Clinical Experimental Obst. & Gynecology
Vol. III, 1-4, 1976 pp. 96-103

Gas chromatographic study of fatty acids in vernix caseosa

by
G. CASTELLO**, F. DIANI*, D. PECORARI*

INTRODUCTION

In the course of previous investigations ([5,6]) on some properties of amniotic fluid to be used as indicators of fetal lung maturity, we observed that results could be strongly influenced by the technical details of the preparations of the sample. For instance, it seemed necessary to centrifuge immediately the sample of amniotic fluid in order to remove all macroscopic and microscopic organic particles (vernix, blood cells, meconium, epithelial cells and their fragments) because their chemical composition is obviously different from that of amniotic fluid, and if they are analysed together with the amniotic fluid, the results will be of reduced diagnostic significance if not meaningless at all.

Vernix caseosa is probably the most important particulate contaminant of amniotic fluid; its presence is constant and it is in a state of physico-chemical equilibrium with the surrounding liquid phase, so that the concentrations of several substances dissolved in amniotic fluid are presumably correlated with the composition of the vernix.

With the scope of contributing to a better understanding of the interrelations between amniotic fluid and vernix caseosa, a small series of samples of vernix have been analysed with the same method previously used for amniotic fluid ([5,6,7]). The first question was to investigate whether the vernix caseosa could be the site of origin of the fatty acids dissolved in amniotic fluid; the second question was to establish the minimal quantity of vernix contaminants which can introduce a significant error into the evaluation of fetal lung maturity by means of analysis of amniotic fluid fatty acids ([1,5,8,,16,17,18]).

As a collateral study, a series of determinations of fatty acids in amniotic fluid after increasing periods of centrifugation and at different temperatures have been performed; work is still in progress and the results will be published in the near future.

MATERIALS AND METHODS

1. *Method for obtaining the vernix caseosa.*

Twenty samples of vernix were obtained from the same number of newborns (see Table 1). Using small soft plastic containers the vernix was removed directly from the skin not later than ten minutes after birth. The sample was sealed immediately and stored at $-20°$ C until the analysis was done.

2. *Methylation of fatty acids.*

The preparations of the methyl esters of the fatty acids contained in the vernix, in order to permit gas-chromatographic (=GC) analysis, have been accomplished by two methods:

* Dept. of Obstetric and Gynaecology I. Faculty of Medicine, University of Genova (Italy).
** Institute of Industrial Chemistry, Faculty of Sciences C.N.R. grant N. 75/0067004.

a. methylation with BF_3;
b. methylation with $Zn\text{-}ZnCl_2$.

The choice between the two described methylation methods depends on the available time, on the number of samples and on the personal experience of the operators. Method (a) allows to obtain the results in a much shorter time, while method (b) permits the recovery and analysis of the volatile esters of the light fatty acids, that, otherwise, are lost by heating. With the above mentioned exception, the results of the two methods are strictly comparable, as can be seen from

Table 1 - *Sex, weight and gestational age of the twenty babies from whom the samples of vernix caseosa have been obtained.*

Case N.	Sex	Weight (grams)	Gestational Age (completed weeks)
1	Male	3550	40°
2	Male	3030	41°
3	Male	3150	41°
4	Male	2850	39°
5	Male	3250	39°
6	Male	3430	37°
7	Female	3800	39°
8	Male	3380	40°
9	Female	3900	41°
10	Female	2200	36°
11	Female	3700	39°
12	Female	3380	40°
13	Male	2300	40°
14	Female	3540	41°
15	Female	3320	39°
16	Female	3110	39°
17	Female	3230	39°
18	Male	3650	40°
19	Male	3050	39°
20	Female	3500	40°

the values listed in Table 2 for samples 1 and 2, where 1a and 2a are the results by the BF_3 method, and 1b, 1c, 2b, 2c are the results by the $Zn\text{-}ZnCl_2$ method in duplicate samples.

a. Methylation with BF_3.

This is the method previously used for the determination of fatty acids in the amniotic fluid ([5]). Therefore, in order to permit a direct comparison of the resuls, the same tecnique has been employed in samples 1 and 2, with only minor modifications as the sample was directly available as a solid.

The sample of vernix caseosa (100 to 200 mg) was treated with 5 ml of NaOH 0,5N in methanol, and heated until the fat globules went into solution. 4 ml of BF_3-methanol solution (20%) were added and the mixture boiled for 2 minutes.

