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Sanfilippo Type C Diagnosis: Assay of Acetyl-CoA: α-Glucosaminide *N*-Acetyltransferase Using [^{14}C]Glucosamine as Substrate and Leukocytes as Enzyme Source

RITA PALLINI, IRWIN G. LEDER,[12] AND PAOLA DI NATALE[13]

Institute of Cellular and Molecular Biochemistry, Second Medical School, University of Naples, Via Pansini 5, Naples, Italy

Summary

We describe an assay for acetyl-CoA: α-glucosaminide *N*-acetyltransferase using purified [^{14}C]glucosamine as substrate, and leukocytes as enzyme source. The assay is suitable for the diagnosis of homozygous and heterozygous carriers of Sanfilippo syndrome type C.

Sanfilippo syndrome comprises a group of genetic diseases that result from a deficiency of specific lysosomal enzymes involved in the degradation of heparan sulfate. They have been designated Sanfilippo A, B, C, and D corresponding to deficiencies of: sulfamidase (8): α-*N*-acetylglucosaminidase (3, 11), acetyl-CoA: α-glucosaminide *N*-acetyltransferase (7), and *N*-acetylglucosamine 6-sulfate sulfatase (9), respectively.

Type C syndrome has been diagnosed by using a complex trisaccharide substrate (5) or tetrasaccharide and disaccharide substrates (4) prepared from heparin. Glucosamine, a monosaccharide that is commerically available, was shown to be a suitable substrate for the diagnosis of this syndrome in fibroblasts (4). In this report we describe the use of purified [^{14}C]glucosamine as substrate, and leukocytes as enzyme source for the diagnosis of homozygous and heterozygous individuals.

MATERIALS AND METHODS

Purification of D-[U-^{14}C]glucosamine hydrochloride. D-[U-^{14}C] glucosamine hydrochloride, 277 mCi/mmol from Amersham was purified through chromatography on Dowex H^+ 50 W-X8 100–200 mesh. Fifty microcuries of the radioactive compound were applied to a column 1 x 5 cm. The column was washed with 50 ml distilled water to eliminate a neutral radioactive contaminant(s) that accounted for 6% of total radioactivity. [^{14}C] Glucosamine was eluted with 15 ml of 0.3 M HCl and then subjected to three to four cycles of evaporation to eliminate HCl. The dried residue was stored overnight in a desiccator over NaOH, then collected with about 10 ml distilled water, and lyophilized. As an alternative procedure to remove excess hydrochloride, the [^{14}C]glucosamine was treated with approximately 0.4 ml of anion exchange resin (Ag 1-X8 OH^-) per ml of solution. After shaking, the resin was centrifuged and washed 5 times with 2 ml cold water. The combined washings were then lyophilized. After lyophilization the labeled glucosamine was dissolved in 2.5 ml of 5% aqueous ethanol and stored in aliquots at −20°C. The solution contained 36×10^6 cpm/ml, 0.105 μmol/ml.

Preparation of leukocyte homogenates. Leukocytes were prepared according to Fallon *et al.* (2). Ten milliliters of venous blood was mixed with 10 ml of a sedimentation fluid which contained 1.5 ml of ACD solution (2.45 g glucose, 2.2 g sodium citrate dihydrate, 0.73 g citric acid, and 0.9 g sodium chloride in 100 ml water), 5 ml of a 6% dextran grade A solution (British Drug Houses), and 3.5 ml of a 5% glucose solution. Erythrocytes were allowed to sediment at room temperature for 45 min. The supernatant was centrifuged for 10 min at 800 *g*. Leukocyte pellet was suspended in 2 ml of 0.9% NaCl to which 8 ml of 0.83% NH_4Cl were added. The mixture was left 5 min at room temperature. After centrifugation at 400 *g* for 5 min the supernatant was discarded and the hemolysis cycle was repeated. The pellet was washed with 10 ml of 0.9% NaCl and stored at −20°C. To prepare the homogenate, the leukocyte pellet was resuspended in 200 μl of 0.9% NaCl plus 20 μl of 1% Triton-X-100 and subjected to seven cycles of freezing and thawing before dialyzing

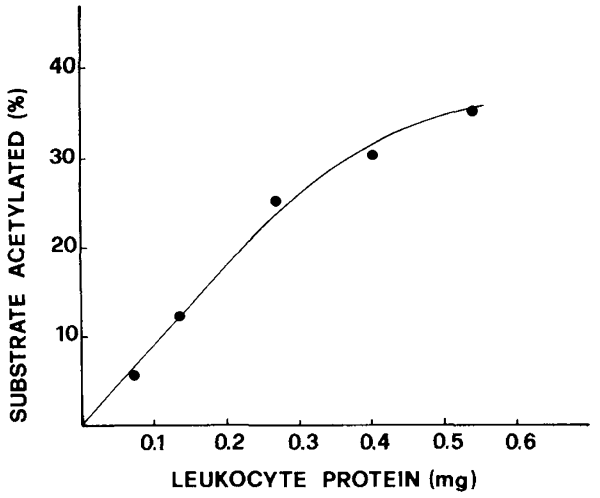

Fig. 1. Acetylation of [^{14}C]glucosamine as a function of leukocyte protein. Incubation was for 4 h at 37°C.

