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CHARACTERIZATION OF NEURAMINIDASE ACTIVITY OF CULTURED HUMAN FIBROBLASTS

GEORGE H. THOMAS *, LINDA W. REYNOLDS and CAROL S. MILLER

The John F. Kennedy Institute and Department of Pediatrics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205 (U.S.A.)

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Summary

Investigations have been carried out to establish the enzymatic properties and specificities of the neuraminidase of cultured human fibroblasts. Homogenates of these cells cleaved the actylated derivative of neuraminic acid from fetuin, *N*-acetylneuraminyllactose and 2-(3′methoxyphenyl)-*N*-acetyl-α-neuraminic acid. Maximum activity occurred between pH 4.2 and 4.6 in sodium acetate buffer. The K_m values were $3.6 \cdot 10^{-4}$ M, $3.0 \cdot 10^{-3}$ M and $1.1 \cdot 10^{-3}$ M, respectively, against fetuin, *N*-acetylneuraminyllactose and 2-(3′methoxyphenyl)-*N*-acetyl-α-neuraminic acid. Against the first two substrates, the rate of hydrolysis fell below the expected value as the cell homogenate was diluted with water or 10 mM NaCl. Dilution with 8 mg/ml bovine serum albumin prevented the deviation and yielded the expected linear decrease. After the first 2-h incubation, the rate of hydrolysis decreased from the initital linear rate. The enzyme(s) was partially or completely inactivated by sonication at 20 kHz, freeze-thaw treatment, incubation at 52°C or storage for 48 h at —70°C. Suspension of the fibroblasts in water for 10 min at room temperature, followed by homogenization with a tissue grinder, yielded preparations that were suitable for the assay of the neuraminidase activity.

Introduction

Neuraminidase(s) (sialidase, α-*N*-acetyl-neuraminosyl glycohydrolase, EC 3.2.1.18) cleaves the terminal sialic acid from glycoproteins, glycopeptides and

* To whom reprint requests should be addressed.
Abbreviation used: MPN, 2-(3′methoxyphenyl)-*N*-acetyl-α-neuraminic acid.

gangliosides and is thus probably responsible for initiating the degradation of the oligosaccharide portion of many of these substances [1].

Recently, several inherited human disorders resulting from, or associated with, neuraminidase deficiencies have been reported. These include mucolipidosis I [2], the cherry-red spot-myoclonus syndrome [3,4] and several additional patients whose classification remains unclear [5—7]. Additionally, alterations in neuraminidase activity have been found in mucolipidosis II [8,9] and III [2,10], however, in these disorders, it is unclear if the defect is a primary or secondary phenomenon.

As a result of these newly discovered disorders, considerable attention has been directed towards the measurement of neuraminidase activity in readily available human tissues for both clinical and scientific purposes. While we [4,8] and others [2,3,6,7] have demonstrated the presence of this enzyme in cultured human fibroblasts, little is known about its properties in these cells. We now report the results of a study of the properties of, and the assay conditions for, human fibroblast neuraminidase against fetuin, *N*-acetylneuraminyllactose and 2-(3′methoxyphenyl)-*N*-acetyl-α-neuraminic acid (MPN).

Materials

Fibroblasts. Human fibroblast cultures were established and maintained in Eagle's minimum essential medium supplemented with L-glutamine, fetal calf serum and antibiotics as previously described [11,12]. The cultures were harvested at confluence (approx. 1 mg protein/75 cm^2 Falcon flask No. 3024) with trypsin containing EDTA, 7 days after subculture [11]. Following trypsin treatment, the cells were washed 3 times with cold phosphate-buffered saline [12], with centrifugation following each wash step. Except where noted, the washed fibroblasts were suspended in deionized water, held at room temperature for 10 min and then homogenized (20 strokes) with a motor-drived 12 × 100 mm glass tissue-grinder and pestle having a 0.13—0.18 mm clearance [4]. The final protein concentration of the cell homogenate was approx. 2—2.5 mg/ml. Unless noted otherwise, cell sonicates were prepared by suspending fibroblasts in cold water and disrupting them at 20 kHz with a Sonifier Cell Disruptor, Model W-185D (Heat Systems-Ultrasonics, Plainview, New York) equipped with a Model No. 420 special microtip. During sonication the cells were maintained in an ice-water bath and sonication was carried out for 3 10-s periods with cooling intervals between each treatment.