The residual liquid was poured into a 50 ml separatory funnel, where 6-7 ml of saturated NaCl solution and 10 ml of petroleum ether were added. The upper organic layer (8-10 ml) was drained through filter paper in a vial and concentrated to 1-2 ml for GC analysis. The whole procedure lasts about one hour.

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Table 2 - *Percent composition of fatty acids in the 20 samples of vernix caseosa*

Fatty acids	Case number 1a	1b	1c	2a	2b	2c	3	4	5	6	7
C_{10}	—	—	—	—	—	—	—	—	—	0.12	—
$C_{10'}$	—	—	—	—	—	—	0.05	—	0.10	0.12	—
C_{11}	0.45	0.50	0.69	0.43	0.61	0.28	0.50	0.46	1.26	1.36	0.19
C_{12}	1.36	1.70	1.95	1.72	1.49	1.60	3.40	0.69	2.03	1.81	0.39
$C_{12'}$	0.45	0.20	0.21	0.53	0.61	0.42	0.62	0.23	0.31	0.12	0.19
	1.02	1.15	1.56	0.21	0.47	0.42	0.50	0.31	0.52	0.19	0.19
C_{13}	0.36	0.53	0.34	0.53	0.68	0.64	0.50	0.46	0.52	0.19	0.19
	2.85	3.33	3.64	3.55	3.54	3.86	2.50	2.51	6.57	10.12	2.97
C_{14}	7.66	7.78	9.55	7.32	9.85	7.58	7.0	5.11	5.84	7.01	7.14
$C_{14'}$	3.37	2.68	2.04	3.87	5.58	5.50	4.55	1.39	1.67	3.63	1.58
	3.19	2.65	1.51	0.93	0.80	0.85	2.40	0.85	1.87	3.18	2.77
C_{15}	4.92	4.59	2.77	8.08	5.58	5.01	6.65	2.79	4.38	5.90	7.14
$C_{15'}$	1.13	0.70	0.52	5.38	4.08	3.22	1.50	0.54	0.83	0.77	0.19
	1.36	0.95	0.86	1.18	1.36	1.28	2.45	1.62	5.36	3.63	2.38
C_{16}	23.30	22.55	30.04	16.37	18.39	22.90	27.62	18.83	26.30	22.20	32.63
$C_{16'}$	14.50	15.83	10.94	20.36	25.34	31.35	24.02	17.82	20.87	19.48	21.42
	1.64	2.12	2.08	9.48	6.82	5.79	2.50	1.93	2.92	1.55	1.38
C_{17}	1.27	1.90	1.82	3.77	2.38	1.78	1.40	1.93	3.34	1.55	1.19
$C_{17'}$	0.15	0.20	0.13	0.64	1.02	0.68	0.05	0.23	0.31	0.32	0.39
	0.29	0.42	0.30	1.72	0.54	0.21	4.01	0.54	1.09	1.55	0.39
C_{18}	3.28	3.21	3.82	2.15	1.70	2.57	2.70	2.09	3.13	2.59	3.96
$C_{18'}$	23.34	22.76	22.27	8.72	8.58	7.58	8.40	12.09	9.18	12.66	12.50
$C_{18''}$	2.73	2.74	2.86	0.64	0.81	0.77	0.67	0.34	1.25	1.36	0.69

b. Methylation with Zn-$ZnCl_2$*.

This is the method adopted for all samples of the present investigation because time was not a problem, as it is usually in the case of diagnostic procedures on amniotic fluid, but mainly because we intended to obtain analytical data on the volatile esters of the light fatty acids too.

The catalyst was prepared by weighing in a flask 7 g of previously melted $ZnCl_2$. 100 ml of anhidrous methanol were added, the flask was shaken to complete solution, and 2 g of finely powdered Zn were added. This methylation reagent can be stored for a long time at low temperature (4° C).

The sample of vernix caseosa (25 to 100 mg) was introduced in a small glass tube of about 2 ml capacity (standard drug vials have been found very satisfactory for this use) and subsequently weighed. The sample can be introduced as a solid, by using a small spatula without touching the neck of the vial, or by adding several drops of ethyl ether to the sample placed in a small glass funnel. In the latter case funnel and the vial neck must be throughly washed with the same solvent, that is eventually evaporated at low temperature in order to avoid a loss of volatile fatty acids.

About 20 drops of the methylation mixture, well shaked before use, were then

* Metodi ufficiali di analisi oli e grassi. Ministero Agricoltura e foreste. Gazzetta Ufficiale Repubblica Italiana N. 320. 10-12-1963.

