated with the size of the foetus, has now taken on a wider meaning.

It appears reasonable to admit that foetal maturity has been attained when the functional capacity of all the organs has reached the minimum level which allows the neonate to adapt to autonomous life.

The well-known factors which affect foetal growth are:

a) the maternal nutritional conditions;
b) placental sufficiency;
c) normal uterine blood flow;
d) the level of foetal insulin increase;

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e) maternal oxygenation;
f) genetic factors;
g) toxic factors;
h) factors related to the maternal constitution ([2]).

After an initial latent period related to the establishment of the placental function, the rate of growth in mammals proceeds cubically in relation to time, according to the following formula:

$$\text{foetal weight} = a\ (t\text{-}t')^3$$

where *a* is a constant which expresses the rate of nutritional supply per unit of foetal surface; *t* is the day of pregnancy; *t'* corresponds to 36 days after the beginning of the last menstrual period. Deviations from this pattern can be observed towards the end of pregnancy, in cases of twins, in women who smoke and in the presence of poor socio-economical conditions; all these circumstances represent a break in the relationship between the foetal requirements and the placental capacity to satisfy them.

It should be noted that other, unknown maternal factors may alter the value of *a* in the above equation ([3]).

The rules mentioned as valid for establishing normal physical growth are not always applicable to an assessment of the functional maturity of the various organs and systems of the foetus.

It is obvious that, in a normal pregnancy, a particular gestational age corresponds to a particular size (e.g. the biparietal diameter, the body length and weight) and to a particular degree of maturity of the various organs and systems. This harmonious anatomical and functional development is certainly ensured by regular placental function.

Consequently, under normal conditions, a close bond exists between the four factors mentioned: gestational age, foetal maturity, foetal dimensions and placental function. This bond is so close that when one of these factors has been established in the normal foetus, it should be possible to determine the others. For example, it would seem reasonable to expect that at the end of a normal pregnancy, without signs of placental insufficiency, a foetus in the 40th week would be of normal weight, length and functional maturity. Under pathological conditions (e.g. in toxoemia, in placental insufficiency, in diabetes, in Rhesus isoimmunisation, etc.), the bond between the four factors mentioned above (gestational age, foetal maturity, foetal dimensions and placental function) does not necessarily exist.

Consequently it is not always valid to establish more or less clearly a direct relationship between maturity and the dimensions of foetus. For example, the single dimensions of the biparietal diameter cannot always be taken as a measurement of foetal maturity.

Therefore, whereas it is fairly easy to establish foetal maturity under normal physiological conditions, it is much less easy to do so in the presence of complications.

In the light of the numerous complex functions of the body, foetal maturity is probably only attained when all the principal functions (and not merely some) have reached a minimum level of efficiency.

Only under normal conditions will a specific period of time spent in the uterus (gestational age) combine with normal placental function and normal maternal

metabolism in a manner which allows the foetus to undergo regular anatomical development and to reach an adequate level of functional maturity.

Under pathological conditions the period spent in the uterus (gestational age) may be used in various ways by the foetus. The less favourable environment may alter in an independent manner the physical development and the functional maturity of the organs. This unfavourable environment affects the foetus as a whole (for example in chronic nutritional placental insufficiency), and also produces sectional maturational changes (for example in pregnancy complicated by diabetes). In nutritional placental insufficiency, foetal maturity may correspond to the gestational age while it does not correspond to the foetal dimensions. In the presence of sectional maturational changes, the degree of maturity does not correspond to the gestational age and to the dimensions, and moreover the metabolically altered environment in which the foetus develops has a greater negative effect on some functions than on others. We thus observe here a discordant functional development of the foetus.

The direct or indirect symptomatological studies which have been suggested in order to determine whether the foetus is ready for extra-uterine life may be traditional ones which are largely indirect and involve the foetus or the mother, or the more modern ones based on examination of the amniotic fluid.