Chemicals. Lyophilized fetal calf serum fetuin (Type II) and bovine colostrum *N*-acetylneurminyllactose (Type II), having 85% (2 → 3) and 15% (2 → 6) isomers, were purchased from the Sigma Chemical Company, (St. Louis, MO, U.S.A.). 2-(3′Methoxyphenyl)-*N*-acetyl-α-neuraminic acid was obtained from the Research Resources Branch of the National Institute of Allergy and Infectious Disease (NIH, Bethesda, MD). Methoxyphenol was obtained from the Eastman Kodak Company, (Rochester, NY). All other chemicals and solvents were obtained commercially and were of reagent grade quality.

Methods

Neuraminidase activity towards *N*-acetylneuraminyllactose and fetuin was measured by following the release of neuraminic acid utilizing a micromodification [4,8] of the thiobarbituric acid procedure [13]. Neuraminidase activity against 2-(3'methoxyphenyl)-*N*-acetyl-α-neuraminic acid was quantitated by measuring the release of toluene extractable methoxyphenol according to Thomas et al. [4]. Other lysosomal enzyme activities were measured against the appropriate artificial substrates [4]. Protein concentrations were determined by the method of Lowry et al. [14]. Except where noted, all enzyme studies were carried out on freshly-prepared homogenates.

Results

Stability

Preliminary studies indicated that the neuraminidase activity of human fibroblasts is inactivated by a number of procedures frequently utilized for the rupture and/or release of enzymes of these cells. As shown in Table I, sonication of intact cells suspended in water resulted in an average reduction in neuraminidase activity of 73 and 79% against 4 mM *N*-acetylneuraminyllactose and fetuin. A similar, but smaller, reduction in activity also occurred when the cells were sonicated in 0.05 M buffer pH 4.2 (Table I). Preincubation of intact fibroblasts in 0.2% Triton X-100 in 0.05 M acetate buffer in the presence of *N*-acetylneuraminyllactose or fetuin for 30 min in an ice-bath followed by a 6-h incubation at 37°C resulted in a 51% reduction in neuraminidase activity. As sonication and homogenization techniques are widely employed as means of rupturing fibroblasts prior to the measurement of enzymes, the effect of these procedures on neuraminidase was compared to the effect on a number of acid lysosomal hydrolases. As indicated in Table II, neuraminidase activity against all three substrates was markedly reduced by sonication and mildly reduced by homogenization. In contrast, the activities of all of the lysosomal enzymes examined were increased when compared to untreated cells suspended in water.

Additional findings indicating enzyme instability include the loss of 43 and

TABLE I

EFFECT OF VARIOUS METHODS OF CELL RUPTURE ON NEURAMINIDASE ACTIVITY OF HUMAN FIBROBLASTS

Enzyme source	*N*-Acetylneuraminyllactose			Fetuin		
	Mean *	Range	n	Mean	Range	n
Whole cell suspension	119.5	79.6—179.4	8	30.1	32—37.1	7
Homogenate in buffer	93.2	—	1	24	—	1
Homogenate in water	106.2	46—176.7	11	25.6	17.4—40.0	10
Sonicate in buffer	48.0	42.5—61.3	4	11.7	7.5—18.3	4
Sonicate in water	32.7	16—59	8	6.2	0.8—8	8

* nmols substrate cleaved/mg protein per 6 h at 37°C in 0.05 M acetate buffer in the presence of 4 mM *N*-acetylneuraminyllactose or 5 mg fetuin/ml.

TABLE II

EFFECTS OF SONICATION AND HOMOGENIZATION ON NEURAMINIDASE AND 7 KNOWN LYSOSOMAL ENZYMES

Enzyme	Activity *	
	Homogenized cells (%)	Sonicated cells (%)
Neuraminidase		
with AcNeu lactose	89	27
with Fetuin	85	21
with MPN	76	0
Arylsulfatase A	110	203
β-Galactosidase	101	124
α-Mannosidase	115	107
N-Acetyl-β-glucosaminidase	121	176
α-Glucosidase	116	122
β-Glucosidase	132	158
α-Glucuronidase	119	100

* Expressed as a percent of the activity found in intact fibroblasts suspended in H_2O and assayed simultaneously.