8	9	10	11	12	13	14	15	16	17	18	19	20
0.14	—	—	—	—	—	0.12	—	—	—	—	—	—
0.07	—	—	0.16	0.34	—	0.12	0.23	—	—	0.21	—	—
0.21	—	—	0.24	1.15	0.43	1.12	0.58	0.41	0.74	1.20	0.41	—
0.88	—	—	0.81	1.15	1.31	1.54	0.70	2.06	0.68	1.31	0.75	—
0.44	—	—	0.73	1.34	0.43	0.77	0.70	0.41	0.55	0.87	1.33	—
0.33	0.34	—	0.65	1.23	1.09	0.77	1.17	0.82	0.12	0.32	1.33	—
0.44	0.79	0.20	0.65	0.07	0.43	0.90	0.70	0.30	0.49	0.21	0.16	0.39
2.80	2.94	4.08	1.62	3.27	7.01	7.23	7.04	8.26	5.20	6.03	4.84	3.34
6.65	4.35	2.85	3.17	5.77	6.14	7.10	4.92	4.64	4.95	7.68	4.67	4.18
4.13	0.90	0.71	1.95	1.34	0.43	3.48	0.46	1.44	0.37	1.64	0.41	0.35
2.66	7.93	0.61	3.70	4.62	1.09	1.93	2.34	0.20	5.57	2.19	2.50	1.40
3.76	6.34	1.78	10.25	4.62	3.28	6.45	2.34	3.09	5.57	7.13	2.50	2.11
3.10	0.68	0.61	3.25	1	0.43	0.96	0.23	0.30	0.49	1.09	0.16	0.17
3.54	2.72	9.49	2.92	3.23	4.38	2.58	4.22	3.09	7.93	3.95	4.01	6.33
22.76	29.81	31.44	21.64	25.56	32.23	28.92	28.16	28.09	22.30	30.73	31.41	34.15
21.65	11.33	12.35	13.42	13.86	15.78	17.55	21.12	24.17	9.54	21.95	16.54	18.48
2.36	0.90	2.29	1.30	1.07	0.43	0.25	0.93	0.61	0.55	0.21	0.83	0.26
2.77	1.36	0.56	5.69	1.73	0.43	0.96	1.40	0.69	2.35	0.98	1	0.44
0.29	1.36	0.66	6.71	1.73	0.43	1.35	0.70	0.72	2.47	1.97	0.91	0.52
0.18	0.68	0.86	1.34	0.38	0.43	0.64	0.93	0.41	0.49	1.09	1.16	0.52
2.99	3.62	6.12	4.06	4.62	4.60	2.06	2.46	2.16	4.46	2.63	4.51	4.04
15.96	19.72	17.35	13.87	17.52	15.78	11.36	15.49	17.45	20.81	11.85	16.29	20.59
1.77	3.96	7.96	1.79	4.31	4.38	1.54	3.05	0.61	3.71	2.30	4.17	2.64

added to each vial; thereafter the vial was flame sealed and heated at 100° C for about 24 hours, then stored at room temperature as long as required.

The vials were opened immediately before the analysis, a few microliters of liquid were aspirated by means of a microsyringe and directly injected into the gas chromatograph.

A fraction of the sample remains undissolved, beeing probably constituted by waxes, unsaponifiable factions, etc., and may contain small amounts of not esterified fatty acids.

Repeated analyses on various samples showed that the percent composition of the fatty acids methyl esters was constant, independently of the amount of solid residue, and therefore it has been deduced that the results are not influenced by the amount of undissolved material.

3. *Gas chromatographic analysis.*

The GC separations have been accomplished by using a stainless steel column, 2 m long, 2 mm internal diameter, filled with 10% ethylenglycol adipate (EGA) on Chromosorb W 60/80 mesh. Similar results can be obtained by using standard columns for the analysis of fatty acids methyl esters (DEGS, DEGA, FFAP) *.

* DEGS=Diethylenglycolsuccinate; DEGA=Diethylenglycoladipate; FFAP=Free Fatty Acids Phase, Varian-Aerograph.

