Coupled-Enzyme System for Measuring Viral Neuraminidase Activity

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A coupled-enzyme assay for determining viral neuraminidase activity is described. All reactants—viral neuraminidase, the initial substrate (fetuin), N-acetylneuraminic acid aldolase, lactic acid dehydrogenase, and reduced nicotinamide adenine dinucleotide—are combined in a single cuvette. Thus, in a single coupled system neuraminidase releases N-acetylneuraminic acid, which is cleaved to N-acetyl-D-mannosamine and pyruvic acid; finally, pyruvate is reduced to lactate as reduced nicotinamide adenine dinucleotide is oxidized. The rate of change of absorbance at 340 nm, as reduced nicotinamide adenine dinucleotide is oxidized, is a measure of the rate of reaction of the coupled system. This procedure, which measures the rate of release of N-acetylneuraminic acid by neuraminidase, is an alternate method for those procedures which require multistep, colorimetric determinations.

Influenza viruses are characterized by identification of specific antigenic viral components; within a type, specific strains are distinguished by their surface antigens, principally hemagglutinins. Complement fixation, hemagglutination, and hemagglutination inhibition tests are the principal serological procedures used in most laboratories. Recently, the importance of myxovirus neuraminidases (N-acetylneuraminyl-hydrolase, EC 3.2.1.18) has been recognized in viral characterization. The neuraminidases of myxoviruses have been shown to be not only an integral component of the viral surface but also distinguishable from the hemagglutinin and complement fixation antigens (4, 5, 9). Additional investigations by Kendal and Madeley (6) have shown that many different influenza virus strains possess neuraminidases which differ from each other in both their antigenic identities and their kinetic characteristics. Investigation of either of these aspects requires quantitative determination of the enzyme activity.

The antigenic identity is determined by measuring the inhibition of the enzyme activity by specific antisera; the kinetic characteristics of virus-associated neuraminidases are ascertained by measuring the rates of enzyme activities in response to various physical conditions. In either case, rates of viral neuraminidase activities must be quantitatively determined.

Neuraminidase is a hydrolase which cleaves the terminal sialic acid from the nonreducing end of polysaccharides, oligosaccharides, and disaccharides. In most investigations neuraminidase activity is measured by allowing the neuraminidase to act on an appropriate substrate. The released sialic acid is then measured by a colorimetric method of either Warren (8) or Aminoff (1). Each of these methods is based upon the oxidative cleavage of the 9-carbon N-acetylneuraminic acid (NANA) to yield β-formylpyruvic acid. The subsequent reaction of this product with thiobarbituric acid forms a chromophore which is extracted by organic solvents. The color production and extraction into organic solvents varies linearly with the concentration of NANA.

Although these methods have been satisfactorily used in many laboratories, the multistep procedures induce variations associated with biphasic extractions and chemical transfers. In addition to this limitation, the methods are not easily suited to the determination of rate reactions. Brunetti et al. (2) and Comb and Roseman (3) described a coupled enzymatic assay for the determination of sialic acid. That assay suggested the development of a coupled system for measuring the neuraminidase activity of viruses. This report describes an alternate coupled enzyme system which permits neuraminidase activity to be measured spec-

trophotometrically as the reaction proceeds. Thus, in a single coupled system neuraminidase releases NANA, which is cleaved to N-acetyl-D-mannosamine and pyruvic acid by NANA-aldolase. Finally, pyruvate is reduced to lactate by lactic acid dehydrogenase (LDH) as reduced nicotinamide adenine dinucleotide (NADH) is converted to nicotinamide adenine ·dinucleotide (NAD). The ultimate determination of the coupled reaction is provided by the rate of change in absorbance at 340 nm as NADH is oxidized.

The method to be described measures neuraminidase activity in a single cuvette. Hence, this procedure can replace current methods which require sequential sampling and multiple chemical determinations to measure rate reactions.