Among the foetal and maternal tests we should mention x-ray esamination, which can reveal the presence of particular ossification nuclei, as well as the foetal dimensions. This technique has marked limitations because of differences in interpretation; the factors which make for the differences are the sex of the foetus, twins, the variability in the pictures even when the foetal weight is the same, and the lack of uniformity in the findings ([4, 5]).

Radiographic visualisation of the ossification centres is therefore not a very reliable index of maturity, although some investigators assert that it permits an even more precise forecast of the circumstances relating to the birth than the menstrual history ([6]).

Another method of investigation which has almost completely replaced radiological examination (if only because of its safety) is the measurement of the principal foetal diameters by ultrasonic echography. If we keep in mind the previously expressed reservations on the relations between maturity and foetal dimensions in the various fields of abnormal pregnancy, there is no doubt that under normal physiological conditions there exists a firm relationship between the gestational age and the foetal development.

Serial measurements of the size of the foetus, which enable us to check on the regular development of growth and to compare divergent findings with normal data, may indicate not only foetal pathology but also maternal or adnexal abnormalities. Delays in growth may indeed result from an inadequate supply to the placenta, or may be due to pathological maternal conditions. This indirect method for measuring placental function is thus comparable in usefulness to that of estrogenuria ([7]).

Foetal growth may also be studied by determining the quantity of amino acids incorporated into the proteins in the foetal liver. A gamma-emitting labelled amino acid, 75-selenomethionine, may be followed in its passage to the foetus by means of external readings ([8]).

Apart from some technical difficulties due to the uncertain pattern of the radiation source, to the possible obesity of the patient, and to the localisation of the placenta (factors which can all alter the distance between the radiation

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source and the external counter), this investigation offers a high degree of display ([9]).

Of major importance among the investigations carried out on the mother are the exact determination of the history in regard to the last menstruation, and the clinical assessment of the uterine dimensions, nor must the importance of the cytological and hormonal interpretation of placental activity be overlooked. As has been said previously, the nutritional supply to the placenta may affect both growth and maturity.

Studies of the amniotic fluid, i.e. an environment which expresses the conditions of the foetus more directly, are of far greater importance, and may become even more so in the future. Preliminary studies of its constituents have recently been extended to reveal its variations during pregnancy, and to establish the correlation with foetal maturity.

One of the constituents of amniotic fluids is *creatinine.* This substance gradually increases from the 34th week on, and after the 37th week it attains a concentration which is two to three times greater than that in the serum. Because of this gradual increase it has been suggested as an indicator of foetal maturity ([10]).

The creatinine contained in the amniotic fluid is thought to originate by a process of secretion through the chorio-amnion from the maternal tissue to the amniotic cavity ([11]) and from foetal urinary excretion. The gradual increase in creatinine, completely filtered by the glomeruli and seabsorbed in the tubuli ([12]), might indicate not only the maturation of the foetal kidneys and in particular the glomerular filtration capacity ([13]), but also the development of the foetal muscle mass ([14]).

In evaluating the findings we must not overlook the limited diffusion of creatinine through the amniotic membrane ([11]) and the decrease in the volume of the amniotic fluid which is observed during the last three to four weeks of pregnancy ([15, 10]).

Under normal physiological conditions a concentration of more than 2 mg/100 ml creatinine in the amniotic fluid is thought to indicate a gestational age of more than 37 weeks, a foetal weight of more than 2500 g ([12]) and foetal maturity ([10, 14]). In the literature available, various critical levels are reported in order to indicate maturity: 1,6 mg % after the 37th week ([16]); 1,7 mg % after the 36th week ([17]); 1,8 mg % after the 36th week ([18]); 1,8 mg % after the 37th week ([12]).

In two cases of twin pregnancy the creatinine level was higt in relation to a relatively low foetal weight, and was thus not in correlation with the sum of the foetal weights ([15]). The average values are normal in foetal erythroblastosis ([10]), higher in prolonged pregnancy ([10]) and normal or raised ([12]) in pregnancy complicated by diabetes. In this case the increase in creatinine may indicate more advanced foetal maturity in relation to the gestational age, as has also been observed in radiological tests ([6]).

