Biosynthesis of acid α-glucosidase in late-onset forms of glycogenosis type II (Pompe's disease)

Friedhelm Steckel, Volkmar Gieselmann, Abdul Waheed, Andrej Hasilik, Kurt von Figura, Ronald Oude Elferink+, Roelof Kalsbeek+ and Joseph M. Tager+

Physiologisch-Chemisches Institut, Westfälische Wilhelms-Universität, Waldeyerstrasse 15, 44 Münster (Westf.), FRG and *+Laboratorium voor Biochemie, Universiteit van Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands*

Received 22 September 1982; revision received 14 October 1982

Cultured human skin fibroblasts from control persons and from patients with the generalized and late-onset forms of Pompe's disease were labelled with radioactive leucine and the incorporation of radioactivity into acid α-glucosidase and cathepsin D was analysed by immunoprecipitation, gel electrophoresis and fluorography. When the labelling was carried out for 6–12 h in the presence of NH_4Cl, the labelling of secreted α-glucosidase relative to that of secreted cathepsin D in fibroblasts from patients with the late-onset form of Pompe's disease was <15% of that in fibroblasts from control persons. However, when the fibroblasts were labelled for <1 h, the relative rate of incorporation of radioactivity into acid α-glucosidase was rather similar in the two types of fibroblasts. In fibroblasts from patients with the generalized form of Pompe's disease no incorporation of radioactivity into acid α-glucosidase could be detected.

Acid α-glucosidase *Pompe's disease* *Lysosomal α-glucosidase* *Glycogenosis type II* *Lysosomal enzyme synthesis*

1. INTRODUCTION

Glycogenosis type II (Pompe's disease) is an inborn error of metabolism in man in which a deficiency of acid α-glucosidase leads to accumulation of glycogen in the lysosomes [1]. The disease is clinically heterogeneous [2]. In the infantile or generalized form glycogen storage is observed in most organs and the patients die within the first 2 years of life [2]. In the late-onset or muscular form glycogen storage is observed mainly in skeletal muscles and death occurs several years after the onset of the disease [2]. The infantile form is characterized by a complete absence of acid α-glucosidase [1,2] and, in most cases, of cross-reactive immunological material [3–7]. On the other hand, residual acid α-glucosidase activity (5–20% of normal) is present in patients with the late-onset form [6–10].

Enzymological and physicochemical properties of the residual acid α-glucosidase in cultured skin fibroblasts [6] and in urine [10] from patients with the late-onset form of the disease are indistinguishable from those of the enzyme from control persons. These studies indicate that patients with the late-onset form of the disease have a smaller number of acid α-glucosidase molecules than normal persons. This reduction may be due either to decreased synthesis or to enhanced breakdown of acid α-glucosidase in the patients [6].

In cultured human skin fibroblasts, acid α-glucosidase is synthesized via a precursor with M_r ~95000 which is subsequently converted to smaller products [11,12]. Neither the precursor nor the product could be detected in fibroblasts from patients with the infantile form of glycogenosis type II [11]. Here, we present results on the synthesis of

Enzymes: cathepsin D (EC 3.4.23.5); α-glucosidase (EC 3.2.1.20)

Published by Elsevier Biomedical Press
001457593/82/0000—0000/$2.75

acid α-glucosidase in fibroblasts from patients with different forms of glycogenosis type II. Some of the results have been presented in abstract form [13].

2. MATERIALS AND METHODS

L-[4,5-^{3}H]Leucine (spec. act. 50 Ci/mmol), L-[^{35}S]methionine (spec. act. 1200 Ci/mmol) and [^{14}C]methylated standards were from New England Nuclear. Immunoprecipitin, a formalin fixed *Staphylococcus* A cell wall suspension (10%) was from Bethesda Research Labs. Antiserum against human liver acid α-glucosidase was raised in a rabbit [10] and against human placental cathepsin D in a goat [11].

2.1. *Cell lines and culture*

The fibroblast cell lines 80RD174, 261, M and B were from control persons; 266 and 248LAD were from patients with the infantile form of Pompe's disease; and G, H, 338LAD and 77RD89 (referred to as RD89 in fig. 2) were from patients with the late-onset form of Pompe's disease.