66% of neuraminidase activity following 24 h and 48 h of storage of homogenates of −68 at −70°C, loss of 49% of enzyme activity following rapid freezing and thawing (10X) in a dry ice-acetone mixture, and loss of 22, 85 and 100% of activity during incubation of cell homogenates at 24, 37 and 52°C for 15 min. The neuraminidase activity in each of the above studies was determined against *N*-acetylneuraminyllactose.

To examine the possibility that some of the apparent reduction in neuraminidase activity was due to an artifact arising from metabolism of the sialic acid (e.g., by neuraminic acid aldolase), 25 nmol free sialic acid were incubated with cell homogenates at pH 4.5 for 6 h at 37°C. In the controls, the sialic acid was added to the homogenate at the end of the incubation period. The average amount of free AcNeu in each was 23.5 nmol. It was, therefore, concluded that AcNeu released by neuraminidase was not metabolized to a non-reactive compound.

Phase contrast microscopy indicated that suspension of harvested fibroblasts in distilled water at room temperature resulted in enlarged, swollen and circular cells. While some cells ruptured within 10 min, a mild homogenization procedure was necessary to routinely obtain satisfactory cell rupture. Following the combined hypotonic-homogenization treatment outlined in the methods section, the preparation consisted of cellular debris, cell ghosts and some intact, swollen cells. While this procedure also caused some reduction in neuraminidase activity (Table II), the preparation was found to be suitable for the assay of neuraminidase and was utilized for the remainder of the study.

Effect of pH

The pH optimums of the enzyme(s) against fetuin, *N*-acetylneuraminyllactose and methoxyphenyl-*N*-acetyl-α-neuraminic acid in acetate buffer are shown in Figs. 1D, 2D and 3D. While the maximum activity in all cases was found to be between 4.2 and 4.6, the optimum against fetuin, *N*-acetyl-

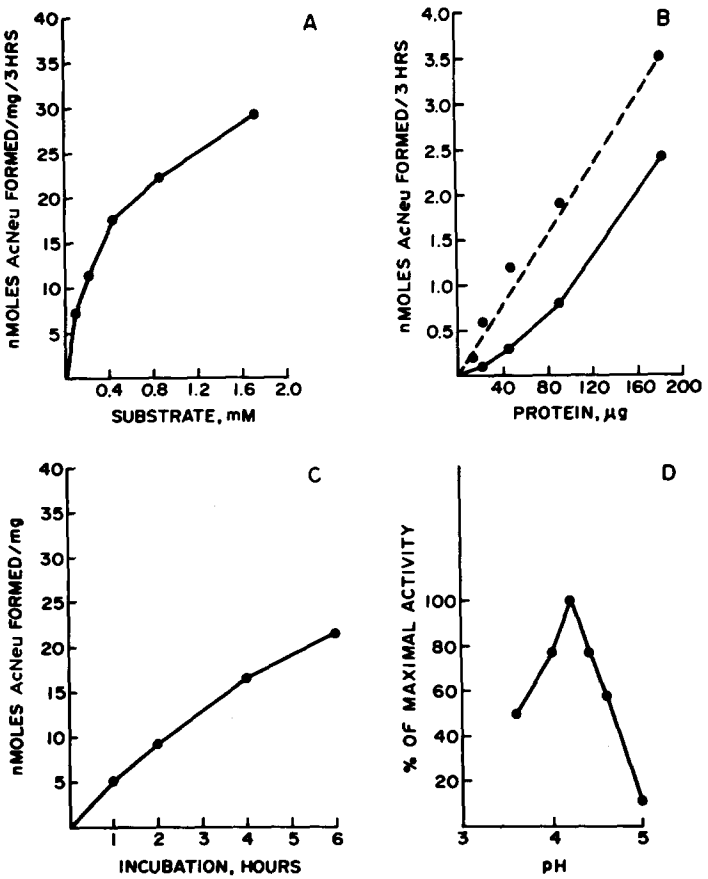

Fig. 1. Neuraminidase activity of homogenates of cultured fibroblasts against fetal calf serum fetuin in 0.05 M sodium acetate buffer. Effect of: A, substrate concentration; B, homogenate concentration; C; incubation time; D, pH. Except as noted, the pH was 4.2, the fetuin concentrations was 5 mg/ml (0.86 mM total sialic acid) and the cell homogenates contained 90—180 µg protein/tube. Incubation times were 3 h for A and B, 6 h for D and as indicated for C. The cell homogenate (Fig. 4B) was diluted with H_2O (●———●) and with 8 mg bovine serum albumin/ml (●- - - - -●). AcNeu, *N*-acetylneuraminic acid.

neuraminyllactose and MPN was, respectively, 4.2, 4.4 and 4.6. With all three substrates, the activity at pH 5.4, the highest values examined, was less than 20% of the maximum value.