MATERIALS AND METHODS

Virus. Influenza virus (A_2/Aichi/2/68[H3N2]) preparations were obtained from the Respiratory Virology Unit, Center for Disease Control, Atlanta, Georgia. The virus was grown in the allantoic cavity of 11-day-old chick embryos. Virus in pooled allantoic fluid was concentrated by clarification at 500 × g and finally by sedimentation at 41,000 × g for 60 min. The pelleted virus was suspended in phosphate-buffered saline (PBS; 0.15 M sodium chloride, 0.05 M phosphate, 0.02% sodium azide, pH 7.1) and stored at 4 C.

Substrate. Fetuin, a serum glycoprotein, was purchased from the Grand Island Biological Co. The substrate was dissolved in PBS adjusted to pH 6.7. In investigations of optimal pH, the buffer was appropriately adjusted.

Enzymes and cofactor. Intermediate enzymes and cofactor used in the coupled-enzyme system were obtained from commercial suppliers; LDH and NADH were obtained from Nutritional Biochemicals Co., Cleveland, Ohio, and NANA-aldolase was obtained from the Sigma Chemical Co., St. Louis, Mo. NANA-aldolase was received as a lyophilized product and was reconstituted to one unit/ml with 0.02 M phosphate buffer (pH 7.2); one unit releases 1 μmole of pyruvate from N-acetylneuraminic acid per min at pH 7.2 at 37 C. The reconstituted enzyme was dispensed in samples sufficient for use during 1 day of laboratory operation and was stored at −20 C.

Stock solutions of both LDH and NADH were prepared weekly and were stored at 0 C. The LDH working stock was diluted in PBS (pH 6.7) to contain 17 units/ml (one unit oxidized 1 μmole of NADH per min at pH 7.4 at 25 C). Stock NADH was diluted in PBS (pH 7.1) to contain 20 μmoles/ml. The working solution of NADH was diluted tenfold in PBS (pH 6.7) immediately before it was added to the reaction mixture.

General procedure. The enzyme determination was performed in microcuvettes (10-mm path length by 2-mm inside dimension), and the total volume of the reaction mixture was 0.2 ml. In developing this procedure, the concentrations of the reagents were varied to determine optimal concentrations. In the standard procedure a near optimal concentration of each reagent was added to each microcuvette. The concentrations were: LDH, 0.050 unit; NADH, 0.06 μmole; NANA-aldolase, 0.025 unit; and fetuin, 0.10 μmole. The last reactant, viral neuraminidase, was diluted in PBS and added to the reaction mixture. A volume of PBS was added to make the final volume of the reaction mixture 0.2 ml. The reaction mixture was mixed with a plastic stirring rod and incubated at 37 C in the spectrophotometer sample compartment.

Despite the fact that the cuvettes and the cuvette carriers were prewarmed to 37 C, the reaction mixtures were allowed to equilibrate for at least 10 min. The rate of reaction, measured as the change of absorbance at 340 nm ($\Delta A340$/min), was determined with a model 2000 Gilford automatic recording spectrophotometer equipped with thermospacers. The temperature was maintained at 37 C within the sample compartment.

RESULTS

Experimental scheme. The complete coupled-enzyme reaction consists of three separate enzymatic reactions. They each proceed sequentially and simultaneously. The enzymatic steps of the coupled reaction are listed in the following scheme: (i) neuraminidase, sialylglycoprotein → NANA + glycoprotein; (ii) NANA-aldolase, NANA → pyruvate + N-acetyl-D-mannosamine; and (iii) LDH, pyruvate + NADH + H^+ → lactate + NAD^+.

When neuraminidase is added to a mixture of the essential reactants (sialylglycoprotein, NANA-aldolase, LDH, and NADH), the hydrolytic product, NANA, of the initial reaction (i, above) is released. The remaining steps of the coupled reaction (ii and iii, above) then proceed sequentially.

Because neuraminidase should be the rate-limiting reactant, the other reactants should be present in optimal concentrations which permit maximal rates of reaction. To determine optimal concentrations, each reactant was added in various concentrations, whereas all other reactants were added in prescribed concentrations.