After intra-uterine death the level of amniotic creatinine falls abruptly ([28]). In pre-eclamptic toxaemia, normal values ([10, 19, 20]) and elevated values ([12, 15]) have been found despite the low foetal rate and the consequent reduction in the muscle mass. This anomaly cannot be explained by increased foetal production, but by an increase in maternal creatinaemia because of diminished glomerular filtration and a consequent increase in secretion through the chorio-amnion. On the other hand, in pre-eclamptic toxaemia, the increase in maternal creatininaemia is not always observed ([21]).

The correlations between amniotic creatinine, foetal rate and gestational age

have been much discussed in regard to slow foetal growth. The low creatinine values are thought to indicate prematurity only when development is normal, and not when it is slow ([22, 13]). Foetuses which are small-for-date have a smaller creatinogenic muscle mass and a lower rate of glomerular filtration. It may therefore be concluded that the titration of amniotic creatinine is least useful in those cases where it is most necessary ([19, 20]).

Another constituent of amniotic fluid which has been suggested for the determination of foetal maturity is *urea.* In the same way as creatinine, this substance increases in the amniotic fluid during the second half of pregnancy, and originates partly from the maternal blood through secretion by the amniotic epithelium, and partly from the foetal urine. The concentration of urea in the amniotic fluid increases during the second half of pregnancy until it exceeds that of the maternal plasma, after the 30th week ([23]). These variations are due to the foetal glomerular filtration, the diffusion through the foetal skin and the filtration of the maternal plasma. The role of the foetal skin diminishes between the 20th and 28th week, while the production of urine increases during this period. Thus, since the concentration of amniotic urea is related to the progression of the pregnancy, it may be used as an indicator of the gestational age. Whereas the maternal serum levels of creatinine remain constant, those of urea show wide variations which have repercussions on the amniotic concentrations by causing the maternal production to vary. In order to eliminate this source of error, which reflects not only the foetal conditions but also the maternal ones, it is preferable to take into account the difference between the urea concentrations in the maternal serum and in the amniotic fluid. This difference is more closely related to the length of gestation ([24]).

During the first pregnancy, the amniotic fluid is isotonic with the maternal and foetal plasma ([25, 26, 77, 28]). Its ionic composition is equivalent to that of an ultrafiltrate of the foetal plasma through the immature skin ([23]). As the pregnancy progresses, we observe a gradual reduction in the solutes, particularly in sodium ([23, 27, 29, 30]). After the 20th week a well-defined osmotic gradient develops between the amniotic fluid and the foetal maternal plasma ([23]). The determination of sodium and of the osmolarity of the amniotic fluid has been studied for the purpose of establishing the gestational age ([23, 31]). Foetal maturity is thought to have been attained at a concentration of solutes equal to or less than 250 mOsm/lithe ([31]).

Many investigations have been carried out on the protein composition of the amniotic fluid, which was thought to be similar to that of the interstitial fluids ([32, 33]). Immunological investigation of the proteins in the amniotic fluids has confirmed this hypothesis ([34]). The separation of the various fractions by means of zone electrophoresis has shown that, in contrast to what occurs in the rat ([35]), in man the concentration of albumin is always greater than that of globulin throughout the whole of the pregnancy ([36]), although the A/G ratio gradually varies as the foetus matures ([37]).

As the gestational age increases, there occurs a gradual decrease in the protein concentration of the amniotic fluid; this has also been studied in relation to Rhesus iso-immunisation ([38]). In the lightest cases the protein values remain essentially normal, whereas in severe cases, with foetal hydrops, there is generally an increase in the protein level independently of the period of pregnancy. If foetal death occurs or in the presence of severe malformations, we note a marked increase in the proteins ([39, 40, 41, 42]). A correlation between the gestational age and the amniotic protein picture may be obtained not by examining

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the total concentration, but only the pre-albuminic fraction which reflects the foetal contribution ([43]). This fraction gradually increases until the 38th week, and subsequently tends to decline in a characteristic manner. Its determination promises to become a useful diagnostic tool ([43]).

