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TWO GENETICALLY DIFFERENT MU-NANA* NEURAMINIDASES IN HUMAN LEUCOCYTES

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Human leucocytes contain two different MU-NANA neuraminidases, which can be distinguished by Concanavalin A binding. The Con A binding form is predominant in lymphocytes (more than 80%) and the non-binding form predominates in granulocytes. The pH optima of both these neuraminidases as well as their subcellular localization as determined by Percoll gradient centrifugation suggest that they are both lysosomal. Immunological studies indicate that the Con A binding form is present in a complex with β-galactosidase whereas the non-binding form is not. Leucocytes from patients with sialidosis or galactosialidosis are deficient in the Con A binding neuraminidase, whereas the non-binding form is normal. In sialolipidosis both forms are normal. These results demonstrate that leucocytes contain at least two genetically different MU-NANA neuraminidases. Thus, the use of leucocytes should be avoided for the diagnosis of sialidosis and galactosialidosis, and isolated lymphocytes should be used to obtain reliable results.

In man several inherited disorders are known with a deficiency of neuraminidase activity (1). With the present biochemical knowledge these disorders can be separated in three distinct groups; within each group clinical heterogeneity is observed. Firstly, there is a group of patients, classified as sialidosis, characterized by a deficiency of a lysosomal neuraminidase specific for glycoproteins and oligosaccharides with terminal sialic acid (2). This group includes disorders previously described as mucolipidosis I and cherry-red spot myoclonus syndrome (1-7). The second group, galactosialidosis, is characterized by a combined deficiency of β-galactosidase and neuraminidase (8,9). The primary defect lies in a glycoprotein which normally prevents degradation of β-galactosidase (9-12). Neuraminidase is deficient as well, which may find its origin in the fact that β-galactosidase and neuraminidase occur as enzyme complex (13). The third group has recently been classified as sialolipidosis (i.e. mucolipi-

*MU-NANA, 4-methylumbelliferyl-α-D-N-acetylneuraminic acid

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dosis IV), in which the enzyme deficiency is a ganglioside specific neuraminidase (14,15).

For the diagnosis of sialidosis and galactosialidosis hydrophilic substrates were employed: glycoproteins or oligosaccharides and 4-methylumbelliferyl α-D-N-acetylneuraminicacid (MU-NANA). Sialolipidosis has been diagnosed using labelled ganglioside substrates. Nearly complete enzyme deficiencies have been detected in cultured fibroblasts from patients with these different disorders (1-9,15). However, using leucocytes as the source of neuraminidase, considerable residual activities (sometimes close to normal) were found in sialidosis and galactosialidosis (16-20).

Recently it was proposed that leucocytes contain two MU-NANA neuraminidases distinguished by slight physical differences (17,20,21). In contrast to these reports, we demonstrate the presence of two neuraminidases with qualitative biochemical differences and have extended the studies to all three disorders. Our results indicate that both MU-NANA neuraminidases are of different genetic origin. The implications for diagnosis of sialidosis and galactosialidosis are discussed.

MATERIALS AND METHODS

Blood cell isolation

Isolation of leucocytes was performed by sedimentation of freshly collected heparinized blood samples in 3% Dextran (T-500, Pharmacia) in 0.9% NaCl, whereafter lymphocytes and granulocytes were separated on Ficoll Paque (Pharmacia). Remaining erythrocytes in leucocytes and granulocytes were lysed during 10 minutes in 50 mM NaCl at 4°C, after which the cells were spun down at 300 x g for 10 min.

Preparation of cell homogenates

Cell homogenates for specific activity determination were made by homogenizing the cells in 60 µl 0.1% sodium taurocholate (Sigma, synthetic) using a glass-teflon Potter homogenizer. Leucocytes derived from 2-3 ml blood samples were used, and lymphocytes and granulocytes from 4-7 ml.

Assay of protein and enzyme activity

Protein was measured by the method of Lowry (22). Neuraminidase was measured with 4-methylumbelliferyl N-acetylneuraminic acid (MU-NANA) (a generous gift of Prof. R. Brossmer, Heidelberg). 20 µl Cell homogenate was added to 10 µl 2 mM substrate solution in 0.25 M sodium acetate buffer pH 4.3. Incubation was at 37°C for one hour. The reaction was terminated by addition of 500 µl, 0.5 M sodium carbonate buffer pH 10.7. β-Galactosidase was assayed in a thirtyfold diluted homogenate as described earlier (23). Fluorescence was measured with a Perkin Elmer fluorimeter.