Human diploid skin fibroblasts were maintained at 37°C under 5% CO_2 in Eagle's minimal essential medium supplemented with antibiotics, non-essential amino acids and 10% fetal calf serum (Boehringer Mannheim GmbH) as in [14]. Fibroblasts from patients affected with glycogenosis type II were kindly provided by Professor Martinus Niermeijer (Division of Clinical Genetics, Erasmus University, Rotterdam) and Dr Kurt Ullrich (Universitäts-Kinderklinik, Münster).

2.2. *Metabolic labelling and isolation of lysosomal enzymes*

Cells grown to confluency in 25 or 75 cm^2 tissue culture flasks were depleted of either leucine or methionine and incubated in a radioactive medium as in [11]. The apparent rate of synthesis of lysosomal enzymes was measured as incorporation of radioactivity in immunoprecipitable material in secretions obtained in the presence of 10 mM NH_4Cl (see [15] for rationale) or in cells after a short labelling period (10 min pulse and 5 min chase) to avoid degradation in lysosomes or en route to lysosomes, respectively. Immunoprecipitates were solubilized in 1% SDS–10 mM dithiothreitol and analysed by gel electrophoresis and fluorography as in [11]. Immunoprecipitates of cells and medium were prepared as in [11] except when cells were pulse-labelled for 10 min. In the latter case samples were suspended in 1% Nonidet NP40, 1% sodium deoxycholate, 0.14 M NaCl and 10 mM sodium phosphate (pH 7.4). After clarification of the extract by centrifugation for 1 h at 45000 × g, α-glucosidase was immunoprecipitated using 0.5 μg purified enzyme as carrier. After removal of the immune complexes, the supernatant was processed for immunoprecipitation of cathepsin D, as follows. Two volumes of immunoprecipitin were added and after 30 min the suspension was centrifuged for 1 h at 45000 × g. The supernatant was mixed with 0.8 vol of buffered detergent (1% Triton X-100, 1% SDS, 0.5% sodium deoxycholate, 5 mg/ml bovine serum albumin, 10 mM sodium phosphate (pH 7.4) and 0.14 M NaCl). Affinity purified anti-cathepsin D antibodies (80 μg immunoglobin able to precipitate 2.7 μg cathepsin D directly) were added and after 0.5 h at 22°C and $\geq$ 16 h at 4°C 140 μl immunoprecipitin was added. After 0.5 h at 4°C with occasional gentle mixing, the cell walls were spun down in an Eppendorf centrifuge (30 s) and washed once with 0.5 ml cold buffered detergent and twice with 0.5 ml cold 0.14 M NaCl–10 mM sodium phosphate (pH 7.4). The adsorbed antigen was solubilized by incubating the immunoprecipitin with 70 μl of a solution containing 1% SDS, 10 mM dithiothreitol, 10% glycerol and 125 mM Tris–HCl (pH 6.8) for 5 min at 95°C. After removal of immunoprecipitin by centrifugation, the supernatant was subjected to electrophoresis as in [15].

The radioactivity in the bands was quantified as in [15].

3. RESULTS AND DISCUSSION

When fibroblasts from patients with the late-onset form of Pompe's disease were cultured in the presence of [^{3}H]leucine for 16 h, or pulse-labelled for 1 h with [^{35}S]methionine and chased for 24 h, a pattern of immunoprecipitated α-glucosidase polypeptides was obtained which closely resembled that of polypeptides immunoprecipitated from normal cells (fig. 1; see [11]). These polypeptides could not be detected in fibroblasts from patients with the infantile form of Pompe's disease (fig. 1),