Effect of viral neuraminidase concentration. The relationship of neuraminidase concentration to the rates of reaction of the coupled-enzyme system is illustrated in Fig. 1. Oxidation of NADH was accompanied by a decreased absorbance at 340 nm. The rate of decrease in absorbance was directly proportional to the amount of neuraminidase added to the reaction system. As the volume of the virus preparation was increased tenfold, the rate of
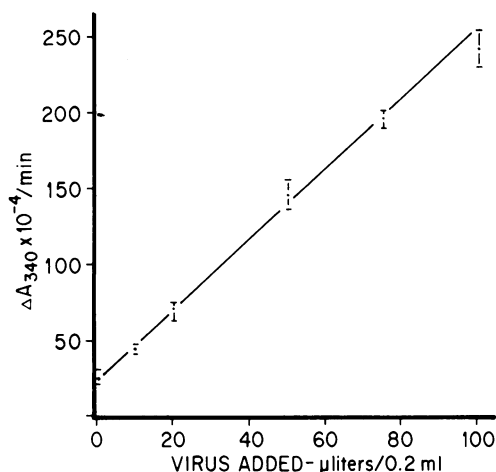
FIG. 1. *Relationship of neuraminidase activity to virus concentration. Neuraminidase activity is expressed as the rate of change of absorbance at 340 nm ($\Delta A340/min \times 10^{-4}$). Each of the plotted values is the mean of four determinations.*
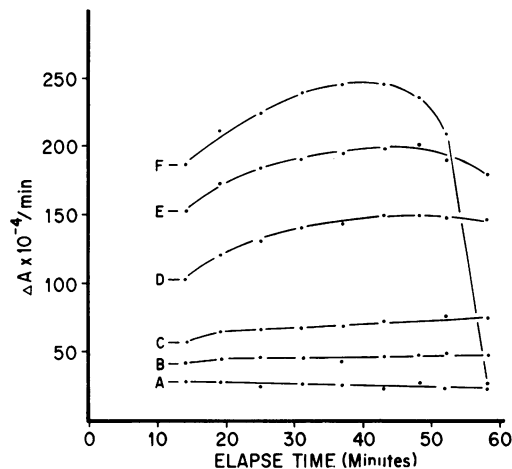

FIG. 2. *Neuraminidase activity (expressed as the rate of change of absorbance ($\Delta A340/min \times 10^{-4}$) of different viral concentrations in a reaction mixture containing standard concentrations of essential factors. Each curve represents the activity of a different amount of influenza virus ($A_2/Aichi/2/68[H3N2]$, 1: 200 dilution). A, Control; B, 10 μliters; C, 20 μliters; D, 50 μliters; E, 75 μliters; and F, 100 μliters.*

change of absorbance ($\Delta A340/min$) increased approximately sixfold, from 0.0040 to 0.0240. These rates are equivalent to the oxidation of NADH at the rates of 0.16 nmole/min to 1.16 nmoles/min.

Variation of rate of reaction with time. In the coupled-enzyme system an amount of viral neuraminidase causing the maximal rate of oxidation of NADH (1.16 nmoles/min) would deplete this coenzyme after 30 to 40 min of incubation. Therefore, the rates of reaction over an extended incubation period were examined. After an initial period of temperature-equilibration, the initial $\Delta A340/min$ rate was low and gradually increased through the first 20 to 30 min. This is illustrated in Fig. 2. After an initial period of 20 min, the $\Delta A340/min$ rate for reactions containing 10 to 20 μliters of a 1:200 dilution of a viral preparation (curves B and C) remained relatively constant for 40 min or longer. For an intermediate amount of virus preparation (50 μliters of a 1:200 dilution, curve D) yielding a $\Delta A340/min$ rate of 0.0100 to 0.0140, the $\Delta A340/min$ rate did not approach a constant rate until after 30 min of incubation. After this period, the rate changed less than 5% during the next 30 min.

Reaction mixtures containing 75 to 100 μliters of a 1:200 dilution (curves E and F) failed to attain a constant $\Delta A340/min$ rate, and after 40 min of incubation the rate of oxidation of NADH declined rapidly. The $\Delta A340/min$ rate decreased precipitously in reaction mixtures containing 100 μliters of a 1:200 dilution of

viral neuraminidase (Fig. 2, curve F). The decrease reflected depletion of NADH because a secondary addition of this factor restored a high $\Delta A340/min$ rate.