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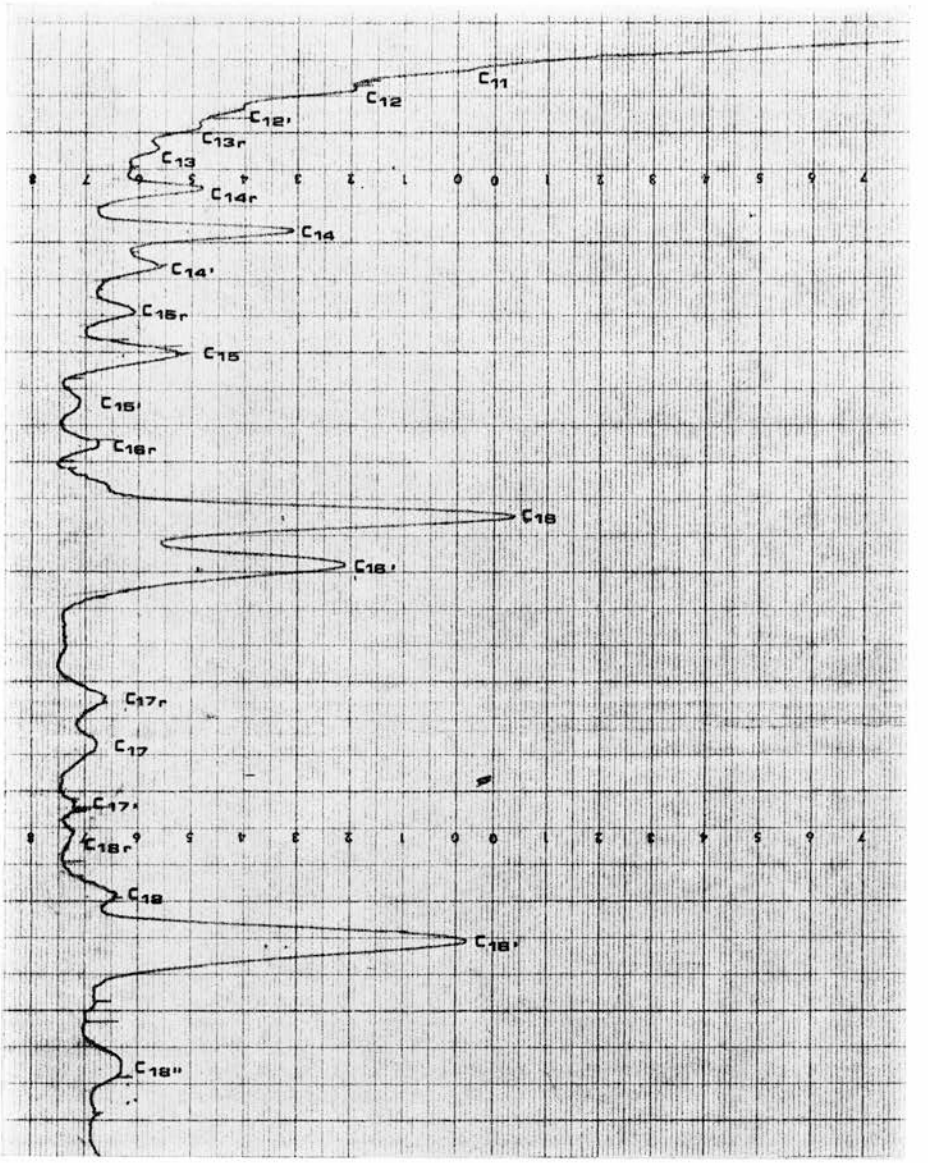

FIG. 1 - Gas chromatogram of fatty acids in sample Ib.
C_{14}: Miristic acid C_{16}: Palmitic acid C_{18}: Stearic acid

A Varian Aerograph dual column chromatograph with flame ionization detectors (FID) was used (Nitrogen flow 20 ml/min, programmed temperature increase at 4° C/min from 100 to 175° C).

The identifications have been made by comparison with authentic samples of methyl esters and with methylated samples from various animal and vegetal fats of known composition.

Quantitative results (see Table 2) are given as percent area, without using the specific correction factors for FID, because they are very similar for all the methyl esters analysed and because the uncertainty due to the sampling method overcomes the small difference due to the use of correction factors.

RESULTS

All the analysed samples gave the same qualitative composition (see Table II and Fig. 1). The comparison of the results of the two methylation methods (samples 1 and 2) has been discussed above. The samples from 3 to 20 have been analysed only by the Zn-$ZnCl_2$ technique and the numbers reported in table II correspond to the mean of two parallel runs. As already stated, the choice of this technique was dictated by our intention to measure also the volatile esters of the light fatty acids.

The volatile esters of the light fatty acids of amniotic fluid have presently no diagnostic use, so that the BF_3 methylation is adequate for clinical purposes. For the present study on the vernix caseosa we have elected to use the Zn-$ZnCl_2$ method because we wanted to assess also the esters of light fatty acids in view of the possibility of further investigations, where the knowledge of the whole spectrum of fatty acids would be necessary.