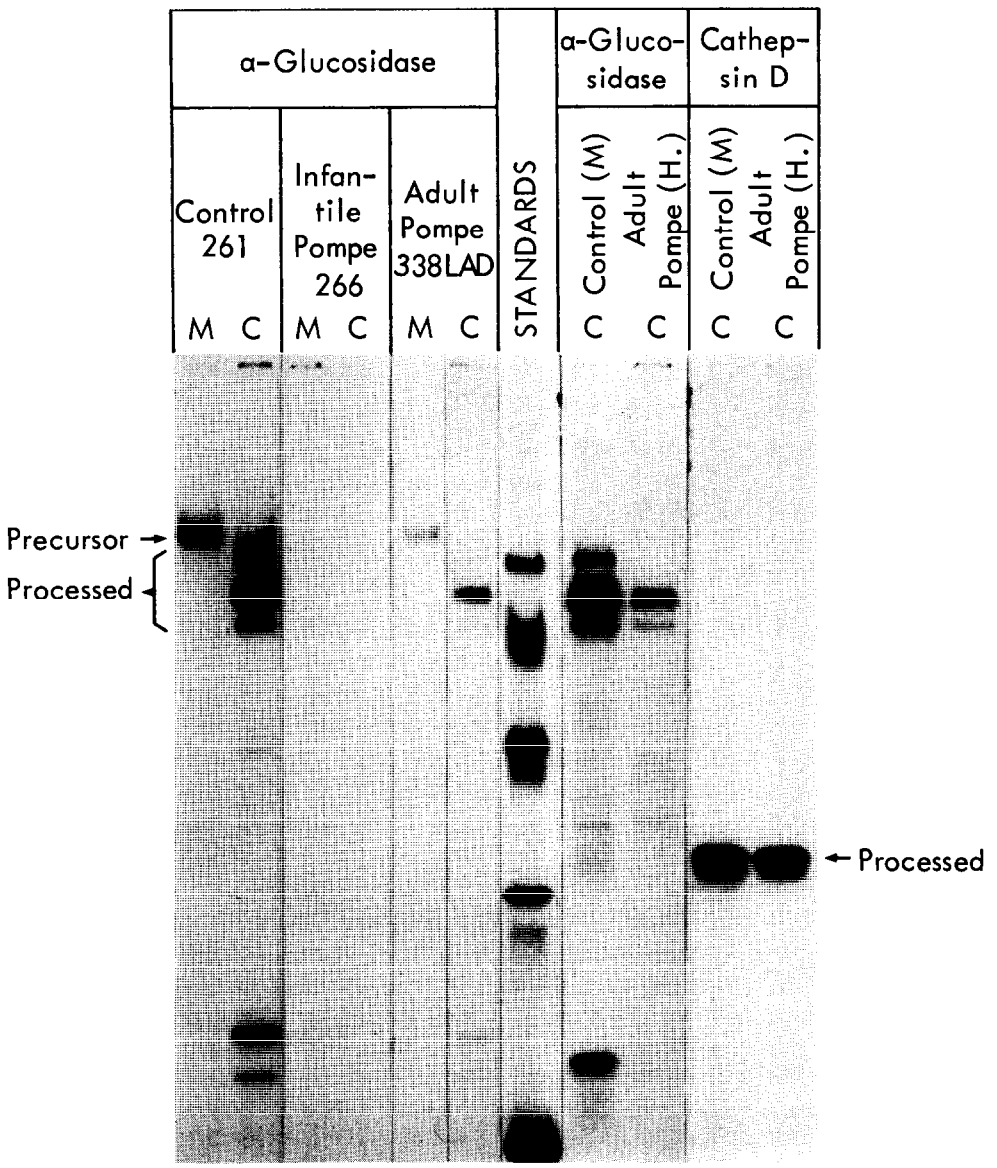

Fig. 1. Incorporation of radioactivity into α-glucosidase and cathepsin D in fibroblasts from control persons and patients with different forms of Pompe's disease. Lanes: (1–6) labelling for 16 h with 0.2 mCi [^{3}H]leucine; (8–11) cells labelled for 1 h with 0.2 mCi [^{35}S]methionine and chased for 24 h in medium free of radioactivity. The ratio of media and cell extracts used for immune precipitation of α-glucosidase was 2:1. *Abbreviations*: C, cells; M, medium.

in agreement with [11]. Fig. 1 shows that there is a difference in the intensity of the labelling of α-glucosidase polypeptides (but not cathepsin D polypeptides) in adult Pompe's disease fibroblasts.

To study the rate of formation of the precursor of α-glucosidase, fibroblasts were cultured for various times in the presence of [^{3}H]leucine together with NH_4Cl; in the presence of certain weak bases like NH_4Cl the processing of the precursors of lysosomal enzymes is prevented and the precursors accumulate in the medium [11,12,16–18]. The results of a typical experiment are presented in fig. 2. Only the precursor forms of acid α-glucosidase and cathepsin D, and not the