Solubilization of neuraminidase and β-galactosidase

Cells derived from 10-25 ml blood were homogenized in 50 µl 20 mM sodium phosphate buffer containing 100 mM NaCl and 1% Zwittergent 3.12 (Calbiochem.) pH 6.0 using a glass-teflon Potter homogenizer. The homogenates were centrifuged at 10000 x g for 15 minutes at 4°C. The supernatant containing solubilized neuraminidase and β-galactosidase was used in the precipitation experiments with Con A-Sepharose and anti placental β-galactosidase IgG.

Con A-Sepharose precipitation

5 µl Supernatant was added to 5 µl 20 mM sodium phosphate buffer pH 6.0 containing 100 mM NaCl, 1% Zwittergent 3.12 and various amounts of Con A-Sepharose-4B (Pharmacia). The tubes were gently rotated at 4°C. After one hour all tubes were centrifuged at 10000 x g for 20 seconds. Supernatants were assayed

for both neuraminidase and β-galactosidase. The incubation volume was reduced to 3 µl and the reaction was terminated with only 200 µl sodium carbonate buffer pH 10.7.

Immunotitration

3 µl Supernatant was added to 2 µl of a serial dilution of IgG (13) in 20 mM sodium phosphate buffer containing 100 mM sodium chloride pH 6.0. After two hours incubation at 4°C, 5 µl of a 1:1 Protein A Sepharose-4B (Pharmacia) suspension in the same buffer was added. After one hour gently rotating at 4°C, all tubes were centrifuged at 10000 x g for 20 seconds. Supernatants were assayed for both neuraminidase and β-galactosidase activity as described under Con A-Sepharose precipitation.

Subcellular fractionation in Percoll gradients

Lymphocytes or granulocytes isolated from 200 ml respectively 100 ml freshly collected blood from healthy donors, were suspended in 0.5 ml 0.25 M sucrose, 10 mM sodium phosphate buffer pH 6.8 and disrupted by 25 strokes in a 2 ml glass-teflon Potter homogenizer. Nuclei and intact cells were removed by centrifugation for 10 min. at 600 x g. About 90% of granulocyte β-hexosaminidase (lysosomal latency >80%) and about 80% of lymphocyte β-hexosaminidase (lysosomal latency >60%) was recovered in the postnuclear supernatant. Percoll (Pharmacia) was made iso-osmotic with 0.25 M sucrose and this solution (100% Percoll) was diluted to a final Percoll concentration of 40% with 0.25 M sucrose, 10 mM sodium phosphate buffer pH 6.8. 0.3 ml Postnuclear supernatant was applied on top of 8 ml of 40% Percoll solution underlayed with 0.5 ml 2.5 M sucrose. The samples were centrifuged at 25000 rpm (~48000 x g) for 45 minutes at 4°C in a Beckman type 50 fixed angle rotor. Fractions of 300 µl were collected with a density-gradient removing apparatus (Auto densi-flow II, Buchler instruments). For neuraminidase assay 15 µl gradient fraction was mixed with 15 µl H_2O and incubated with 10 µl substrate containing 0.4% taurocholate and 4% Zwittergent 3.12. Recovery of neuraminidase activity after the Percoll centrifugation was >70%. Alkaline phosphatase activity was measured with 1.5 mM methylumbelliferyl phosphate (Koch-Light) in 1 mM $MgCl_2$ and 75 mM 2-amino-2 methyl-1-propanol/HCl pH 10.1 in the presence of 0.1% triton X-100 (24).

RESULTS

Total leucocytes and isolated lymphocytes and granulocytes from patients with sialidosis (patient of Dr. H.D. Bakker, (9)), galactosialidosis (patient of Dr. M.C.B. Loonen, (9,25)) and sialolipidosis (15), were tested for neuraminidase and β-galactosidase activity (Table 1). Leucocyte neuraminidase activities of sialidosis and galactosialidosis patients were found to be close to the lowest control value (up to 39% of the normal mean value). Leucocytes from a patient with sialolipidosis had neuraminidase activities in the control range. However, isolated lymphocytes from sialidosis and galactosialidosis patients were clearly deficient in neuraminidase activity (<15% of the mean control value), whereas lymphocytes from the patient with sialolipidosis had normal activity. In contrast granulocytes from all different types of patients showed normal neuraminidase activities. As expected, β-galactosidase activities were normal in all cell types of the sialidosis and sialolipidosis patients and clearly deficient in all cell types of the galactosialidosis patient. The fact that neuraminidase activity in both sialidosis and galactosialidosis, which are two genetically distinct diseases, is deficient in lymphocytes, but normal in granulocytes, suggests that there are at least two genetically different neuraminidases.

