zene, washed several times with the same, and dried *in vacuo*, to give a colorless product, mp 178–180°.

Anal. Calcd for $C_6H_5O_5Br_2P$: Br, 46.0. Found: Br, 45.2.

Oxidation of XIII with N-Bromosuccinimide.—In anhydrous acetonitrile, XIII is oxidized slowly by *N*-bromosuccinimide to 2,6-dibromo-*p*-quinone, 24 hours being sufficient for completion of the reaction. In a mixture of acetonitrile–*t*-butanol (1:1), the reaction follows the same course, but is complete in 3–4 hours.

When XIII was oxidized with 1.5 equivalents of *N*-bromosuccinimide in methanol (dried over Mg), the reaction mixture, after 1.5 hours, showed a main spectral peak at 255 mμ and a minor peak at 285 mμ. Judging from the spectra of the pure quinone ketal, XI, and 2,6-dibromo-*p*-quinone (XII), the mixture contained 80% of the former and 20% of the latter. A portion of the material was analyzed on a preparative thin-layer plate and, after elution of the individual bands with acetonitrile, the relative amounts of XI and XII were again found spectroscopically to be 80:20. In two additional runs, ratios of 89:11 and 87:13 were obtained. In methanol containing 10% water (by volume), the ratio of XI:XII was found to be 59:41. Using the monotriethylammonium salt of XIII, the ratio in absolute methanol was 79:21. The presence of methyl phosphate was demonstrated qualitatively in several of these runs by paper chromatographic comparison (Ukita *et al.*, 1958), with an authentic sample, prepared by methylation of dibenzyl phosphate with diazomethane and catalytic hydrogenolysis of the product.

Thin-layer Chromatography.—All chromatograms were run on silica gel G. Compounds were located by spraying the plates with 50% sulfuric acid, followed by heating at 100° for 15 minutes. With dimethylformamide as solvent, dibenzyl phosphate showed an R_F value of 0.44 on silica gel G. Identification of products was performed by comparison with authentic samples run on the same plates.

TABLE I
R_F VALUES ON SILICA GEL

Compound	Solvent System[a] A	B	C
VI	0.20		
VIII	0.63		
X	0.33		
XI	0.72	0.60	
XII	0.80	0.69	0.48
2-Br-*p*-quinone			0.34
p-Quinone	0.39		0.18

[a] A = ether, B = ether-cyclohexane (4:1), C = benzene.

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Inhibition of *N*-Acetylneuraminic Acid Aldolase by 3-Fluorosialic Acid*

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3-Fluoropyruvic acid was condensed with *N*-acetylglucosamine or *N*-acetylmannosamine in alkaline aqueous solution to give 3-fluorosialic acid. The compound was isolated in a yield of 1.5 and 3.0%, respectively, by chromatography on Dowex-1 and carbon. The molar absorbancy index of the chromophore in the Ehrlich assay was 8175 at 536 mμ. $[\alpha]_D^{20} = -19.2°$ (H_2O 1.1%). Neutralization equivalent, 330. Infrared spectra were nearly identical with *N*-acetylneuraminic acid except in the C-F absorbing area. The compound reduced ferricyanide. The products from *N*-acetylglucosamine and *N*-acetylmannosamine were identical in several chromatographic systems. Both products were competitive inhibitors and inactivators of *N*-acetylneuraminic acid aldolase. The K_I is 2.5×10^{-3} M for both products compared with a K_m of 1×10^{-3} M. Evidence is presented to show that metals and sulfhydryl groups are not involved in the inhibition.

The sialic acids belong to a family of compounds that have the basic nine-carbon-atom structure of neuraminic acid but differ as to the type and extent of substitution. Neuraminic acid may be regarded as a condensation product of pyruvic acid and *N*-acetyl-D-mannosamine (Comb and Roseman, 1960). The sialic acids occur widely in mammalian tissue, principally in mucoids, mucins, and glycoproteins in association with D-galactose or galactosamine (Castellani

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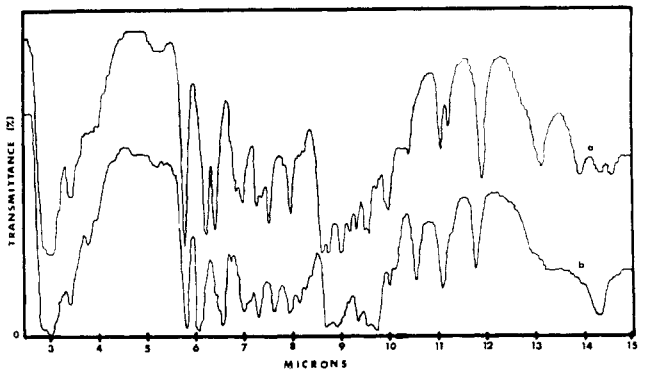
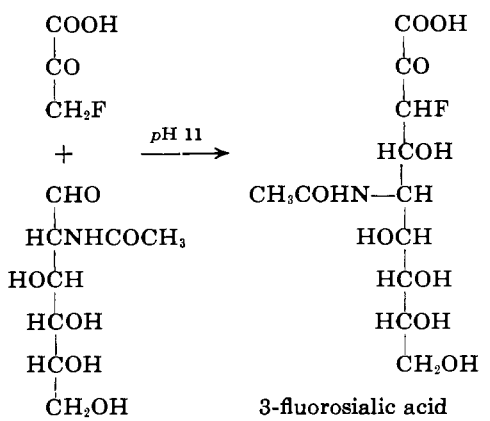


FIG. 1.—Comparison of the infrared spectra of 3-fluorosialic acid (a) and *N*-acetylneuraminic acid (b).

et al., 1959; Zilliken and Whitehouse, 1958). These compounds are also present in some bacteria (Aaronson and Lessie, 1960; Barry, 1958; Watson *et al.*, 1958) and in milk as constituents of oligosaccharides. Little is known about their physiological function, though they have been implicated in a variety of biochemical reactions (Gottschalk, 1960).

In view of the role metabolic inhibitors have played in the elucidation of biological function and metabolic pathways, it was expected that biologically active analogs of *N*-acetylneuraminic acid would help to elucidate the biological function of the sialic acids. With this end in mind, 3-fluorosialic acid was synthesized by condensing fluoropyruvic acid and *N*-acetylglucosamine in a basic solution by a procedure analogous to the original synthesis of *N*-acetylneuraminic acid (Cornforth *et al.*, 1957). Its effect on the enzyme, *N*-acetylneuraminic acid aldolase, which



3-fluorosialic acid

cleaves *N*-acetylneuraminic acid to pyruvic acid and *N*-acetylmannosamine (Comb and Roseman, 1960) was then studied.