These results indicate that addition of viral neuraminidase should not exceed the limits of the system. Therefore, in a reaction mixture containing 0.06 μmole of NADH/0.2 ml, neuraminidase should be added in amounts which yield a $\Delta A340/min$ rate of 0.0100 to 0.0140 or less.

Effect of substrate concentration. A variety of substrates, sialyldisaccharides, sialyloligosaccharides, and sialylpolysaccharides, are acted on by neuraminidase; however, only fetuin was used as the substrate in these studies. The results obtained with different fetuin concentrations when all other reactants, including neuraminidase, were added in constant amounts is illustrated in Fig. 3a. The concentrations of NANA-aldolase, LDH, and NADH were identical to the amounts established in the standard procedure. The rate of reaction ($\Delta A340/min$) increased with increasing concentrations of fetuin until a concentration of 0.15 μmole/0.2 ml was reached. Concentrations exceeding 0.15 μmole/0.2 ml neither increased nor inhibited the rate of reaction. However, because of the high molecular weight and physical character of the fetuin preparation, it was not feasible to test higher concentrations.
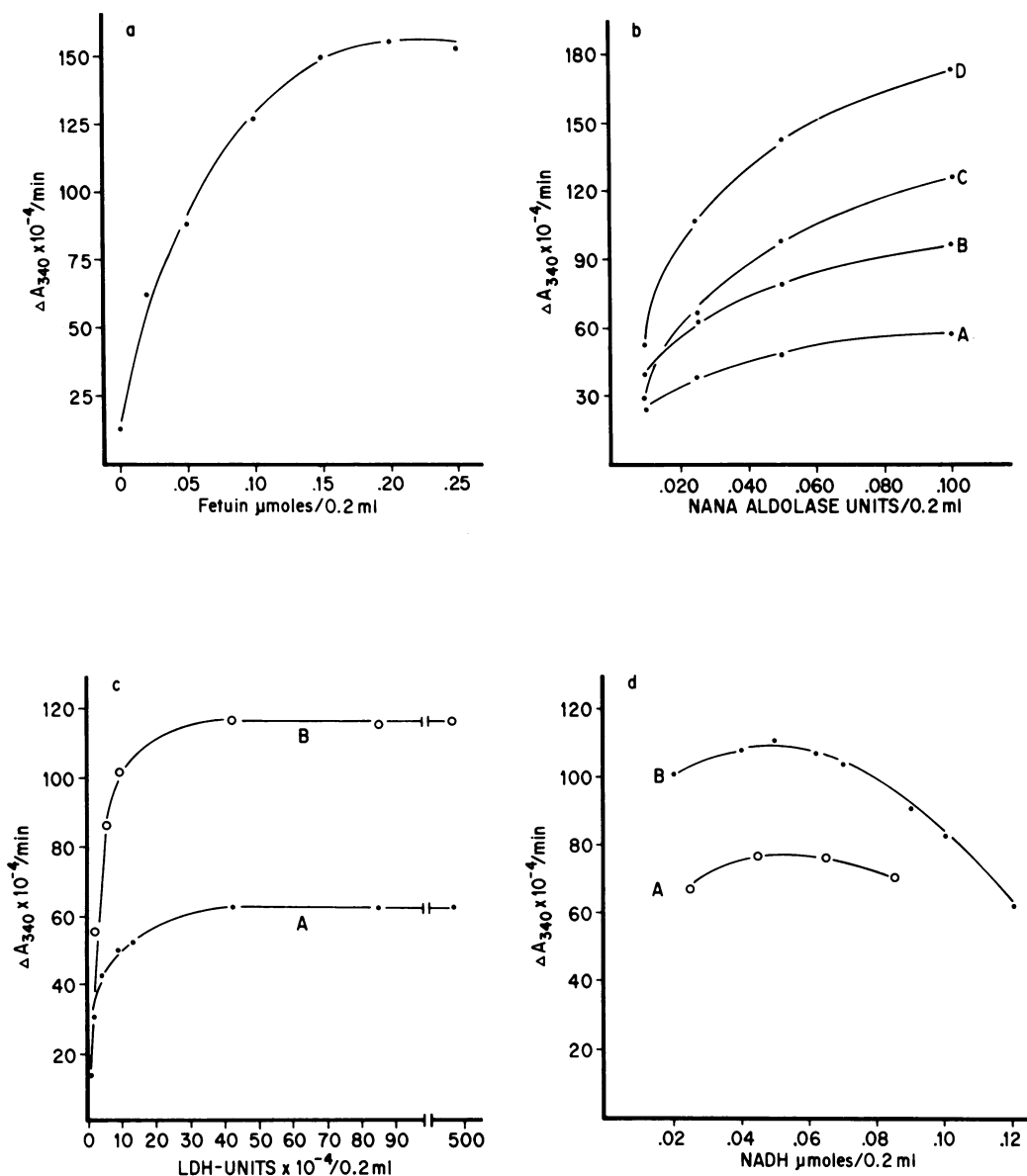
FIG. 3. *Dose response relationships of essential factors (fetuin, N-acetylneuraminic acid [NANA])-aldolase, lactic acid dehydrogenase (LDH), and reduced nicotinamide adenine dinucleotide (NADH) to the rate of activity of the coupled neuraminidase system. Neuraminidase activity is expressed as the rate of change of absorbance at 340 nm ($\Delta A340/min \times 10^{-4}$). Influenza virus ($A_2/Aichi/2/68[H3N2]$) was the neuraminidase source in all trials. The concentration of each factor was varied individually while the remaining factors were added as standard concentrations (fetuin, 0.1 $\mu mole/0.2$ ml; NANA-aldolase, 0.025 unit/0.2 ml; LDH, 0.05 unit/0.2 ml; NADH, 0.06 $\mu mole/0.2$ ml). a, Fetuin as variable. Plotted values are the mean of duplicate determinations. b, NANA-aldolase as variable. Equal volumes of different dilutions of influenza virus ($A_2/Aichi/2/68[H3N2]$) were added. A, 1:600; B, 1:400; C, 1:200; and D, 1:100. c, LDH as variable. Equal volumes of different dilutions of influenza virus ($A_2/Aichi/2/68[H3N2]$) were added. A, 1:200; B, 1:100. d, NADH as variable. Different lots of influenza virus ($A_2/Aichi/2/68[H3N2]$). A, lot no. 1, 1:200; B, lot no. 2, 1:100.*