In order to compare the results of these determinations with the values obtained by analysis of amniotic fluid ([5,6,7]) the ratios between the amount of palmitic and stearic acid have been calculated and listed in Table 3.

Among fatty acids, palmitic acid has been selected because it is the one present in greatest amount; stearic acid has been selected because during the second half of pregnancy its concentration in amniotic fluid is reasonably constant ([1]). In all samples examined (see Table 3) the ratio palmitic acid/stearic acid was always

Table 3 - *Palmitic acid/Stearic acid (C_{16}/C_{18}) ratio and Miristic acid/Stearic acid (C_{14}/C_{18}) ratio of the 20 samples of vernix caseosa*

Case N.	Ratio Palmitic acid/ Stearic acid	Miristic acid/ Stearic acid
1	7.1	2.3
2	7.6	2.6
3	10.2	2.5
4	9	2.5
5	8.4	1.8
6	8.5	2.7
7	8.2	1.9
8	7.6	2.2
9	8	1.2
10	5.1	1
11	6.8	1
12	5.5	1.2
13	7	1.1
14	14	3.4
15	11.4	2
16	12.9	1.9
17	5.2	1.2
18	11.6	2.4
19	6.9	1.1
20	8.4	1.1
Mean	8.5	1.8

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greater than 5, which is supposed to be the critical value in amniotic fluid for assuming that fetal pulmonary maturity has been reached ([8]).

The corresponding values for the ratios miristic acid/stearic acid are also reported in the Table 3; in fact, in a previous paper ([7]) it has been shown that in amniotic fluid this ratio is lower but roughly parallel to palmitic acid/stearic acid ratio and can be used as subsidiary discriminant in dubious cases; more exactly, when palmitic acid/stearic acid ratio in amniotic fluid was 5 or more, miristic acid/stearic acid ratio was neverless than 0,5.

Finally, the meaning of the results obtained in samples 10 and 11 must be stressed, because data on amniotic fluid obtained by means of high puncture of the membranes (Drew-Smythe catheter) at the beginning of labour are also available and a direct comparison is possible. In these two cases the ratio palmitic acid/stearic acid in amniotic fluid was 7,1 (case 10) and 5,1 (case 11), while the same ratio in the vernix was 5,1 (case 10) and 6,8 (case 11). It is relevant to point out that the amniotic fluid samples were centrifuged as described in the paper by Castello *et al.*, ([5]) immediately after having been obtained, and therefore were free of suspended contaminants.

DISCUSSION AND CONCLUSION

As a preliminary observation it should be stressed that there are no obvious differences between the composition of the vernix of female and male babies.

More important, however, is the comparison of our results with the data of Kärkkäinen *et al.*, ([10]) who studied the composition of all lipids present in the vernix caseosa, and of Nicolaides *et al.* ([11, 12, 13, 14, 15]), who studied by means of gas chromatography not only the composition of the vernix caseosa, but also the composition of the fats present on the human skin and on the skin of various animals.

Actually, our data are in good agreement with those of the Authors quoted above; furthermore it is interesting to observe that the average palmitic acid/stearic acid ratio in our samples of vernix caseosa from mature human babies (8,5) is very close also to the figure found by Nicolaides *et al.* for the same ratio in sebum of adult human skin (8,9).

A problem still open to investigation is whether the composition of fatty acids of amniotic fluid is well correlated to the composition of fatty acids of the vernix caseosa not only in term or near term cases with already attained pulmonary maturity (as in the present series), but also in pre-term cases with incomplete maturation of the fetal lungs.

Palmitic acid/stearic acid ratio of the vernix caseosa of all infants of the present series was always higher than 5, which is the discriminant value for amniotic fluid indicating that fetal lung maturity has been reached. A good correlation in term cases can therefore be assumed, as evidenced also by case 10 and 11 of the present series, where data on amniotic fluid and on vernix caseosa were available.

If there is a good correlation between the two compositions (vernix and amniotic fluid) also in pre-term cases, contamination of amniotic fluid with vernix caseosa would not significantly influence the diagnostic accuracy of gas chromatographic determination of palmitic acid/stearic acid ratio as indicator of fetal lung maturity; however, contamination with vernix caseosa would simulate a higher fetal lung maturity if it is evaluated with the method of Warren and Holton ([17, 18]), based on quantitative determinations of amniotic fluid palmitic acid only.