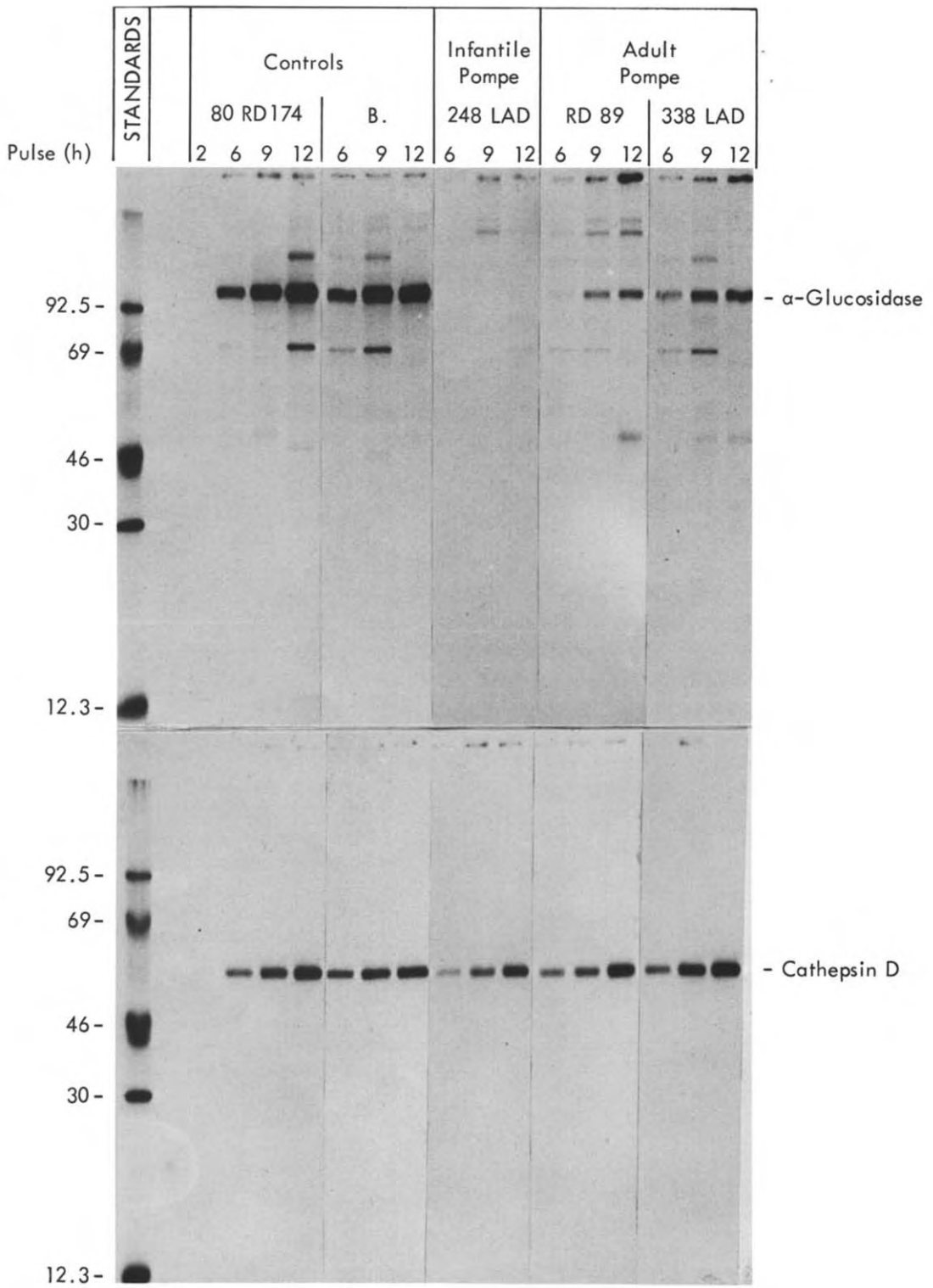

Fig. 2. Radioactivity in acid α-glucosidase and cathepsin D in medium after culture of fibroblasts for various times with 0.4 mCi [^{3}H]leucine and 10 mM NH_4Cl. α-Glucosidase and cathepsin D were immunoprecipitated from 9/10 and 1/10 aliquots of the medium, respectively.

processed forms, were found in the medium, in agreement with [11]. The α-glucosidase polypeptide in the medium of adult Pompe's disease fibroblasts had the same M_r as that in control fibroblasts but was present in lower concentrations. No α-glucosidase polypeptides could be detected in the medium of the infantile Pompe fibroblasts.

The increase in incorporated radioactivity with time was quantified as in [15]. Table 1 shows that the rate of secretion of the precursor of α-glucosidase relative to that of the precursor of cathepsin D in adult Pompe fibroblasts was 3–10% of that in control fibroblasts. In this experiment, incorporation of radioactivity into cathepsin D in the medium was lower than that usually observed. However, a similar picture emerged when arylsulphatase A was used as the reference enzyme.