MATERIALS AND METHODS

Synthetic Materials.—The synthesis of fluoropyruvic acid was carried out as described by Nair and Busch (1958) except that the distillation apparatus was wrapped in heating tape and kept at 85° to prevent solidification of the fluoropyruvic acid, and that the receiving flask was cooled in a dry ice–acetone bath. *N*-Acetylmannosamine was prepared by the alkaline epimerization (Carroll and Cornforth, 1960) of *N*-acetylglucosamine. The final product had a melting point of 125–130° (lit., 128–129°) and contained trace amounts of *N*-acetylglucosamine as shown by paper chromatography (Spivak and Roseman, 1959). The charcoal adsorption was carried out on unground activated Nuchar C treated with 6 N hydrochloric acid and placed under a vacuum overnight. Before use the

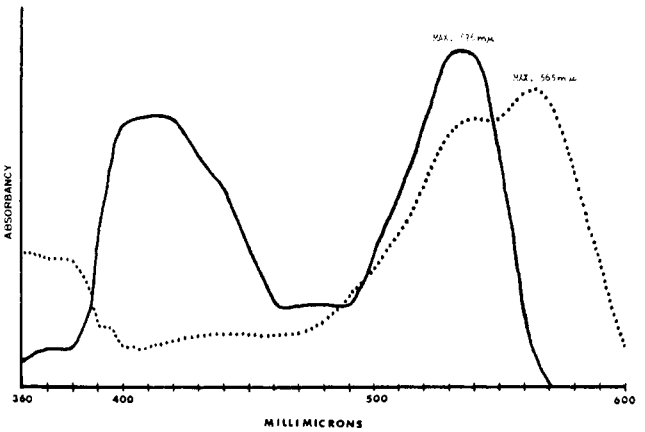


FIG. 2.—Comparison of the spectra of the chromophores of 3-fluorosialic acid (———) and *N*-acetylneuraminic acid (. . . . .) in the direct Ehrlich reaction.

column was washed with water until the effluent was about *p*H 5, and then washed with 10 bed volumes of 0.3 N formic acid and again washed with water.

N-Acetylneuraminic acid was prepared from colominic acid by the method of McGuire and Binkley (1964).

Preparation of N-Acetylneuraminic Acid Aldolase.—*Clostridium perfringens* (A.T.C.C. 10543, Cassidy strain, kindly provided by Dr. Saul Roseman) was grown and the enzyme was isolated as described by Comb and Roseman (1960) with minor modifications. The procedure was scaled down to 9 liters and the medium was inoculated with a 15-ml culture grown on thioglycolate for 16 hours. The bacteria were collected by continuous-flow centrifugation at 21,000 × *g* in a Sorvall RC 2 centrifuge at 0°. The cells were suspended in 40 ml of 0.15 M potassium chloride and ruptured by means of a French press at a pressure of 19,000–20,000 psi. Debris and unbroken cells were removed by centrifugation, and solid ammonium sulfate (18.5 g) was added to the supernatant solution with continuous stirring over a period of 15 minutes at 0°. After it had been stirred an additional 30 minutes the solution was clarified by centrifugation and the precipitate was discarded. The solution then was saturated with ammonium sulfate, and the precipitate was collected by centrifugation and dissolved in 3 ml of 0.02 M potassium phosphate buffer, *p*H 7.2, and dialyzed overnight in 500 ml of the same buffer at 4°. The enzyme solution was stored in a number of small portions at −16° and was stable for at least 12 months. The specific activity at 0.01 M substrate concentration was 0.87 μmole of pyruvate formed per minute per mg protein. All dilutions were made in 0.02 M potassium phosphate buffer, *p*H 7.2. Protein determinations were made by the procedure described by Lowry *et al.* (1951).

Enzyme Assay.—*N*-Acetylneuraminic acid aldolase catalyzes the cleavage of the substrate to *N*-acetylmannosamine and pyruvic acid. The rate of formation of pyruvic acid was measured by the decrease in absorbancy at 340 mμ in an incubation mixture containing NADH (reduced nicotinamide adenine dinucleotide, Sigma, St. Louis, Mo.) and muscle lactic acid dehydrogenase (Sigma, 2 × recrystallized, ammonium sulfate suspension). In contrast to the analogous preparation described in the literature (Comb and Roseman, 1960), no detectable NADH oxidase activity was found in this preparation. Therefore a single-step assay was used to measure the reaction rate. The standard assay solution consisted of 0.3 ml of 0.2 M potassium phosphate buffer, *p*H 7.2, 0.025 ml lactic dehydrogenase (0.05 mg protein), 0.01 ml enzyme solution (usually

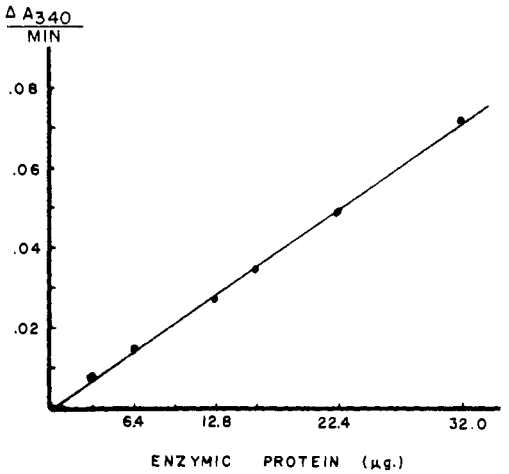


FIG. 3.—Relationship between enzyme concentration and initial velocity. The incubation mixtures contained 1 μmole of substrate under the standard assay conditions.