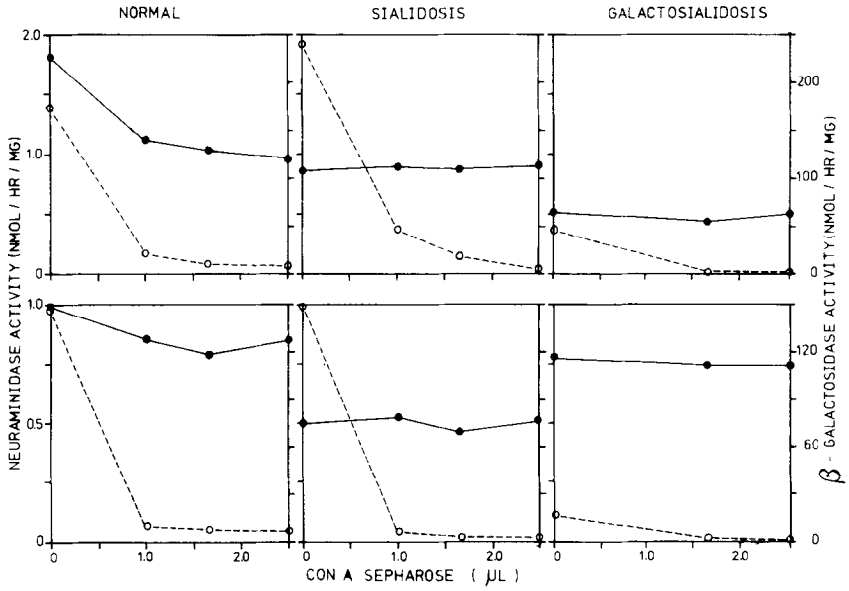

Fig. 1. Con A-sepharose precipitation of neuraminidase and β-galactosidase from normal, sialidosis and galactosialidosis cells.
Upper graphs: lymphocytes
Lower graphs: granulocytes
● Neuraminidase
○ β-Galactosidase

To pursue this hypothesis the neuraminidases were further characterized. Neuraminidase, which is insoluble under our standard assay conditions using sodium taurocholate, was solubilized with a zwitterionic detergent. In normal lymphocytes 40 to 70% of the total activity could be solubilized, whereas in granulocytes the solubilization was 85 to 100%. From the solubilized neuraminidase activity of normal human lymphocytes only 40 to 60% could be precipitated with Con A (Fig. 1). Granulocytes contain only minor amounts of Con A precipitable neuraminidase activity. β-Galactosidase activity was precipitated completely. In sialidosis and galactosialidosis the residual neuraminidase activity of lymphocytes and the normal activity of granulocytes could not be precipitated, whereas β-galactosidase activity could be precipitated normally. Apparently two different neuraminidases are present in leucocytes: a Con A binding form, deficient in sialidosis and galactosialidosis, and a non-binding form, normal in sialidosis and galactosialidosis.

It has been demonstrated that acid neuraminidase and β-galactosidase form an enzyme complex in bovine testis (13). To investigate whether the neuraminidases from human leucocytes are also complexed with β-galactosidase, immunotitration using an antiserum raised against purified β-galactosidase was performed (fig. 2). In normal lymphocytes 40 to 60% of the solubilized neuraminidase activity coprecipitated with β-galactosidase. In granulocytes only minor amounts could be coprecipitated. The residual neuraminidase activity of neither