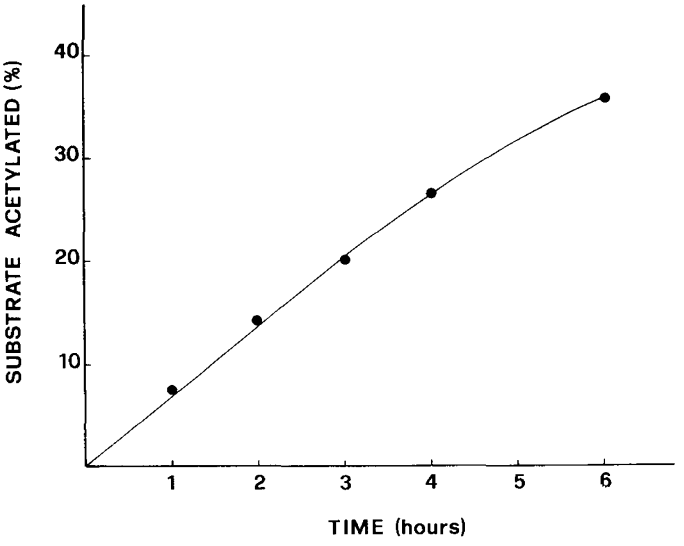

Fig. 2. Acetylation of [^{14}C]glucosamine as a function of time. The incubation mixture contained 0.27 mg of leukocyte protein.

overnight at 4°C against 0.9% NaCl (one change, 2 L each). After dialysis the material was homogenized in minipotter and protein was determined by the Lowry method (10).

Determination of acetyl-CoA: α-glucosaminide N-*acetyltransferase activity.* Determination of enzyme activity was carried out in a total volume of 0.1 ml. The standard mixture consisted of 0.1 M sodium phosphate buffer pH 6.0, 0.15 M NaCl, 4 mM NaN_3, 2 mM acetyl-CoA, 3.5 μM [^{14}C]glucosamine (1.2 x 10^5 count/min), 5 μM cold glucosamine, and leukocyte homogenate (0.1–0.3 mg protein). A control tube without homogenate served as a blank. After 4 h at 37°C, the reaction mixtures were diluted with 0.5 ml H_2O and applied to columns (0.7 x 1.1 cm) of Dowex 50 W-X8 cation exchange resin (H^+ form). A scintillation vial was placed under each column to catch the effluent. Each tube was further washed with 0.5 ml of H_2O which was also applied to the column. An additional 4 ml of water was used to wash out the [^{14}C]*N*-acetylglucosamine produced. Ten milliliters of Lumagel (Supelchem) was added to each vial which was then counted. The results are corrected for values given by the control without homogenate. A unit of enzyme activity is defined as catalyzing the acetylation of 1% of the substrate per hour.

RESULTS AND DISCUSSION

Standard assay conditions for *N*-acetyltransferase activity were determined using normal leukocyte homogenates. Enzyme activity is proportional to protein concentration in the range of 0.1–0.3 mg (Fig. 1) and is linear for 4 hr of incubation (Fig. 2). The leukocyte enzyme shows a broad pH optimum, between pH 5.5 and 7.5, similar to fibroblast enzyme (Fig. 3) whereas no activity was detectable in Sanfilippo C fibroblasts at all the pH values examined (Fig. 3). The K_m for [^{14}C]glucosamine was calculated to be 3.5 μM (Fig. 4). It is 28 times lower than the K_m reported for fibroblast enzyme (4).

We recommend the use of least 10^5 count/min of substrate per assay mixture and that the substrate concentration not exceed