Analogous results have been described in [19]; fibroblasts were cultured in the presence of NH_4Cl and measured acid α-glucosidase activity in the medium; the rate of appearance of the activity in the medium in mutant fibroblasts was ~10% of that in control fibroblasts.

The reduced secretion of newly synthesized α-glucosidase in adult Pompe fibroblasts was not due to retention of the enzyme in the cells; in the presence of 10 mM NH_4Cl, the same proportion of newly synthesized α-gluosidase (~50%) was found intracellularly in control fibroblasts (see also [11]) as in adult Pompe fibroblasts. It should be noted that the proportion of newly synthesized α-glucosidase found intracellularly when fibroblasts were cultured in the presence of 10 mM NH_4Cl was higher than that of newly synthesized β-hexosaminidase (≤20%; see also [11]) or arylsulphatase A (≤25%).

The results presented so far indicated that *net* formation of the precursor of acid α-glucosidase was reduced in fibroblasts from patients with the late-onset form of Pompe's disease. The question arose of whether this was due to a reduction in the rate of synthesis of acid α-glucosidase or to enhanced breakdown of newly synthesized enzyme. We therefore incubated fibroblasts with [^{35}S]methionine, in the absence of NH_4Cl, for a

Table 1

Rate of appearance in medium of radioactively labelled precursors of α-glucosidase and cathepsin D during culture of fibroblasts in the presence of [^{3}H]leucine and 10 mM NH_4Cl

Cell line	Rate of incorporation of [^{3}H]leucine (cpm/h) into precursor of		*A/B*
	Acid α-glucosidase (*A*)	Cathepsin D (*B*)	
Controls			
80RD174	95	170	0.56
B.	73	133	0.55
Infantile form of Pompe			
266LAD	<0.5	186	<0.003
248LAD	<0.5	95	<0.005
Late-onset form of Pompe			
77RD89	9	132	0.068
338LAD	12	182	0.066
H.	3	152	0.020

Data from fig. 1

Table 2

Incorporation of radioactivity into α-glucosidase and cathepsin D in various fibroblast lines after a 10 min pulse with 0.6 mCi [^{35}S]methionine followed by a 5 min chase

Cell line	Radioactivity (cpm) in		*A/B*
	Acid α-glucosidase (*A*)	Cathepsin D (*B*)	
Control			
80RD174	23	336	0.068
Infantile Pompe			
266	<1	511	<0.002
248LAD	<1	452	<0.002
Late-onset Pompe			
338LAD	19	692	0.027
H.	21	983	0.021
G.	14	344	0.041