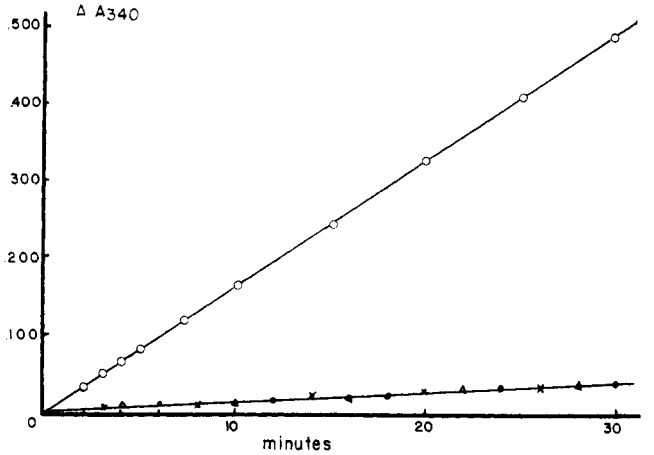


FIG. 4.—Action of the enzyme preparation on NADH and demonstration that 3-fluorosialic acid is not cleaved to 3-fluoropyruvic acid. (Δ), enzyme, NADH; (●), enzyme, NADH, 3-fluorosialic acid; (X), NADH, 3-fluorosialic acid; (O), enzyme, *N*-acetylneuraminic acid, NADH.

6.4 μg of protein), 0.1 ml NADH (0.002 M), 0.01 ml substrate, and 0.02 M potassium phosphate buffer, *p*H 7.2, to give 1 ml total volume. The assay solution minus the aldolase was incubated at 37° for at least 12 minutes in a Beckman DU spectrophotometer. The reaction was started by the introduction of the enzyme with a 10-μl pipet and by blowing through the pipet to effect mixing. Pyruvate formed (μmoles) was calculated from the change in absorbancy using 6.22×10^3 as molar absorbancy index of NADH at 340 mμ.

N-Acetylneuraminic Acid Assay.—*N*-Acetylneuraminic acid was determined by the thiobarbituric acid procedure (Warren, 1959) or by a modified direct Ehrlich reaction (Werner and Odin, 1952). The Ehrlich reagent consisted of 2 g of 2 × recrystallized *p*-dimethylaminobenzaldehyde dissolved in 20 ml of concentrated hydrochloric acid and 80 ml of ethylene glycol. The ratio of reagent to sample was 1:2 and the heating time was 30 minutes in a boiling-water bath. The tubes were read in a Coleman Junior spectrophotometer at 565 mμ.

A control for the nonenzymatic oxidation of NADH (0.00116 absorbancy/minute), which consisted of the complete mixture minus the substrate, was subtracted from each optical density reading. The readings were corrected for a small initial drop in optical density which probably resulted from the mixing procedure.

EXPERIMENTAL RESULTS

Synthesis of 3-Fluorosialic Acid.—Fluoropyruvic acid (0.7 g) was dissolved in water (15 ml) and the *p*H was adjusted to 9 with 6 N sodium hydroxide. *N*-Acetylglucosamine (5 g) was added, and the *p*H was raised and maintained at 11 with 10 N sodium hydroxide for 6 hours. After 2 and 4 hours 0.2-g portions of fluoropyruvic acid were added. After 6 hours the reaction mixture was neutralized with glacial acetic acid. The sodium ions were removed by passing the solution through an Amberlite IR-120 (H^+ form) column (44 × 2.5 cm) and washing the column with water until the effluent was neutral. The effluent was then passed through a Dowex-1 × 8 (formate form) column (30 × 3 cm) and washed with water until the direct Ehrlich test was negative. The column was eluted with 0.3 M formic acid and 20-ml samples were collected. The fractions from the major peak (tubes 28–37) that gave a positive direct Ehrlich test were combined and dried by lyophilization; yield, 80–90 mg. A small peak amounting to about 5% of the major peak was obtained at tube 20 in some experiments. The resulting pale-yellow powder was dissolved in water and the solution was passed through a charcoal column (1 × 20 cm). The column was eluted with 0.3 M formic acid and 20-ml samples were collected. The fractions (tubes 2–15) that gave a positive direct Ehrlich test were combined and dried by lyophilization; yield, 30 mg. The yield, based on fluoropyruvic acid, varied between 1 and 2%, averaging 1.5%. The final product was an amorphous white powder.

Anal. Calcd for $C_{11}H_{18}O_9NF$: C, 40.3; H, 5.52; N, 4.28; F, 5.81. Found: C, 40.15; H, 5.66; N, 4.06; F, 5.70 (Spang Microanalytical Laboratory, Ann Arbor, Mich.). Neutralization equivalent, 330; calculated 327.

The compound reduced ferricyanide, indicating the presence of a carbonyl group. The infrared spectra of *N*-acetylneuraminic acid and of 3-fluorosialic acid were nearly identical except for differences in the carbon-fluorine-absorbing area of 9–10 μ (Fig. 1). $[\alpha]_D^{20} = -19.2°$ (H_2O 1.1%). The molar absorbancy index of the chromophore formed in the direct Ehrlich assay was 8175 at 536 mμ. Figure 2 shows a comparison of the spectrum of the chromophore of *N*-acetylneuraminic acid with that of 3-fluorosialic acid.

The same product was obtained in a 3.0% yield when *N*-acetylmannosamine was substituted for *N*-acetylglucosamine. In addition to giving the same physical properties, it inhibited *N*-acetylneuraminic acid aldolase in an identical manner. Both products gave one spot and the same R_F in several chromatographic systems on both paper and thin-layer silica-gel-G plates. The Ehrlich spray was used to detect the spots in both methods. The sulfuric acid–sodium dichromate charring procedure also was used on the silica-gel plates to detect possible contaminants. None was found. The solvent systems, R_F, R_N (distance moved relative to *N*-acetylneuraminic acid), and number of experiments averaged ( ) were as follows: methyl ethyl ketone–acetone–water–formic acid, 30:10:10:1, 0.17, 0.86 (4); ethyl acetate–pyridine–acetic acid–water, 5:5:1:3, 0.45, 0.90 (4); butanol-acetic acid-water, 4:1:5 (upper phase), 0.12, 0.85 (14); 1-butanol–propanol–0.1 *N* hydrochloric acid, 1:2:1, 0.30, 1.0 (5); *sec*-butyl alcohol-acetic acid–water, 4:1:5, 0.65, 1.0 (16); and ethanol-water–ammonia, 80:20:1, 0.56, 0.92 (8). The product did not move from the origin in butanol saturated with water or methyl ethyl ketone saturated with water.