Despite the fact that 0.15 μmole/0.2 ml of fetuin was the lowest concentration permitting the maximal rate of reaction, a concentration of 0.10 μmole/0.2 ml was used in the standard procedures. This was necessary because fetuin concentrations greater than 0.10 μmole/0.2 ml caused opalescence and excessively high optical densities in the reaction mixture. The use of 0.10 μmole/0.2 ml, although less than optimal, yielded a linear relationship between $\Delta A340$/min and the amount of viral neuraminidase (Fig. 1).

Variation of rates of reaction with NANA-aldolase concentration. Rates of reaction of the coupled-enzyme system varied with the concentration of the intermediate enzyme, NANA-aldolase. The results obtained with various aldolase concentrations and different amounts of viral neuraminidase are shown in Fig. 3b. At each of the virus concentrations (1: 600, 1:400, 1:200, and 1:100; curves A, B, C, and D, respectively), the rates of reaction increased with increasing amounts of NANA-aldolase. However, an optimal concentration of aldolase was not attained. The results indicate that concentrations of NANA-aldolase used did not permit maximal reaction rates with high concentrations of viral neuraminidase (1:200 and 1:100). Maximal reaction rates were approached only in reaction mixtures containing less-concentrated virus dilutions (1:600 and 1:400).

To assure maximum velocity of the coupled reaction, excess NANA-aldolase would be desirable. However, the lack of purity and the high cost of the enzyme preparations prohibit the use of sufficiently high enzyme concentrations. Therefore, to conserve the relatively expensive NANA-aldolase, a 0.025 unit/0.2 ml concentration was used in the standard procedure. Although this concentration was not optimal, it yielded a linear relationship between ΔA and the amount of viral neuraminidase (Fig. 1).