Increasing the final acetate concentration from 0.025 M to 0.2 M resulted in an 11% decrease in enzyme activity against *N*-acetylneuraminyllactose.

Effect of substrate concentration

The effect of varying the concentration of the various substrates on the reaction rate was determined by incubating cell homogenates for 3 h at 37°C in the presence of acetate buffer and appropriate substrate. In each case, the optimal pH for the particular substrate under study was utilized. The apparent Michaelis-Menten constants (K_m were $3.6 \cdot 10^{-4}$ M, $3.0 \cdot 10^{-3}$ M and $1.1 \cdot 10^{-3}$ M, respectively , for fetuin, *N*-acetylneuraminyllactose and MPN (Figs.

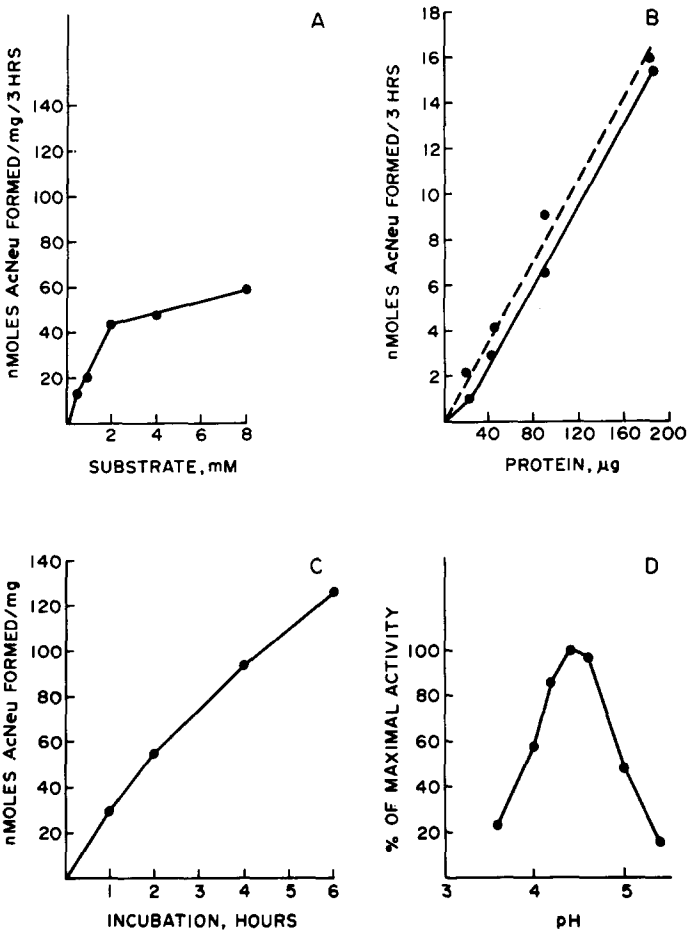

Fig. 2. Neuraminidase activity of homogenates of cultured fibroblasts against *N*-acetylneuraminyllactose in 0.05 M sodium acetate buffer. Effect of: A, substrate concentrations; B, homogenate concentration; C, incubation time; D, pH. Except as noted, the pH was 4.5, the substrate concentration was 4 mM and the cell homogenates contained 90—116 µg protein/tube. Incubation times were 3 h for A and B, 6 h for D and as indicated for C. The cell homogenate (Fig. 4B) was diluted with H_2O (●———●) and with 8 mg/ml bovine serum albumin (●- - - - -●). All values were corrected by subtracting cell homogenate and substrate blank values.

1A, 2A and 3A). For fetuin, the calculation was based on the total amount of sialic acid available, as judged from the sialic acid released following treatment in 0.1 N H_2SO_4 at 80° C for 1 h.