Inhibition Studies.—Figure 3 is a graph showing the production of pyruvate from *N*-acetylneuraminic acid

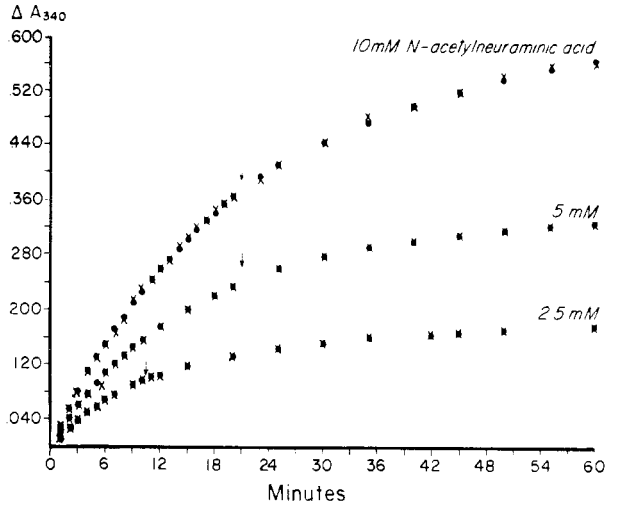


FIG. 5.—Course of reaction in presence of 2.5 mM 3-fluorosialic acid. The substrate was varied as shown. (●), observed experimental values; (X), values calculated from empirical equations (see Discussion); (↓) denotes the time at which the equation of the earlier time was changed to the equation of the later time period.

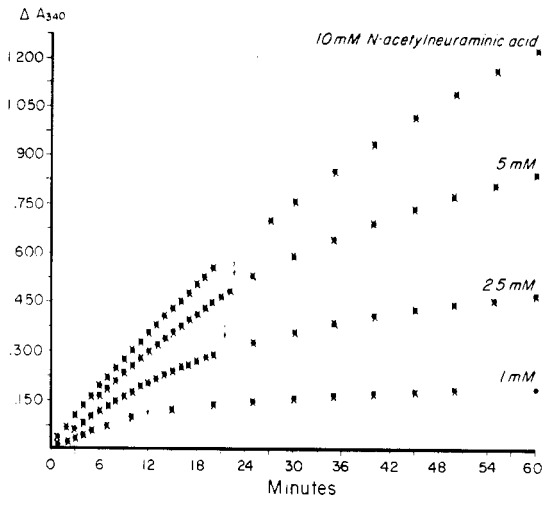


FIG. 6.—Course of reaction in presence of 10 mM 3-fluorosialic acid. The substrate was varied as shown. (●), observed experimental values; (X), values calculated from the empirical equations (see Discussion); (↓) denotes the time at which the equation of the earlier time was changed to the equation of the later time period.

as a function of protein concentration. The initial velocities were calculated from the slope of the line through the plots of the optical density changes observed versus time. Readings were taken at 1, 2, 3, 4, 5, 7, 10, 15, 20, 25, and 30 minutes. With 32 μg protein the rate was linear up to 10 minutes but decreased thereafter. With 22.4 and 16.0 μg, the rate began to decrease at 20 and 25 minutes, respectively. The other rates were linear throughout the 30-minute observation period. Under the same conditions the substrate, *N*-acetylneuraminic acid, was varied keeping the enzyme concentration constant. The initial velocities were calculated in the same manner as before and all the rates were linear during the 30-minute observation period. The K_m was estimated to be 1×10^{-3} M.

The stoichiometry of the reaction was tested in order to show that the appearance of pyruvate would account for the disappearance of *N*-acetylneuraminic acid. The reaction mixture contained 0.1 ml of 0.2 M phosphate buffer, 0.1 ml of 0.02 M phosphate buffer, 1 μmole of substrate, and 32 μg of protein in a total volume of 0.235 ml, and was incubated at 37° for 15 minutes. The reaction mixture was placed in a boiling-water bath for 2 minutes to stop the reaction. Then 0.3 ml NADH (0.002 M), 0.5 ml potassium phosphate buffer (0.2 M, *p*H 7.2), and water were added. The final volume was 3.0 ml. The turbid solution was clarified by centrifugation and the absorbancy was observed. Then 0.01 ml of lactic dehydrogenase was added and the absorbancy was observed after 15 minutes. For a control, the incubation mixture was placed in a boiling-water bath for 2 minutes before the substrate was added. The decrease in *N*-acetylneuraminic acid was measured by the Warren method. For each 0.1 ml of solution 2.25 μmoles of substrate was utilized and 2.88 μmoles of pyruvate appeared. It was concluded that the substrate disappearing could be accounted for by the appearance of pyruvate.

It has been shown (Avi-Dor and Mager, 1956), and confirmed in our laboratory, that lactic dehydrogenase rapidly reduces fluoropyruvic acid to 3-fluorolactic acid in the presence of NADH. To demonstrate that 3-fluorosialic acid is not cleaved to fluoropyruvic acid and *N*-acetylmannosamine, 2.5 μmoles of 3-fluorosialic acid was incubated with *N*-acetylneuraminic acid aldolase. Three controls were run; the first contained no enzyme, the second contained no 3-fluorosialic acid, and the third contained 2.5 μmoles of *N*-acetylneuraminic acid, enzyme, but no 3-fluorosialic acid. The results show that no detectable fluoropyruvic acid is produced from 3-fluorosialic acid (Fig. 4).

Since 3-fluorosialic acid is not cleaved to fluoropyruvic acid, this enzymic assay can be used to study the effect of 3-fluorosialic acid on the cleavage of *N*-acetylneuraminic acid by the aldolase. This study was carried out with two inhibitor concentrations and several substrate concentrations as shown in Figures 5 and 6. It is readily apparent from these figures that the rate of production of pyruvic acid, as measured by the disappearance of NADH, decreases with time. At the lower substrate concentrations it is almost zero after 60 minutes, whereas in the absence of 3-fluorosialic acid the rates are linear over this time period.

In order to show that the decreased rate of oxidation of NADH was caused by a decreased rate of pyruvic acid production and not by an inhibition of the assay system, the experiment shown in Figure 7 was performed. In this experiment the reaction was followed for 30 minutes in the presence of 3-fluorosialic acid, the enzyme was again added to the same cuvet, and the reaction was followed for another 30 minutes. Within 10 seconds after the addition of the second portion of enzyme the absorbancy was noted, and that point was considered the second zero point. At the end of the second 30-minute period 0.25 μmole of sodium pyruvate was added. From the figure it is clear that the assay system was intact and that the decreased rate of disappearance of NADH was caused by a decrease in the production of pyruvic acid.