Variation of rates of reaction with changes in LDH concentration. The effect of LDH concentration was measured in the coupled reaction by varying LDH concentration with all other reactant constants. The results shown in Fig. 3c indicate that a 0.004 unit/0.2 ml concentration or greater permits maximal velocity of the coupled reaction. Concentrations of LDH which permitted maximal velocity were approximately the same for the two concentrations of virus (curve A, 0.025 ml of 1:200 dilution; curve B, 0.025 ml of 1:100 dilution).

The highest concentration of LDH tested,

0.05 unit/0.2 ml, neither increased nor inhibited the maximal velocity. Although 0.05 unit/0.2 ml of LDH is approximately tenfold greater than the concentration permitting maximal velocity, it was used in the standard procedure. This concentration permitted the pH of the coupled reaction to be adjusted to values considerably less than optimal for LDH.

Variation of rates of reaction with concentration of NADH. The change of rates of reaction with different concentrations of NADH was examined to determine an optimal concentration. Although excess NADH was desirable in the coupled system, the concentration of this coenzyme was found to be critical. The concentration range of 0.02 to 0.12 μmole/0.2 ml was evaluated with viral enzyme concentrations yielding a $\Delta A340$/min rate of 0.0080 to 0.0100.

The results are shown in Fig. 3d. During an incubation of 50 min, the lowest concentration, 0.02 μmoles/0.2 ml, was rate-limiting, whereas the highest concentrations, 0.08 to 0.12 μmole/0.2 ml, were inhibitory. Concentrations of 0.04 to 0.07 μmole/0.2 ml were adequate to maintain maximal rates of reaction when the $\Delta A340$/min rate was approximately 0.0080 to 0.0100. A concentration of 0.06 μmole/0.2 ml was used in the standard procedure. This provides adequate NADH for the range of activity anticipated in this analytic procedure. To minimize the degradation of NADH at pH 6.7, the coenzyme was added to the reaction mixture just before incubation of the complete reaction system.

Effect of pH on the coupled-enzyme reaction. The presence of three different enzymes in the reaction mixture makes it impossible to perform the procedure at a pH value which is optimal for each of the enzymes. Both NANA-aldolase and LDH have optima above pH 7.0, whereas viral neuraminidases generally have optima below pH 7.0. The rates of reaction of the coupled enzyme system were measured from pH 6.4 to 7.1, and the results are illustrated in Fig. 4. From pH 6.4 to 6.7 little change in the rates of reaction occurred; however, from pH 6.9 to 7.1 a marked reduction in the rate of activity occurred.

The pH optima for the neuraminidases of different viruses probably would vary somewhat from the optimal pH for A_2/Aichi/2/68 (H3N2). However, Kendal and Madeley (6) and Rafelson (7) reported that most influenza virus neuraminidases function at near maximal rates between pH 6.5 to 6.7. In our investigation we used pH 6.7 in the standard assay procedure.
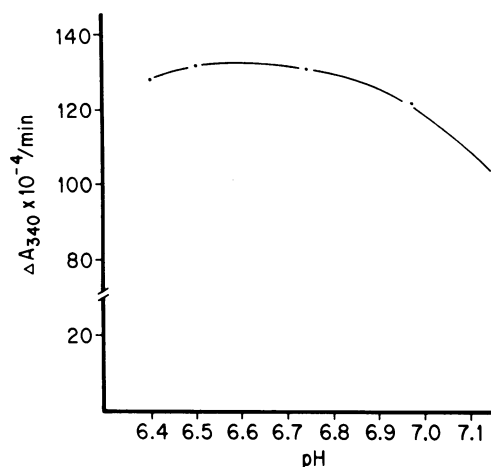
FIG. 4. *Response of coupled neuraminidase reaction to variation of pH. Rates of neuraminidase activity are expressed as the rate of change of absorbance at 340 nm (ΔA340/min × 10⁻⁴). All reactants were present in concentrations of the standard procedure. Viral neuraminidase was 0.025 ml of a 1:100 dilution of influenza virus (A₂/Aichi/2/68[H3N2]).*

DISCUSSION

The procedure proposed in this investigation is a coupled-enzyme method which permits determination of rates of reaction in a single-reaction system; it is a one-step procedure. All of the components of the reaction are mixed in a single cuvette, and the rate of reaction is measured directly by determining the change in absorbance per min at 340 nm.