Effect of enzyme concentration

Activity against MPN was linear with increasing enzyme concentration from 80 to 400 µg of cell protein, the highest concentration examined (Fig. 3B). With the sialylactose (Fig. 2B), the reaction was linear between 30 and 180 µg cell protein (the latter being the highest concentration studied). With this sub-

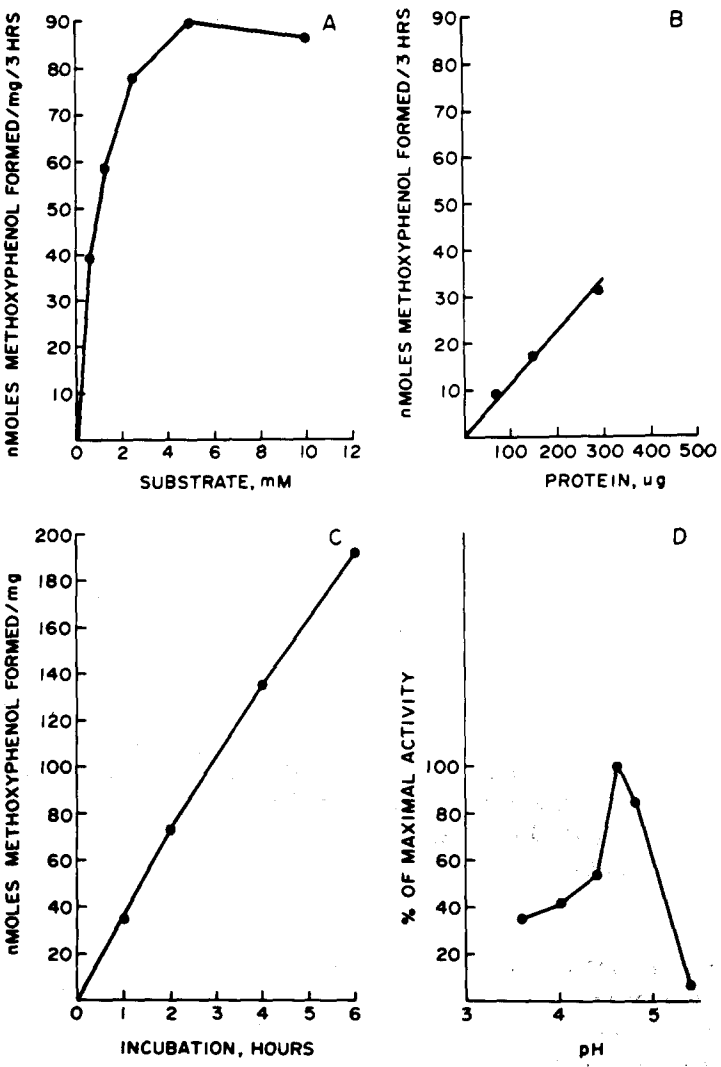

Fig. 3. Neuraminidase activity of homogenates of cultured fibroblasts against 2-(3′methoxyphenyl)-*N*-acetyl-α-neuraminic acid in 0.2 M acetate buffer. Effect of: A, substrate concentrations; B, homogenate concentration; C, incubation time; D, pH. Except as noted, the pH was 4.6, the substrate concentration was 5 mM and the cell homogenates contained 136—353 μg protein/tube. Incubation times were 3 h for A and B, 6 h for D and as indicated for C. All values were corrected by subtracting cell homogenate and substrate blank values.

strate, however, the projected curve did not pass through the zero point, thus indicating nonlinearity at lower levels of the enzyme (Fig. 2B). Similar results were obtained when the cell homogenate was diluted with 10 mM NaCl. A similar finding was also obtained with fetuin.

Dilution of cell homogenates with 8 mg bovine serum albumin/ml, in place of water, prevented the loss of specific activity, at the lower enzyme concentrations, against both substrates (Figs. 1B and 2B). Thus, in the presence of bovine

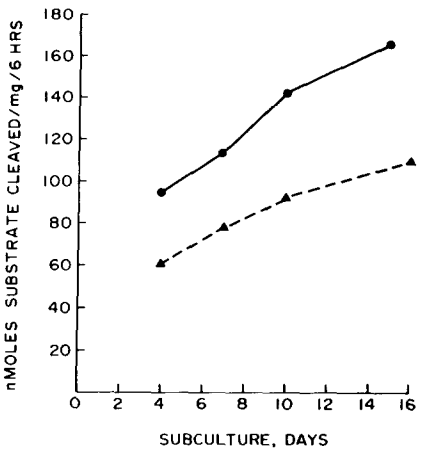

Fig. 4. Effect of tissue culture time on the specific activity of sialidase. Enzyme activity was measured in cell homogenates in the presence of 2 mM MPN in 0.2 M acetate buffer, pH 4.6 (●———●) and 4 mM *N*-acetylneuraminyllactose in 0.05 M acetate buffer, pH 4.5 (▲- - - - -▲).

serum albumin, there was a linear decrease in activity with increasing dilution of cell homogenates.