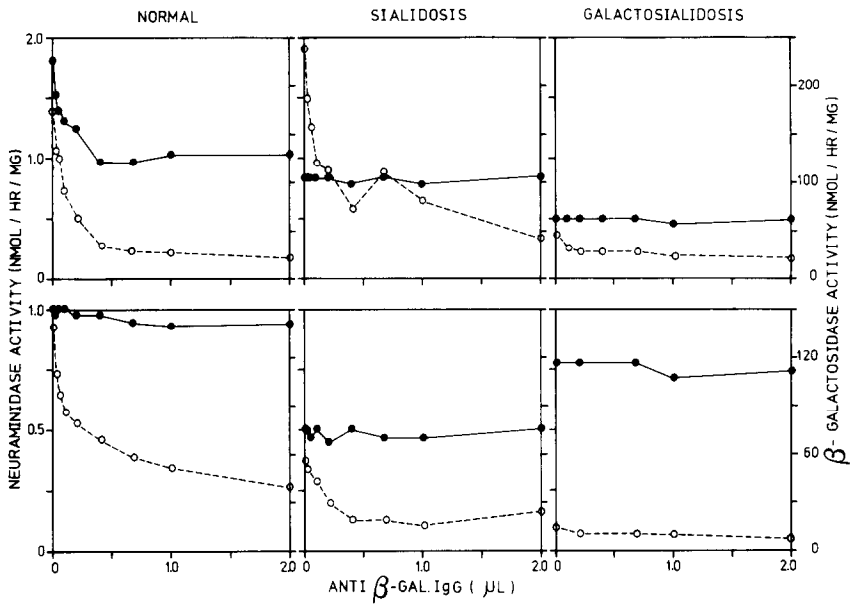

Fig. 2. Immunotitration of neuraminidase and ß-galactosidase from normal, sialidosis and galactosialidosis cells.
Upper graphs: lymphocytes
Lower graphs: granulocytes
● Neuraminidase
○ ß-Galactosidase

lymphocytes nor granulocytes from the patients coprecipitated with ß-galactosidase. Apparently the Con A binding form of neuraminidase is complexed with ß-galactosidase while the non-binding form is not.

We investigated whether both forms of neuraminidase are lysosomal or in contrast might be plasma membrane associated. Fig. 3 shows the results of Percoll gradient centrifugation studies and it is evident that lysosomes and plasma membranes are clearly separated. In both lymphocytes and granulocytes neuraminidase sedimented in a single band, coinciding with the lysosomal marker.

Finally a number of biochemical properties of neuraminidase from lymphocytes and granulocytes were analysed (Table 2). No differences were observed in pH optimum and K_m for the MU-NANA substrate. In lymphocytes neuraminidase was somewhat more labile than in granulocytes during sonication, freeze-thawing and heat denaturation. Cu^{2+}, which is reported to be an inhibitor of ganglioside neuraminidase (26), inhibited both lymphocyte and granulocyte neuraminidase to the same extent. Similar results were obtained with 2,3-dehydro-2-deoxyNANA, a possible inhibitor of the lysosomal glycoprotein and oligosaccharide specific neuraminidase (26).

DISCUSSION

Our results demonstrate the presence of two different neuraminidases in human leucocytes: a Con A binding form, complexed with ß-galactosidase and a

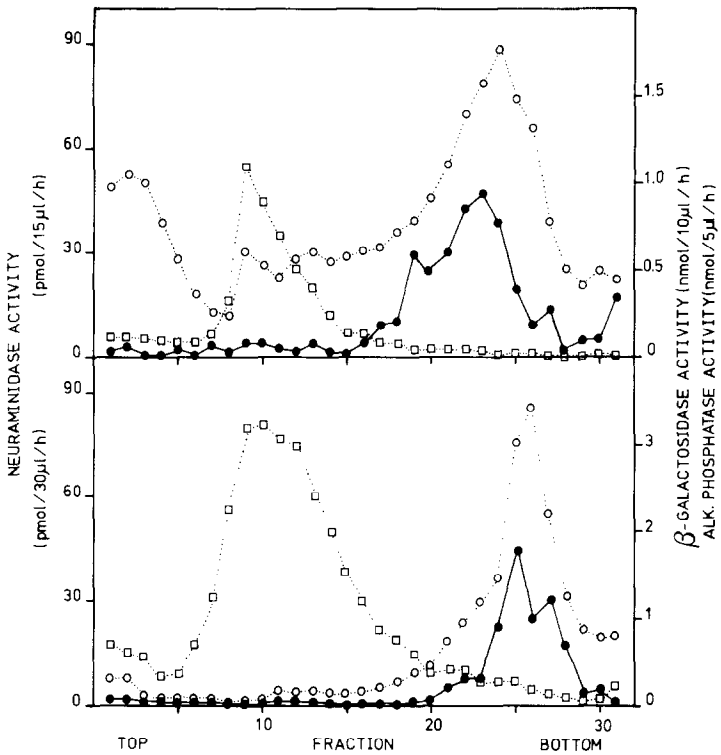

Fig. 3. Percoll density gradient centrifugation of subcellular components from normal lymphocytes (upper figure) and normal granulocytes (lower figure).
● Neuraminidase
□ Alkaline phosphatase (Plasma membrane marker)
○ ß-Galactosidase (Lysosomal marker)