Three enzymatic reactions proceed sequentially in the coupled system. In the initial reaction, viral neuraminidase acts on an appropriate substrate releasing NANA. This product then becomes available to the secondary enzyme, NANA-aldolase; pyruvate, the product of this reaction, is reduced to lactate by LDH as NADH is converted to NAD. Burnetti et al. (2) reported that the equilibrium constant of the NANA-aldolase reaction is Keq = 0.096 M. Therefore, at substrate concentrations of 0.001 M or less, all of the NANA will be cleaved, releasing pyruvate to be acted on by the final enzymatic step. The overall rate of reaction will be dependent on the neuraminidase activity, if all other components are in excess. Thus, the rate of NADH oxidation in the final step is a measure of the neuraminidase activity. NADH has an absorption peak of 340 nm, whereas NAD does not absorb energy at this wavelength. Therefore, continuous monitoring of the reaction at 340 nm in a recording spectrophotometer allows the rate of reaction to be determined semi-automatically.

In spite of the fact that the optimal or near optimal concentrations of each of the reactants was determined, it was impractical to add optimal amounts of fetuin and NANA-aldolase. Fetuin, a natural product isolated from fetal calf blood, has an approximate molecular weight of 50,000. Preparations of the highest purity available produce viscous solutions; hence, the amount of fetuin which may be added to the reaction mixture is limited. Consequently, less than an optimal amount of fetuin was used. Although 0.15 to 0.25 μmole/0.2 ml produced optimal rates (Fig. 3a) of reaction, a fetuin concentration of 0.1 μmole/0.2 ml was used for the routine assay procedure.

The intermediate enzyme, NANA-alsolase, was obtained commerically or isolated in our laboratory. In either case, the enzyme was extracted from mass cultures of *Clostridium perfringens;* in the best preparations both the purity and specific activity were relatively low. Therefore, it was impractical to add sufficient NANA-aldolase to produce maximal rates of reaction when large amounts of neuraminidase were present in the coupled system. In addition to the low specific activity of the NANA-aldolase preparations, the cost of the enzyme likewise limits the use of large amounts of the enzyme in routine procedures.

Despite the limitation of less than optimal concentrations of both fetuin and NANA-aldolase, the data presented in Fig. 1 show that a linear response, over an eightfold range, was obtained as viral neuraminidase was added. If NANA-aldolase preparations of greater specific activity become available, the range of the linear response may be increased.

The remaining reactants of the coupled system are available as purified materials of high specific activity. Thus, the amounts of LDH and NADH which could be added to the reaction mixture did not constitute limitations. However, the optimal concentrations of these reactants, as determined in this investigation, may not be valid if different experimental conditions are introduced. Some lots of NANA-aldolase contain NADH-oxidase as a contaminant and optimal pH values for some viral neuraminidases are lower than the pH values used in this investigation. Thus, both oxidation of NADH by NADH-oxidase and degradation of the coenzyme in acidic mixture contribute to the total decrease in absorbance. Therefore, markedly varied conditions may require alteration of the concentration of NADH in the assay system. In any case, the test system must include controls to obviate nonspecific NADH consumption.

The value of neuraminidase characterization in myxovirus identification has been amply discussed by Rafelson et al. (7) and Kendal and Madeley (6); however, the available techniques for neuraminidase determinations of Warren (8) and Aminoff (1) have retarded investigations of viral neuraminidases because they are not readily adaptable to the determination of rates of enzyme activities. The coupled-enzyme system described in this report provides an alternate method for neuraminidase characterization. It permits continuous rate-reaction determinations in a single-reaction system instead of a procedure requiring multiple sampling and multiple analyses. The procedure is readily adaptable to the determination of enzyme-substrate kinetics, and it is being adapted to measure the neuraminidase-neutralizing activity of specific antisera.

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