For experimental details see section 2

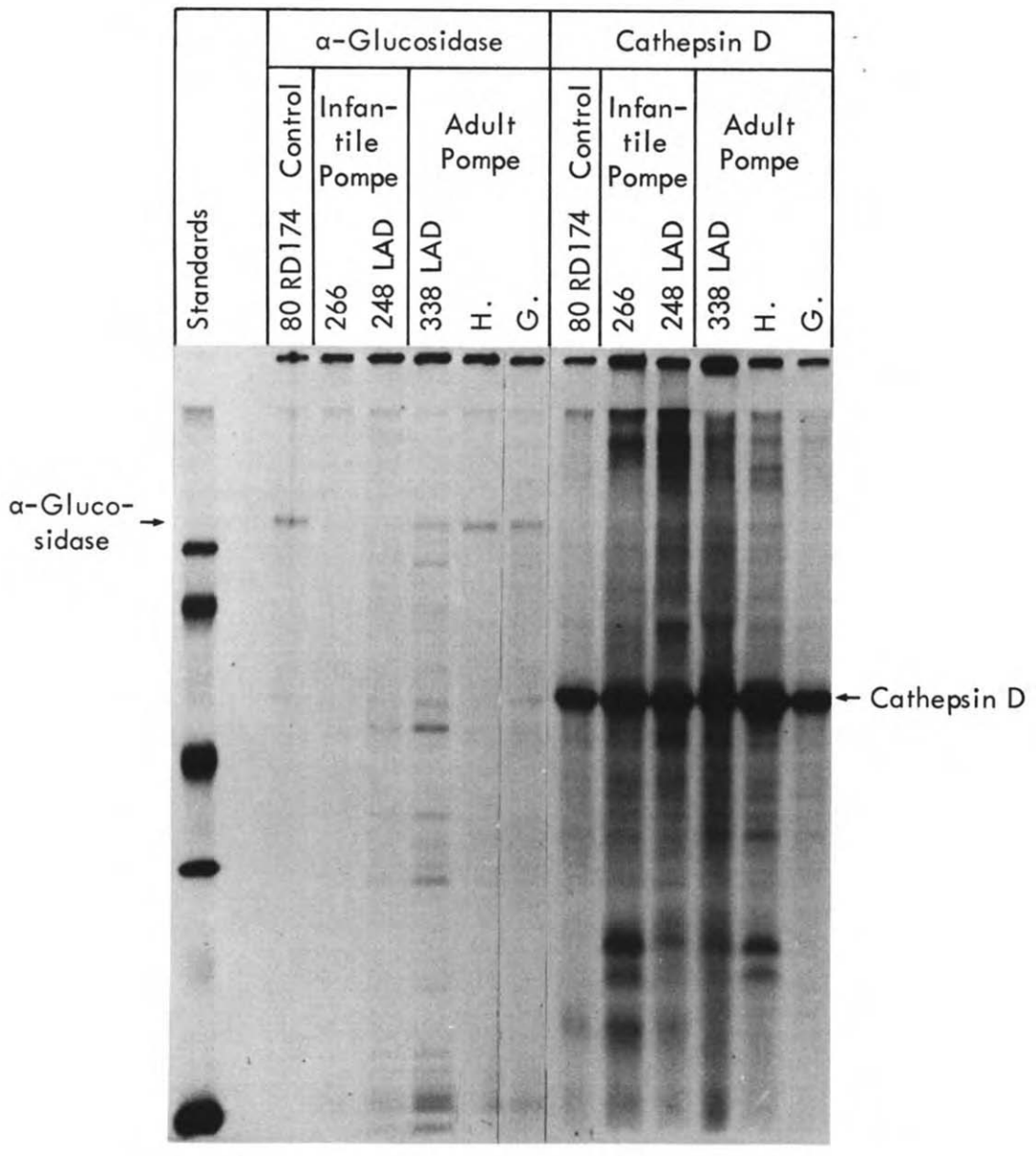

Fig. 3. Radioactivity in acid α-glucosidase and cathepsin D in fibroblasts after a 10 min pulse with 0.6 mCi [^{35}S]methionine and a 5 min chase. For experimental details see section 2.

relatively short period (10 min pulse + 5 min chase). The results of a typical experiment are shown in fig. 3. The M_r of the radioactive α-glucosidase polypeptide was the same in adult Pompe fibroblasts as in control fibroblasts. Furthermore, the difference in the intensity of the α-glucosidase band in adult Pompe and control fibroblasts was much less than in the experiments where cells were pulsed for 3–16 h (cf. fig. 1,2). Indeed, the quantification in table 2 shows that the initial rate of synthesis of α-glucosidase in adult Pompe fibroblasts was 30–60% of that in control cells. In an experiment in which cells were pulse-labelled with [^{35}S]methionine for 0.5, 1, 1.5 and 2 h the amount of ^{35}S-labelled α-glucosidase relative to that of ^{35}S-labelled cathepsin D in an adult Pompe

cell line (338LAD) was 70, 85, 40 and 30%, respectively, of that in a control line (80RD174). Similar results were obtained when the incorporation of radioactivity into α-glucosidase was related to that into arylsulphatase A.

The stability of acid α-glucosidase in lysosomes has been estimated by allowing purified enzyme to be endocytosed by fibroblasts from patients with the infantile form of Pompe's disease and monitoring the enzyme activity in the cells [19]. α-Glucosidase purified from fibroblasts from patients with the late-onset form of Pompe's disease had about the same half-life as that from secretions of control fibroblasts [19].

4. CONCLUDING REMARKS

Three observations suggested that the rate of synthesis of α-glucosidase may be much lower in fibroblasts from patients with the late-onset form of Pompe's disease than in control fibroblasts:

(i) The residual enzyme activity is low (5–20% of that in controls [6–10];

(ii) The amount of radioactivity recovered in processed α-glucosidase after prolonged incubation is low (<15% of that in controls; this paper);

(iii) The rate of NH_4Cl-induced secretion of radioactively labelled precursor is <15% of that in controls (table 1).