non-binding form which is not complexed. Only the binding form is deficient in sialidosis and galactosialidosis. The residual activities of neuraminidase in cell homogenates of white blood cells from patients (Table 1) indicate that normal lymphocytes contain predominantly (>80%) the binding form, whereas normal granulocytes contain mainly the non-binding form. It should be noted that studies with solubilized neuraminidase (Con A binding and immunotitration experiments) are not informative for the relative amounts of the two neuraminidases present in leucocytes, since different degrees of solubilization were observed for both forms.

Diagnoses of sialidosis and galactosialidosis, using total leucocytes, have been reported and often very high residual neuraminidase activities were found (1,16-20) similar to the activities found in our cases (Table 1). This is caused by the normal activity of the non-binding neuraminidase in these patients. For this reason granulocytes and hence total leucocytes should not be used for diagnosis. On the other hand we show that lymphocytes can be used satisfactorily.

Multiple forms of neuraminidase have been described previously. Nguyen Hong et al. (27) reported on two neuraminidases in human leucocytes, a heat stabile

TABLE I

ENZYME ACTIVITIES IN VARIOUS WHITE BLOOD CELLS IN SIALIDOSIS, GALACTOSIALIDOSIS AND SIALOLIPIDOSIS

	NEURAMINIDASE*				β-GALACTOSIDASE*				
	Normal(n=19)		Sial.	Gal.sial.	Sialolip.	Normal(n=19)		Sial.	Gal.sial.	Sialolip.
	range	mean				range	mean			
Leucocytes	1.4-4.3	2.8	1.1	0.74	2.3	95-200	133	129	9.2	98
Lymphocytes	3.9-11	7.4	1.1	0.84	4.3	135-280	203	132	13	191
Granulocytes	0.46-1.2	0.94	1.3	0.59	1.2	65-150	102	284	6.5	148

* enzyme activity in nmol/h/mg protein

and labile form, both deficient in sialidosis patients. This indicates that both forms are related to our binding form. Tsuji et al. (20) and Suzuki et al. (17) distinguished two neuraminidase activities in leucocytes with different sonication and freeze-thaw stability. Only the more labile form was deficient in their galactosialidosis patients. Our binding and non-binding neuraminidase exhibit comparable freeze-thaw and sonication stability and are probably the same enzymes as those described (17,20).

TABLE 2

PROPERTIES OF NEURAMINIDASE IN NORMAL LYMPHOCYTES AND GRANULOCYTES

	LYMPHOCYTES	GRANULOCYTES
pH optimum	4.1	4.0
K_M for 4MU-NANA (mM)	0.18	0.26
Inhibition by 5mM Cu^{2+}	46%	51%
Inhibition by 0.05 mM 2,3 dehydro-2-deoxy-NANA	69%	76%
Sonication stability ($t_{\frac{1}{2}}$)	15 sec	58 sec
Freeze/thaw stability*	32%	55%
Heat stability pH7/37°C ($t_{\frac{1}{2}}$)	13 min	50 min

* residual enzyme activity after 10 freeze/thaw cycles.

Subcellular fractionation showed that both neuraminidase activity of lymphocytes (>80% Con A binding form) and neuraminidase activity of granulocytes (mainly non-binding form) sedimented in a peak coinciding with the lysosomes clearly separate from the plasma membranes. Together with the acid pH optima, this suggests lysosomal localization of both forms. This deviates from the report by Yamada et al. (21) proposing a plasma membrane localization of their analogue of our non-binding neuraminidase.

Since in sialidosis as well as galactosialidosis only the Con A binding neuraminidase is deficient we conclude that the binding and non-binding neuraminidases are of different genetic origin. The physiological significance of the lysosomal Con A binding neuraminidase and the ganglioside neuraminidase is emphasized by the existence of lysosomal storage disorders caused by a deficiency of one of these neuraminidases. The function of the non-binding neuraminidase is still unknown and future studies on the substrate specificity should help to reveal its physiological importance. It is unlikely that this enzyme represents ganglioside neuraminidase since a patient with sialolipidosis with a proven deficiency of ganglioside neuraminidase (15) had a normal non-binding neuraminidase activity. The present results may lead to the discovery of a lysosomal storage disorder which is associated with an abnormal non-binding neuraminidase.