Among the most recently proposed methods for the establishment of foetal maturity is the radio-immunological determination of alpha-fetoprotein in the amniotic fluids. As we have seen, most of the proteins in the amniotic fluid come from the maternal blood; this does not mean that no proteins of foetal origin are present, and among these we find alpha-fetoprotein.

This protein is also found in the maternal serum in minute quantities ([44]), which gradually increase as the pregnancy advances ([45]). It is also a component of the foetal serum ([46]) and is synthetised by the liver ([47, 48]). It appears early in the amniotic fluid and it the foetal urine, and its amniotic concentration gradually decreases in such a manner that a statistically significant correlation can be established between this concentration and the gestational age ([49]).

Spectrophotometric analysis of the amniotic fluid has shown that the difference in optical density at 450 millimicrons (peak of absorption for bilirubin) gradually decreases as the gestational age increases, in cases where immunisation is absent ([50]). This observation has suggested the use of the bilirubin concentration in the amniotic fluids as a measurement of foetal maturity ([51]). The pigment, present from the 12th week of pregnancy, is reduced gradually to zero by the 36th week. The absence of a peak at 450 millimicrons would therefore indicate that foetal maturity is practically certain ([52]). This finding might be correlated with the maturation of a conjugating enzymatic system of the foetal liver, which causes the free bilirubin to disappear at that time ([53]). However, the large-scale application of the bilirubin method in order to determine the gestational age has yielded disappointing results ([54]). In contrast, the analysis of the phospholipids in the amniotic fluid is taking on great importance. The mitochondria of the alveolar epithelial cells produce a tensio-active lipoprotein with an active phospholipid component, which lines the internal surfaces of the alveoli and is indispensable for alveolar stability ([55]).

The concentration of this lipoprotein falls below critical levels in premature newborn or in those who have a « hyaline membrane » ([56]). Not all phospholipids have a tensio-active effect, but only a lecithin fraction which can be precipitated with acetone and represents initially approximately 11% of the total lecithin. This fraction can be extracted from the pulmonary parenchyma long before it appears in the fluid which bathes the alveoli.

An hour after birth, as respiratory activity becomes established, the tensio-active lecithin attains 50% of the total; this increase is much slower in premature newborn, who develop the neonatal respiratory syndrome ([57]). During the last part of pregnancy, the tensio-active lecithin accumulates in the cells and subsequently passes into the alveolar lumen ([58]).

The tensio-active effect is thought to be associated with the fatty acids composition of the phospholipids; the presence of saturated fatty acids in the alpha position ([59]) and in the beta position ([60]) is said to be particularly important.

With the foetal respiratory movements, the phospholipids from the alveolar fluid pass into the amniotic fluid ([61]). Consequently the lipid content of the amniotic fluid ultimately reflects the alveolar situation. Indeed, in prematurity or in neonatal respiratory syndrome, the amniotic fluid also shows a reduction in phospholipids and in lecithin in particular ([62]). Pulmonary maturity is attained after the