Several different experiments were performed to minimize the possibility that the 3-fluorosialic acid was acting as a nonspecific denaturing agent or that it might be contaminated by small amounts of heavy-metal ions which may inactivate the enzyme. First, the complete reaction mixture, containing substrate, inhibitor, assay components, and bovine serum albumin (6.5 mg), was incubated at 37° for 15 minutes. Then the *N*-acetylneuraminic acid aldolase (6.4 μg) was added and the reaction was followed for 30 minutes. The rate of inactivation in the presence of protein (1000 times the concentration of the enzyme protein) was indistinguishable from the rate in the absence of pro-

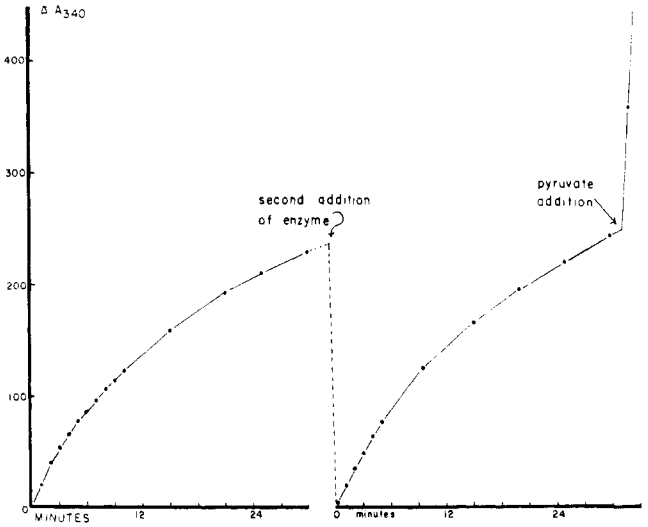


FIG. 7.—Demonstration of the integrity of the assay system. In the presence of 1 μmole of substrate and 2.5 μmoles of 3-fluorosialic acid in the standard assay mixture, 6.4 μg of *N*-acetylneuraminic acid aldolase was added. After 30 minutes, 6.4 μg of the enzyme was added again (second zero point) and the reaction followed an additional 30 minutes. At the end of the experiment 0.25 μmole of sodium pyruvate was added.

tein. Second, a sample of 3-fluorosialic acid (200 mg) was passed through a Dowex-50 (H^+) column and the effluent was dried by lyophilization. The residue was tested and was found to inactivate the aldolase in an identical manner. Third, a 12-mg sample of 3-fluorosialic acid was ashed and dissolved in 0.5 ml water, and the entire solution was introduced into the usual assay solution and its effect on the reaction rate was tested. The rate was found to be linear over a 30-minute period. From these experiments it was concluded that the inactivation was not likely to be caused by a heavy metal contaminant.

To test the possibility that the enzyme might be sensitive to fluoride ion that was in some manner split from the 3-fluorosialic acid, a reaction mixture containing 2.5 μmoles of potassium fluoride and 2.5 μmoles of substrate was incubated in the standard manner with 6.5 μg of enzymic protein. The reaction rate in the presence of fluoride was linear over the 30-minute observation period and the slope (0.02425 absorbancy unit/min) was equal to that of the control (0.02450 absorbancy unit/min).

Though no metal requirement has been demonstrated for *N*-acetylneuraminic acid aldolase, the possibility exists that 3-fluorosialic acid could remove small amounts of a required metal by chelation. The oxygen atoms on the carboxyl group are sterically favorable for the chelation of divalent metal ions with the fluorine in the 3-deoxy position forming a stable six-membered ring. To minimize this possibility, a reaction was carried out in EDTA buffer with and without inhibitor. If the inhibitor inactivates the enzyme by chelation of a required metal, then a comparatively large concentration of EDTA might be expected to remove the metal and inactivate the enzyme. Furthermore, if the inactivation is caused by a heavy divalent cation contaminant, the EDTA would be expected to decrease the inactivation rate. To test this, the reaction was carried out in 0.05 M EDTA at *p*H 7.2. In the absence of inhibitor, the rate was found to be linear with time, and in the presence of the inhibitor the inactivation followed the same course as in the absence of the chelating agent.

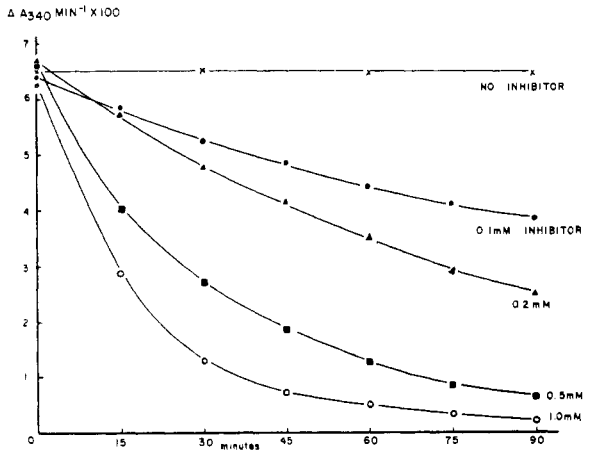


FIG. 8.—Various concentrations of 3-fluorosialic acid were preincubated with 640 μg of enzymic protein at *p*H 7.2. Aliquots were assayed as explained in text, and the rates were plotted as a function of the time incubated with inhibitor.

Conceivably, another enzyme may be present in the protein preparation which converts the 3-fluorosialic acid to an *N*-acetylneuraminic acid aldolase inactivator. To examine this possibility, 32 μg of enzymic protein, 2.5 μmoles of 3-fluorosialic acid, and the standard assay components were incubated at 37° for 1 hour. Then 1 μmole of substrate was added to determine residual aldolase activity. None was observed during a 15-minute interval. Then 6.4 μg of enzyme was added and the reaction was followed for 45 minutes. For the control, an identical solution without the enzyme was incubated 15 minutes and then the 6.4 μg of protein was incubated with 10 μmoles of 3-fluorosialic acid at 37° for 50 minutes. Under these conditions there was no detectable enzymic activity whereas the control lost no activity. The solution was then dialyzed in 1 liter 0.02 M potassium phosphate buffer, *p*H 7.2, at 4° with vigorous stirring. The buffer was changed five times, and after 30 hours the enzyme solution was found to be completely inactive. The control retained about 87.5% of its initial activity (not corrected for dilution during dialysis).