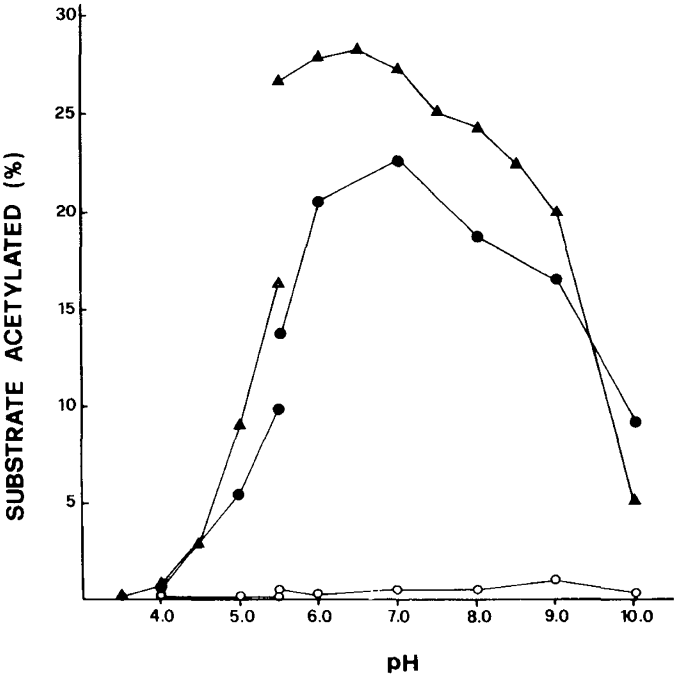

Fig. 3. Transferase activity as a function of pH. Leukocyte protein (0.27 mg) (▲——▲), 0.095 mg of normal fibroblast protein (●——●) and 0.08 mg of Sanfilippo C fibroblast protein (○——○) were incubated 4 h at 37°C in the presence of 0.1 M sodium acetate buffers (pH 3.5–5.5), 0.1 M sodium phosphate buffers (pH 5.5–8.0), and 0.1 M glycine-NaOH buffers (pH 9–10).

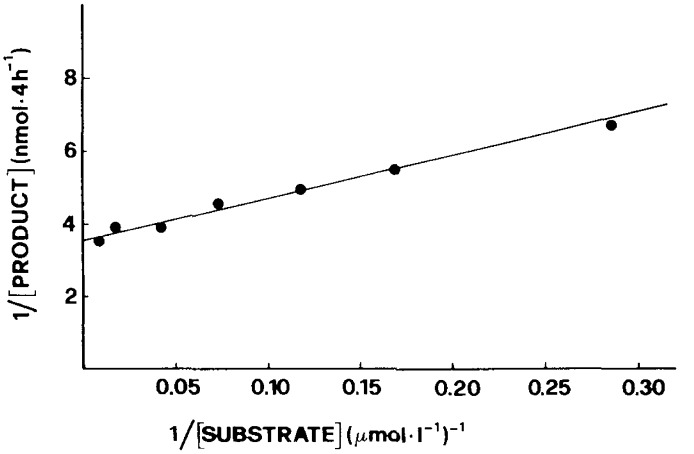

Fig. 4. Determination of K_m of N-acetyltransferase. Leukocyte protein (0.27 mg) was incubated 4 h in the presence of [^{14}C]glucosamine. Unlabeled glucosamine was used to adjust this substrate to the desired concentration.

Table 1. *Specific activity of* N-*acetyltransferase in homogenates of leukocytes from normal subjects, homozygous and obligate heterozygous of Sanfilippo C syndrome*

Subjects	*N*-acetyltransferase activity	
	$(U \cdot mg^{-1})$*	$(nmol \cdot min^{-1} \cdot g^{-1})$†
Normal individuals ($n = 10$)		
Range	21–54	3.0–7.74
Mean	34.42	4.95
SD	11.22	1.62
Homozygous ($n = 2$)		
Mean	0.3	0.004
Obligate heterozygous ($n = 4$)		
Range	7–27	1–3.92
Mean	16.67	2.42
SD	9.38	1.35

* One unit of *N*-acetyltransferase activity is defined as the amount of enzyme catalyzing the acetylation of 1% of the radioactive substrate per hour.

† Values are expressed according to ref. 8.

8.5 μM, that is 2.5 times the apparent K_m value. Increasing substrate concentration would increase the amount of product formed, but would also reduce the number of counts in the product and hence the sensitivity of the test. Purification of [^{14}C] glucosamine reduces substrate blank from 6 to 1.5% and this is particularly valuable for carrier diagnosis. In our assay conditions, using 0.1 mg protein, the mean normal activity corresponds to 12.7% acetylation of substrate and the mean obligate heterozygote to 8% acetylation, with a range 4.8–13%. Using non purified glucosamine, substrate blank values are 20–40% of the enzymatic value.

The assay was applied to the detection of homozygous and heterozygous carriers of Sanfilippo syndrome type C (Table 1). Leukocyte homogenates prepared from patients have no detectable activity even though incubation of homogenate continued for up to 24 h. One of the two patients with Sanfilippo C syndrome was diagnosed on the basis of a fibroblast test in Professor Kresse's laboratory using [^{3}H]trisaccharide substrate (7).

The specific activity of *N*-acetyltransferase allowed a good discrimination between normal individuals and heterozygotes of the Sanfilippo C syndrome (Table 1). As shown in the Table, the mean activity of obligate heterozygotes is 50% of the mean activity of normal subjects. As expected in this type of diagnosis (1) the 2 standard deviation range of normals and heterozygotes showed overlapping. No improvement was found if the *N*-acetyltransferase activity was related to that of β-glucuronidase (6).

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12. On Leave from: Laboratory of Biochemistry and Metabolism, National Institutes of Health Bethesda, Md., U.S.A.
13. Requests for reprints should be addressed to: Prof. Paola di Natale, Institute of Cellular and Molecular Biochemistry, Second Medical School, University of Naples, Via Pansini 5, Naples, Italy.

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