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REFERENCES

1. Lowden, J.A. and O'Brien, J.S. (1979) Am. J. Hum. Gen. 31, 1-18.
2. Cantz, M. and Messer, H. (1979) Eur. J. Biochem. 97, 113-118.
3. Cantz, M., Gehler, J. and Spranger, J. (1977) Biochem. Biophys. Res. Comm. 74, 732-738.
4. Durand, P., Gatti, R., Cavalieri, S., Borrone, C., Tondeur, M., Michalski, J.-C. and Strecker, G. (1977) Helv. Paediat. Acta 32, 391-400.
5. O'Brien, J.S. (1977) Biochem. Biophys. Res. Comm. 79, 1136-1141.
6. Kelly, T.E. and Graetz, G. (1977) Am. J. Med. Genet. 1, 31-46.
7. Thomas, G.H., Tipton, R.E., Ch'ien, L.T., Reynolds, L.W. and Miller, C. (1978) 13, 369-379.
8. Wenger, D.A., Tarby, T.J. and Wharton, C. (1978) Biochem. Biophys. Res. Comm. 82, 589-595.
9. Hoogeveen, A.T., Verheijen, F.W., d'Azzo, A. and Galjaard, H. (1980) Nature 285, 500-502.
10. van Diggelen, O.P., Hoogeveen, A.T., Smith. P.J., Reuser, A.J.J. and Galjaard, H. (1982) Biochim. Biophys. Acta 703, 69-76.
11. d'Azzo, A., Hoogeveen, A., Reuser, A.J.J., Robinson, D. and Galjaard, H. (1982) Proc. Natl. Acad. Sci. USA 79, 4535-4539.
12. Hoogeveen, A.T., Verheijen, F.W. and Galjaard, H. (1983) J. Biol. Chem., in press.

13. Verheijen, F., Brossmer, R. and Galjaard, H. (1982) Biochem. Biophys. Res. Comm. 108, 868-875.
14. Bach, G., Zeigler, M., Schaap, T. and Kohn, G. (1979) Biochem. Biophys. Res. Comm. 90, 1341-1347.
15. Caimi, L., Tettamanti, G., Berra, B., Omodeo sale, F., Borrone, C., Gatti, R., Durand, P. and Martin, J.J. (1982) J. Inher. Metab. Dis. 5, 218-224.
16. Mueller, O.T. and Wenger, D.A. (1981) Clin. Chim. Acta 109, 313-324.
17. Suzuki, Y., Sakuraba, H., Potier, M., Akagi, M., Sakai, M. and Beppu, H. (1981) Hum. Genet. 58, 387-389.
18. Kuriyama, M., Someya, F., Miyatake, T. and Koseki, M. (1981) Biochim. Biophys. Acta 662, 220-225.
19. Matsuo, T., Egawa, I., Okada, S., Suetsugu, M., Yamamoto, K. and Watanabe, M. (1983) J. Neur. Scienc. 58, 45-55.
20. Tsuji, S., Yamada, T., Tsutsumi, A. and Miyatake, T. (1982) Ann. Neurol. 11, 541-543.
21. Yamada, T., Tsuji, S., Ariga, T. and Miyatake, T. (1983) Biochim. Biophys. Acta 755, 106-111.
22. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
23. Galjaard, H. (1980) Genetic Metabolic Diseases, early diagnosis and prenatal analysis. Elsevier/North Holland, Amsterdam.
24. Vanneuville, F.J. and Leroy, J.G. (1981) J. Inher. Metab. Dis. 4, 129-130.
25. Loonen, M.C.B., van der Lugt, L. and Francke, C.L. (1974) Lancet ii, 785.
26. Veh, R.W. and Sander, M. (1981) in: Perspectives in Inherited Metabolic Diseases (Tettamanti, G., Durand, P., Di Donato, D., eds.) Vol. 4, pp. 71-109.
27. Nguyen Hong, V., Beauregard, G., Potier, M., Bélisle, M., Mameli, L., Gatti, R. and Durand, P. (1980) Biochim. Biophys. Acta 616, 259-270.