However, the results of the short-time incubations (< 1 h) described here indicate that the residual rate of formation of α-glucosidase in the mutant fibroblasts is 30–85% of that in control fibroblasts, which is in apparent contradiction to the three observations above. This apparent discrepancy may be explained by assuming that the α-glucosidase initially synthesized in fibroblasts from patients with the late-onset form of Pompe's disease has a decreased stability in compartments that are located proximal to the site where ammonium chloride interferes with the segregation of lysosomal enzymes. In compartments distal to this site, the enzyme appears to be stable (fig. 1 and [19]).

In fibroblasts from patients with the generalized form of Pompe's disease no incorporation of radioactivity into α-glucosidase could be detected. In these fibroblasts either no protein immunologically related to α-glucosidase is synthesized or it is broken down immediately after synthesis.

ACKNOWLEDGEMENTS

The authors are grateful to Professor Martinus Niermeijer (Division of Clinical Genetics, Erasmus University, Rotterdam) and Dr Kurt Ullrich (Universitäts-Kinderklinik, Münster) for providing the cell lines used, to Dr Marja Hollemans, Dr Flip de Groot and Mr Frans Tegelaers for stimulating discussions, to Ms Wendy van Noppen for her help in the preparation of the manuscript and to Ms Anneke Strijland for assistance in culturing the cells. Supported by grants from the Deutsche Forschungsgemeinschaft (SFB 104) and the Netherlands Organization for the Advancement of Pure Research (ZWO) under the auspices of the Netherlands Foundation for Fundamental Medical Research (FUNGO).

REFERENCES

[1] Hers, H.G. (1963) Biochem. J. 86, 11–16.
[2] Hers, H.G. and De Barsy, Th. (1973) in: Lysosomes and Storage Diseases (Hers, H.G. and Van Hoof, F. eds) pp. 147–216, Academic Press, New York.
[3] De Barsy, Th., Jacquemin, P., Devos, P. and Hers, H.G. (1972) Eur. J. Biochem. 31, 156–165.
[4] Koster, J.F. and Slee, R. (1977) Biochim. Biophys. Acta 482, 89–97.
[5] Murray, A.K., Brown, B.I. and Brown, D.H. (1978) Arch. Biochem. Biophys. 185, 511–524.
[6] Reuser, A.J.J., Koster, J.F., Hoogeveen, A. and Galjaard, H. (1978) Am. J. Hum. Genet. 30, 132–143.
[7] Beratis, N.G., LaBadie, G.U. and Hirschhorn, K. (1978) J. Clin. Invest. 62, 1264–1274.
[8] Martin, J.J., De Barsy, Th. and Den Tandt, W. (1976) J. Neurol. 203, 105–118.
[9] Mehler, M. and DiMauro, S. (1976) Arch. Neurol. (Chic.) 33, 692–695.
[10] Schram, A.W., Brouwer-Kelder, B., Donker-Koopman, W.E., Loonen, C., Hamers, M.N. and Tager, J.M. (1979) Biochim. Biophys. Acta 576, 370–383.
[11] Hasilik, A. and Neufeld, E.F. (1980) J. Biol. Chem. 255, 4937–4945.
[12] Hasilik, A. and Neufeld, E.F. (1980) J. Biol. Chem. 255, 4946–4950.

[13] Steckel, F., Waheed, A., Hasilik, A., Von Figura, K., Oude Elferink, R.P.J., Kalsbeek, R. and Tager, J.M. (1982) Hoppe-Seyler's Z. Physiol. 363, 1040.
[14] Cantz, M., Kresse, H., Barton, R.W. and Neufeld, E.F. (1972) Methods Enzymol. 28, 884–896.
[15] Waheed, A., Hasilik, A. and Von Figura, K. (1982) Eur. J. Biochem. 123, 317–321.
[16] Gonzales-Noriega, A., Grubb, J.H., Talkad, V. and Sly, W.S. (1980) J. Cell Biol. 85, 839–852.
[17] Sly, W.S., Natowicz, M., Gonzales-Noriega, A., Grubb, J.H. and Fischer, H.D. (1981) in: Lysosomes and Lysosomal Storage Diseases (Callahan, J.W. and Lowden, J.A., eds) pp. 131–146, Raven, New York.
[18] Neufeld, E.F. (1981) in: Lysosomes and Lysosomal Storage Diseases (Callahan, J.W. and Lowden, J.A. eds) pp. 115–129, Raven, New York.
[19] Reuser, A.J.J. and Kroos, M. (1982) FEBS Lett. 146, 361–364.