35th week, when the concentration of lecithin alone suddenly increases in the amniotic fluid. There is a biochemical explanation for this occurrence: from the 22nd to the 35th week the synthesis of lecithin takes place slowly through the methylation of phosphatidylethanolamine; the lecithin produced has palmitic acid in the alpha position and myristic acid in the beta position. After the 35th week another biosynthetic process for lecithin is activated, starting with citidin-diphosphocholine and with alpha, beta diglyceride. The lecithin produced has palmitic acid in both the alpha and the beta positions ([63]). Thus, after extraction of the lipids from the amniotic fluid and subsequent thin-layer chromatographic analysis, it is possible to establish a diagnosis of foetal pulmonary maturity. In particular, maturity has been attained when the concentration of lecithinic phosphorus is greater than 100 microgrammes/100 cc^3 of amniotic fluid ([64]). Maturity is probably also indicated by a ratio of more than 2 between the concentration of lecithin and that of sphingomyelin. Recent studies have shown that the determination of lecithin alone is the more important one ([64, 65]), since a variation is sphingomyelin may alter the L/S ratio. The introduction of this ratio was based on the hypothesis that the concentration of sphingomyelin remains uniform; this hypothesis has since been proven incorrect. A simplification which may be of clinical interest has been proposed: the use of the « foam test » ([66]). Although the concentration of triglycerides in the amniotic fluid shows characteristic variations, it is of limited usefulness for establishing foetal maturity ([67]). One of the most interesting constituents of the amniotic fluid is an estrogen, estriol, which is produced in large quantities by the combined action of the liver and the placenta. Estriol is initially found in the foetal circulation. Its presence in the amniotic fluid may be due to a direct passage from the latter, from the foetal urine or from the maternal circulation ([68]). Its concentration in the amniotic fluid is regulated by a distribution balance between the maternal and the foetal compartments. Nevertheless it reflects the foetal metabolism more directly than the maternal one ([69]). Estrioluria gradually increases as the pregnancy progresses. In the final weeks this increase is much more marked ([70]). An explanation of this finding must include the following points:

1) The entry and exit routes for the estriol from the amniotic fluid vary during the pregnancy.
2) The concentration in the amniotic fluid is determined not only by the concentration in the neighbouring maternal and foetal compartments, but also by the chemical form which conditions the passage from one compartment to the other.
3) The growth of the foetus brings about a gradual increase in the urinary excretion by the foetus.
4) The estriol in the amniotic fluid is found not only in free form, but also in conjugated form, in various combinations with sulphuric acid and glycuronic acid.

These chemical forms of estriol have different modes and rates of passage through the cellular membrane. The degree of conjugation of the estriol may reflect foetal maturation. The sudden increase in estriol after the 36th week is probably due both to increased foetal urinary excretion and to a low removal rate of the conjugated estriol from the amniotic fluid ([71]).

The above shows that amniotic estriol determination may act as an indicator of foetal maturity and of foetal conditions ([72]).

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Indeed, a good correlation has been found between estrioluria and the week of pregnancy.

On the other hand, an amniotic hormone level clearly below the norm, such as is found in severe Rh isoimmunisation in toxaemia and in diabetes, indicates a poor prognosis ([73]).

The correlation between estrioluria and foetal dimensions have been discussed ([70, 73]).

Estrioluria is thought to indicate the state of maturity of the foetal adrenal cortex, irrespectively of the gestational age ([70]).

Staining of the cells containing the amniotic fluid with Nile blue sulphate provides useful information in regard to assessment of foetal maturity ([74]). The number of orange stained anuclear cells increases together with the maturity of the foetus. After the 38th week of pregnancy we should always find that number of orange cells exceeds 50%. However, our experiments show that more than 65% of the pregnancies which have gone beyond 38th week show far less than 50% of orange cells in the amniotic fluid. This is due to the fact ([75]) that the orange stained cells appear so only because lipid material adheres to them. The degree of adhesion may vary greatly ([76]), which explains the disparity in the findings. Moreover, the sebaceous glands of the foetal skin are wholly secretory glands. This means that in proximity of the excretory ducts of the glands the cells dissolve completely and a mixture of fat, of keratohyalin granules, of keratin and of cellular debris is formed; this is sebum. Thus, the wholly secretory glands do not excrete cells (into the amniotic fluid) but, in the case of the foetal skin, excrete amorphous fatty material to which is added that produced by the amniotic epithelium and by the foetal lungs.

The drawbacks of the cytochemical method described by Brosens and Gordon were overcome by two means. Lind ([24]) suggested a score system in accordance with the gestational age, based on hematoxylin and eosin staining of the smear. The scoring was based on the reading of the smears according to the following criteria:

Three groups of gestational ages can be distinguished on the basis of the cellular morphology:

Early (32nd week or less): before the 30th week the few cells present are basal or pre-cornified, in almost equal proportion. After the 32nd week the pre-cornified cells predominate.