An experiment was performed to test the possibility that the inactivation of the enzyme can be reversed by increasing the substrate concentration. The reaction was started in the presence of 1 μmole substrate and 2.5 μmoles inhibitor. At 15 and 25 minutes, respectively, 2.5 and 5.0 μmoles substrate were added and the reaction rate was followed. The inactivation proceeded even in the presence of the highest substrate concentration.

A series of experiments was carried out to study the inactivation rate at various inhibitor concentrations in the absence of substrate. One ml of enzyme solution containing 640 μg protein in 0.02 M potassium phosphate buffer, *p*H 7.2, was incubated at 37° with inhibitor. The enzyme activity was measured at 15-minute intervals for 90 minutes by removing 0.025-ml aliquots and assaying in the standard manner, except that 2.5 μmoles of substrate and 15 μg of enzymic protein were used. The inhibitor transferred from the enzyme solution with the enzyme was ignored because the highest concentration was 0.025 μmole/ml in the presence of substrate 100 times more concentrated. This procedure was justified because all the rates were linear over the 10-minute period observed, and the calculated K_i (see discussion) is 2.5×10^{-3} M while the estimated K_m is 1×10^{-3} M. It can be seen from the results shown in Figure 8 that the inactivation is dependent on the

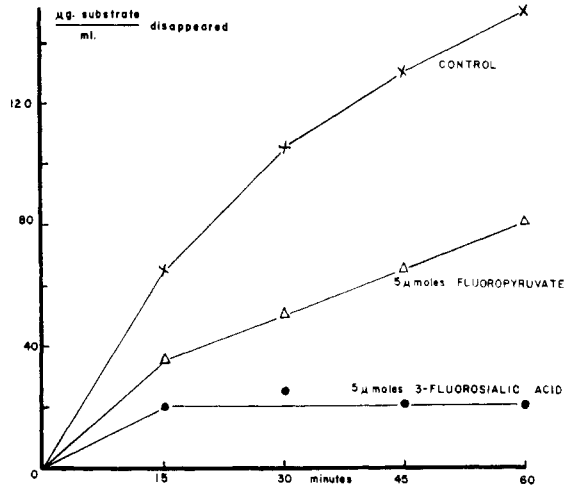


FIG. 9.—Comparison of the effects of fluoropyruvic acid and 3-fluorosialic acid. Each reaction mixture contained 1 μmole of substrate (309 μg), 60 mmoles of potassium phosphate, pH 7.2, and 64 μg of enzymic protein plus the fluoro compound indicated, in a total volume of 1 ml. The reaction was incubated at 37° and N-acetylneuraminic acid was determined by the thiobarbituric acid assay.

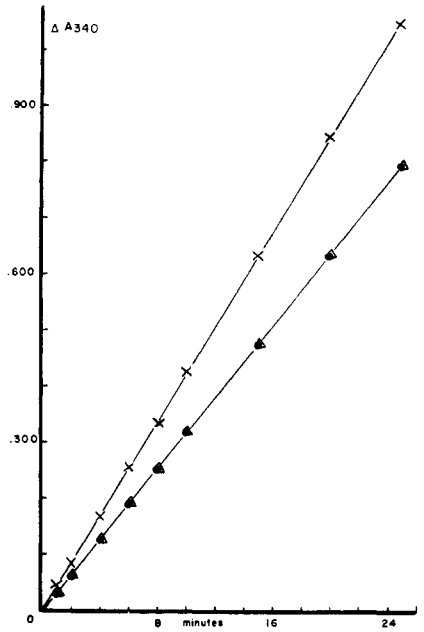


FIG. 10.—Incubation of fluoropyruvate (Δ) or fluoropyruvate plus N-acetylmannosamine (●) with N-acetylneuraminic acid aldolase for 2 hours at 37°. The solution was then dialyzed and tested for its activity. The control (X) was run identically to the others in the absence of the fluoropyruvate and N-acetylmannosamine.

inhibitor concentration. This will be discussed more fully.

Two experiments were designed to determine if fluoropyruvate inactivated the enzyme. In the first experiment, the disappearance of N-acetylneuraminic acid was measured in the presence of fluoropyruvate or 3-fluorosialic acid. It can be seen in Figure 9 that the 3-fluorosialic acid completely inactivated the enzyme in less than 15 minutes and that the fluoropyruvate inhibited the enzyme but did not inactivate it to the same degree as did the 3-fluorosialic acid. If the mechanism of inactivation by 3-fluorosialic acid proceeds through an intermediate of the cleavage process, then fluoropyruvic acid might inactivate the enzyme in the presence of N-acetylmannosamine by a reversal of the cleavage process to give the inactivating intermediates. This possibility was tested by incubating the enzymic protein (640 μg) with 10 μmoles each of fluoropyruvic acid and N-acetylmannosamine at 37° for 2 hours. A second reaction mixture contained only fluoropyruvate and a third contained neither. After incubation, the three mixtures were dialyzed separately in 1 liter of 0.02 M phosphate buffer, pH 7.2, at 4°. The buffer was changed after 4 and 8 hours. After 26 hours of dialysis the activities were measured using 16 μg of protein and 2.5 μmoles of substrate in the standard assay. The results plotted in Figure 10 show that the addition of N-acetylmannosamine did not make a detectable difference and that inactivation by fluoropyruvate was small in comparison to that by 3-fluorosialic acid.

The effect of various divalent metal ions on the inactivation rate was tested. Tris buffer was substituted for phosphate buffer as required. Magnesium concentrations of 2.5×10^{-3} M and 2.5×10^{-2} M showed no difference from the controls. No effect was observed with nickel chloride · 6 H_2O (2.5×10^{-3} M), manganese chloride · 4 H_2O (2.5×10^{-3} M) or ferrous sulfate · 7 H_2O (1×10^{-4} M). Calcium chloride (2.5×10^{-3} M) showed a slight inactivation of the enzyme but did not affect the inactivation by the inhibitor. Zinc chloride at 1×10^{-4} M was found to be a potent inhibitor of the aldolase both in the presence and absence of 3-fluorosialic acid, and 1×10^{-5} M Zn^{2+} reduced the activity to 72.5% of the control. Cobalt chloride (2.5×10^{-3} M) was found to give a small but reproducible protection against inactivation. This result could reflect a stimulation of the enzyme activity by cobalt ion, but no stimulation was found in the absence of inhibitor. Increasing the cobalt concentration to 5×10^{-3} M did not enhance the protective effect.