On the contrary, if there is in pre-term cases a marked difference between the palmitic acid/stearic acid ratio of vernix caseosa and the one of amniotic fluid,

the high content of palmitic acid in the vernix can simulate an apparent increase of palmitic acid/stearic acid ratio in amniotic fluid contaminated even with minimal amounts of vernix, taking into account the difference between the absolute quantities of fatty acids contained in the vernix and the absolute quantities contained in amniotic fluid. According to the data from the literature and according to the results of our investigations, fatty acids concentration in the vernix caseosa is of the order of 20%, while in amniotic fluid the habitual order of magnitude is less than 0,01%. For example it can be calculated that as little as 0,3 mg of vernix (mean palmitic acid/stearic acid ratio: 8,5) suspended in 1 ml of amniotic fluid with a palmitic acid/stearic acid ratio of 4 (indicating poor lung maturity) would increase the ratio of the contaminated sample to a value of 5 (indicating attained maturity), and possibly given origin to erroneous clinical decisions.

Work is in progress in order to obtain pertinent informations on this question in a sufficient number of pre-term cases.

AKNOWLEDGEMENT

Professor G. Vecchietti, presently Chairman of the Dept. of Obstetrics and Gynaecology of the School of Medicine of the University of Verona (Italy), suggested to one of us (D.P.) already in 1959 to investigate the clinical problems related to the composition of the vernix caseosa. The stimulus provided by this suggestion is gratefully aknowledged.

We also aknowledge the kind cooperation of Dr. Carmen Clerici, Genova, in the preparation of the cathalitic mixture and in the identification of light fatty acids.

SUMMARY

Gas chromatographic analysis of fatty acids of vernix caseosa has been performed in 20 normal newborns at term. Fatty acids pattern of vernix caseosa is very similar to the pattern already described in adult sebum and in amniotic fluid at term.

Owing to the great amount of fatty acids present in the vernix, even small vernix particles contaminating amniotic fluid samples for prenatal evaluation of fetal lung maturity can be the cause of false positive results. Therefore an accurate centrifugation of amniotic fluids samples is essential before doing any diagnostic study of fetal lung maturity.

BIBLIOGRAPHY

1. Alcindor L. G., Bereziat G., Vielh J. P., Gautray J. P.: *Clin. chim. Acta, 50,* 31, 1974. - 2. Ansari M. N. A., Fu H. G., Micolaides N.: *Lipidis, 5,* 31, 1970. - 3. Bhagwanani S. G., Fahmy D., Turnbull A. C.: *Lancet, 1,* 159, 1972. - 4. Bhagwanani S. G., Fahmy D., Trunbull A. C.: *Lancet, 2,* 66, 1972. - 5. Castello G., Diani F., Pecorari D.: *Min. Gin., 28,* 789, 1976. - 6. Conte N., Vicino P., Diani F.: *Riv. Ost. Gin. prat. Med. perinat., 56,* 317, 1976. - 7. Diani F., Ciangherotti F.: *Quad. Clin. ost. gin., 31,* 23, 1976. - 8. Gautray J. P., Vielh J. P.: Critères de maturité foetale et décision obstétricale. In: Etienne J. P., Rapin M.: *Retard de croissance intrautérin.* Editions Glaxo, Paris, 1974. - 9. Gluck L., Kulovich M. V., Borer R. C., Brenner P. H., Anderson G., Spellacy W. N.: *Am. J. Obst. Gyn., 109,* 440, 1971. - 10. Karkkainen J., Nikkari T., Ruponen S., Haahti E.: *J. Invest. Dermatol., 44,* 333, 1965. - 11. Miettinen T. A., Luukkainen T.: *Acta chim. scandinav., 22,* 2603, 1968. - 12. Nicolaides N.: *Lipids, 6,* 901, 1971. - 13. Nicolaides N., Ansari M. N. A.: *Lipids, 3,* 403, 1968. - 14. Nicolaides N., Fu H. C., Ansari M. N. A., Rice R.: *Lipids, 7,* 506, 1972. - 15. Nicolaides N.: Skin Lipids: *Science, 186,* 19, 1974. - 16. Schirar A., Vielh J. P., Alcindor L. G., Gautray J. P.: *Am. J. Obst. Gyn., 121,* 653, 1975. - 17. Warren C., Allen J. T., Holton J. B.: *Clin. chim. Acta, 44,* 457, 1973. - 18. Warren C., Holton J. B., Allen J. T.: *Brit. med. J., 1,* 94, 1974.