Effect of time

The activity of the neuraminidase(s) against fetuin, *N*-acetylneuraminyllactose and MPN increased in a linear fashion for the first 2 h (Figs. 1C, 2C and 3C). Following this initial period, there was a continued but non-linear increase in activity against each substrate.

Effect of culture time

As is shown in Fig. 4, there was a 1.7- to 1.8-fold increase in specific neuraminidase activity between the 4th and 15th day from subculture against each of the substrates examined. During this period, the culture media was replaced every 3 or 4 days. The cell culture became confluent on the 6—7 day from subculture.

Discussion

These data indicate that cultured human fibroblasts contain at least one (and perhaps more) acidic enzymes capable of cleaving the acylated derivative of neuraminic acid from a number of substrates. These include *N*-acetylneuraminyllactose, fetuin and 2-(3′methoxyphenyl)-*N*-acetyl-α-neuraminic acid. While this neuraminidase activity has some of the characteristics of a lysosomal enzyme, e.g., acidic pH optimum, properties similar to known lysosomal neuraminidase [15,16] and presence of a progressive storage disorder in its absence [2,7], direct proof of its lysosomal origin is lacking.

In contrast to most lysosomal enzymes that have been examined, the neuraminidase(s) of human fibroblasts appears to be a labile enzyme (Table I). These results are in agreement with those of others who have previously shown

neuraminidase(s), from a variety of sources, to be an unstable enzyme [15,16]. One of the practical consequences of this lability is that certain conventional means of rupturing cells and/or releasing enzymes, e.g., sonication, freeze-thaw or Triton X-100 treatment, cannot be utilized for the study of neuraminidase.

In the presence of fetuin or *N*-acetylneuraminyllactose, neuraminidase activity could be detected by measuring the release of free AcNeu by the thiobarbituric acid method of Warren [13]. With MPN the release of the aglycone, 3-methoxyphenol, was measured with diluted phenol reagent. While it has been previously suggested that MPN could serve as a substrate for mammalian neuraminidase, little data has appeared regarding its utilization for this purpose [17]. This appears to be due, in part, to the problem of interference with the color reaction by protein. In this study, this potential problem was overcome by extracting the reaction product with toluene. This procedure yielded absorption values of the water, substrate and cell blanks that were generally 20% or less of the value obtained from the complete reaction mixture. Indirect evidence that MPN is cleaved by the same enzyme that hydrolyzes fetuin and *N*-acetylneuraminyllactose is provided by the observation that enzyme activities against each of these substrates are similarly reduced in a specific human genetic disorder affecting neuraminidase activity [4].

As is shown in Fig. 4, the specific neuraminidase activity increased between the 4th and 16th day following subculture. While an explanation for this is currently lacking, it is of note that changes in the specific activity of a variety of enzymes following subculture of several cell types have been reported [18—20]. It is thus suggested that the changes in neuraminidase levels following subculture of cells in vitro is part of a general phenomenon and not a unique property of this enzyme.

The measurements of neuraminidase activity in fibroblasts have been successfully utilized for the detection of several patients with inherited neuraminidase deficiencies [1—7]. In most of these cases, however, the cell preparation and/or assay conditions were not optimal. For example, Thomas et al. [8], Kelly and Graetz [6] and O'Brien [3] utilized sonication as a means of cell rupture. The data presented here clearly indicate that this technique results in a reduction in neuraminidase activity. In addition, both these and other investigators have employed prolonged incubation times [4,6] and/or sub-optimal substrate concentrations [4,6,7]. While the major conclusions derived from these investigations are unaffected by these factors, a direct comparison of the enzyme results reported by different investigators is presently impossible. It will, therefore, be necessary to reexamine these patients under more uniform and satisfactory enzyme conditions of the type outlined in this report.

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