Because of the known stabilizing effect of sulfhydryl-containing compounds on many enzymes, several experiments were conducted to determine the effect of these compounds on the inactivation. A 0.1 M solution, pH 7.0, of reduced glutathione or L-cysteine was freshly prepared for each experiment. The standard assay solution containing 2.5 μmoles of inhibitor, 1 μmole of substrate, and 10 μmoles of either L-cysteine or reduced glutathione was incubated at 37° and the reaction was started with 6.4 μg of enzymic protein. Under the conditions of these experiments there was a small apparent protective effect, with cysteine being somewhat more active than glutathione.

DISCUSSION

The neutralization equivalent, elementary analysis, reducing test, and the infrared spectrum of the product of the condensation of fluoropyruvate and N-acetylglucosamine indicate that a fluorosialic acid was formed. However, this leaves the stereochemistry around asymmetric carbon atoms 3, 4, and 5 unresolved. Carbon atoms 3 and 4 become asymmetric upon condensation, and carbon atom 5 readily epimerizes in the alkaline solution before condensation (Comb and Roseman, 1960). Probably most of the possible isomers are present in the lyophilized preparation, but one would expect one or two isomers to predominate based on the rule of "steric control of asymmetric induction" (Cram and Elhafez, 1952).

Invoking this rule, one would predict that the N-acetyl group on carbon atom 5 in N-acetylneuraminic acid would be opposite the hydroxyl group on carbon atom 4 in the Fischer projection formula, as indeed has been recently shown (Kuhn and Baschang, 1962). However this does not explain a preference for the mannose configuration (carbon atoms 5 through 8) over

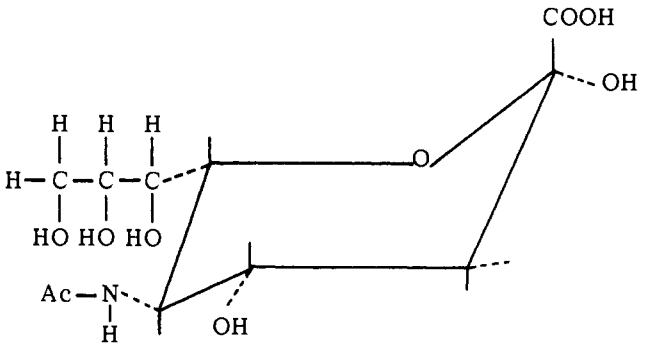


FIG. 11.—*N*-Acetylneuraminic acid in the 1C conformation. This conformation gives the thermodynamically most stable configuration in which all the bulkier groups are in the equatorial position.

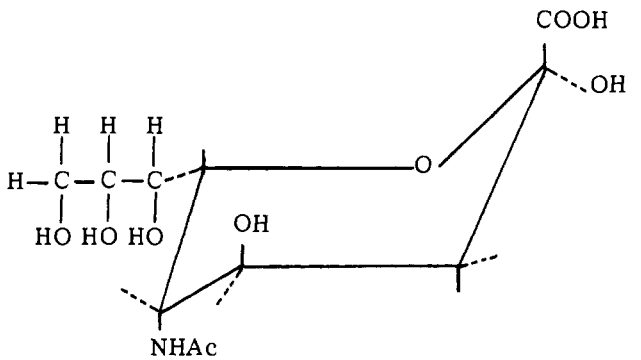


FIG. 12.—A 1C conformation of the condensation product which has the glucose configuration around carbon atoms 5, 6, 7, and 8. This configuration results in the bulkier groups at carbon atoms 4 and 5 assuming the axial position.

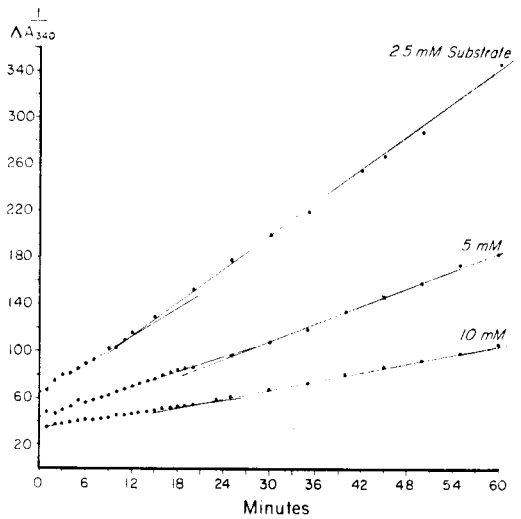


FIG. 13.—A rectified plot of the inactivation curves described in Fig. 5. The time divided by the optical density change versus the time gives two straight lines for the three lower substrate concentrations and one straight line for the highest substrate concentration.

the glucose configuration. This preference can be rationalized by a consideration of the 9-carbon sugar in its ring form (Fig. 11). After the condensation, the keto group forms a pyranose ring with the hydroxyl on carbon atom 6 (Blix *et al.*, 1956). If the chair conformation is assumed, and the large group containing carbon atoms 7, 8, and 9 is dominant, then the 1C conformation is the dominant species. When the molecules condense in the mannose configuration, all the large groups are equatorial (Fig. 11). However if the glucose configura-

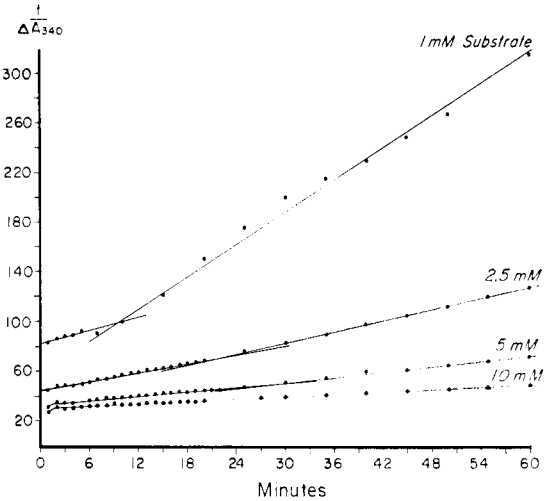


FIG. 14.—A rectified plot of the inactivation curves described in Fig. 6. The time divided by the optical density change versus the time gives two straight lines for each substrate concentration.