Intermediate (32nd-36th week): the number of cells increases and the basal cells diminish until complete disappearance around the 35th week. After the 36th week the cornified cells appear. The characteristic picture consists of 20% cornified cells, 80% pre-cornified cells, and an occasional basal cell.

Late (37th week and beyond): an equal number of pre-cornified and cornified cells is found during the 37th-38th week; the anuclear squamae indicate the approach of term.

Since our experience has shown that this method entails a margin of error, we concentrated our attention on a different approach, i.e. we studied the preparations by fluorescence microscopy. This was possible because Nile blue sulphate acts in the same way as fluorochrome ([77]). This method provides further data to supplement the findings obtained in the study of the orange coloured cells (because of their lipid coating) with the ordinary light microscope. Indeed, the gradual increase in large accumulations of lipid cells and of extra-ecllular lipid drops towards the end of pregnancy can be put to use, by enabling us

to estimate at a glance the « total quantity » of lipid material. It is this « total quantity of lipid material » present on the smears, rather than merely the number of lipid cells, which is of principal importance in determining the degree of maturity. Our method has two further advantages: it completely cancels out the background (which consists of lipid material) and allows a simultaneous double assessment (under ordinary light and by fluorescence microscopy) of the same preparation.

In order to demonstrate the validity of certain biological and cytological variables of the amniotic fluid, we tested some of the methods described above. In a normal pregnancy which terminates with the birth of a completely normal foetus in regard to physical and functional development, in the light of the points discussed above, foetal maturity should correspond to gestational age, which is always carefully recorded. Foetal maturity was not forecast in weekly terms because this does not have real clinical significance. Consequently we chose four groups of gestational ages: below the 30th week, between the 31st and 34th week, between the 35th and 37th week, beyond the 38th week. The creatinine level of the amniotic fluid, the difference in urea concentration of the amniotic fluid and of the maternal serum, and the cytological findings interpreted according to Lind's method were used to establish a score which, according to the author, should enable us to place 72% of the cases in their exact gestational age group. Instead of the percentage of diagnostic accuracy we calculated the mean error, in weeks, of foetal maturity on the basis of the aforementioned criteria (Table 1).

Table I. *Mean error for the biochemical variables in amniotic fluid and maternal serum and foetal maturity score with advancing gestation.*
(Gestational age is expressed in completed weeks)

MEAN ERROR IN WEEKS	DURATION OF GESTATION IN WEEKS			
	30 or Less	31-34	35-37	38-40
Amniotic creatinine	0	0	0	0
Creatinine (amniotic fluid-maternal serum)	0	0.5	0	0
Amniotic urea	0	−7.5	−12.0	−14.0
Urea (amniotic fluid-maternal serum)	+6.0	+0.5	+ 1.0	− 3.0
Amniotic sodium	0	+3.5	− 4.0	+ 2.0
Amniotic osmolality	0	−8.0	—	− 1.0
Scoring System	+2.5	+1.0	+ 3.0	0

We found that among the various methods, examination fo the creatinine level in the amniotic fluid is the one which most accurately enables us to place each case in one of the four gestational age groups. The amniotic urea concentration indicates a much lower degree of foetal maturity than is the case in

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reality. The difference in urea content between the amniotic fluid and the maternal serum, the sodium concentration and the osmolarity of the amniotic fluid are also markedly inaccurate indicators in this regard. The score system tends to indicate a more advanced degree of foetal maturity than is actually the case. Serial determinations, during the second half of pregnancy, of various substances contained in the amniotic fluid will undoubtedly lead to increasingly accurate determinations of foetal maturity in the future. Surely it will become possible to monitor foetal development by studying the composition of the amniotic fluid, in the same way as we can now accurately follow foetal growth by ultrasonic echography, but at present neither method is of reliable diagnostic significance.

SUMMARY

The assessment of foetal maturity particularly in relation with biochemical and cytological modifications of the amniotic fluid are rewiewed. The accuracy of various methods is compared.

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