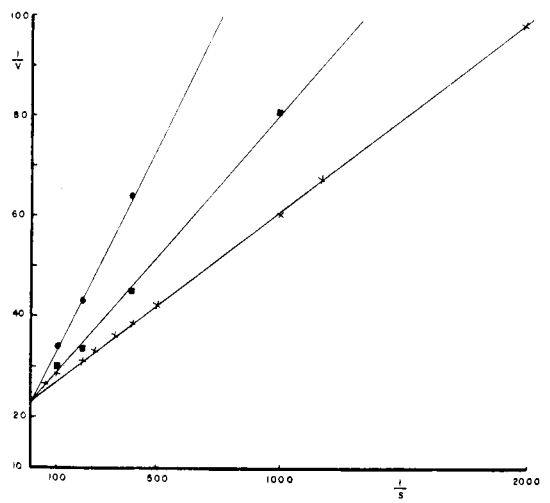


FIG. 15.—A reciprocal plot of the velocity at zero time versus the substrate concentration. (X), no inhibitor present; (■), 2.5 mM 3-fluorosialic acid; (●), 10 mM 3-fluorosialic acid.

tion is formed in the condensation, the large groups on carbon atoms 4 and 5 will be axial, and the large group (C atoms 7, 8, and 9) will be equatorial (Fig. 12); in the Cl conformation the large groups on carbon atoms 4 and 5 will be equatorial, but the group containing carbon atoms 7, 8, and 9 will be axial. A boat form may have less strain, but the most stable compound would have the 1C conformation with all the groups equatorial.

This rationalization is consistent with the larger yields obtained with *N*-acetylmannosamine (10%) as compared with *N*-acetylglucosamine (2–3%) (Cornforth *et al.*, 1957, 1958; Carroll and Cornforth, 1960). It was also noted that the yields were doubled in the fluoropyruvate condensation when *N*-acetylmannosamine was used (3%) as compared with *N*-acetylglucosamine (1.5%) under identical conditions.

Since the structure of 3-fluorosialic acid is very close to that of *N*-acetylneuraminic acid, it would be expected to be a competitive inhibitor of *N*-acetylneuraminic acid aldolase. Because the inhibitor inactivates the enzyme, reaction velocities at time zero must be used. In order to obtain the empirical equations the curves in Figures 5 and 6 were rectified by plotting time divided by absorbancy change (x/y) versus time (x). From

TABLE I
EMPIRICAL EQUATIONS OF THE INACTIVATION CURVES

Substrate Concn	Inhibitor Concn	Equation at Earlier Time Period[a]	Equation at Later Time Period
1 mM	2.5 mM	$y = \frac{x}{1.88x + 81}$	$y = \frac{x}{4.22x + 61}$
2.5 mM	2.5 mM	$y = \frac{x}{1.2x + 45}$	$y = \frac{x}{1.44x + 40}$
5.0 mM	2.5 mM	$y = \frac{x}{0.54x + 33.6}$	$y = \frac{x}{0.68x + 30.3}$
10 mM	2.5 mM	$y = \frac{x}{0.31x + 30.15}$[b]	
2.5 mM	10 mM	$y = \frac{x}{3.96x + 64}$	$y = \frac{x}{4.73x + 56}$
5 mM	10 mM	$y = \frac{x}{2.15x + 43}$	$y = \frac{x}{2.51x + 33}$
10 mM	10 mM	$y = \frac{x}{1.04x + 34}$	$y = \frac{x}{1.31x + 28}$

[a] x = time, y = absorbancy change at 340 mμ. [b] One equation fits the entire curve observed.

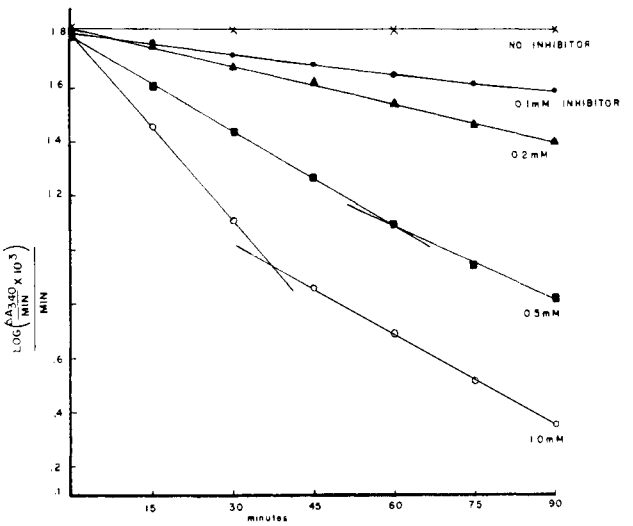


FIG. 16.—A plot of the log of the velocity versus time from the data in Fig. 8.

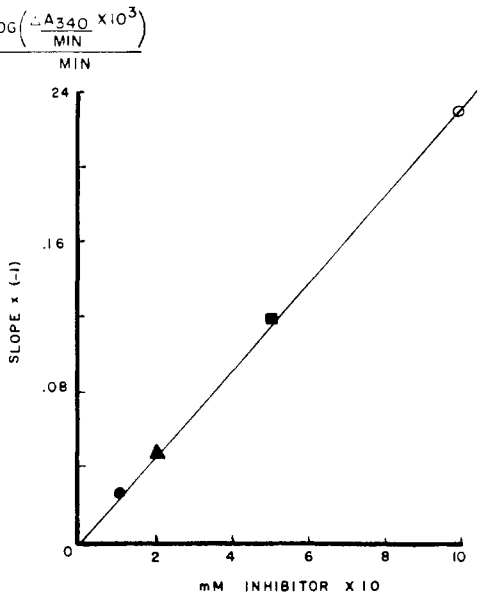


FIG. 17.—A graph of the slopes (times minus 1) of the log plot in Fig. 16 versus inhibition concentrations. The two points at the higher concentrations are the slopes at the earlier times in Fig. 16.

the results shown in Figures 13 and 14, it can be seen that the course of the inactivation can be represented by two equations (except for the case where the substrate-to-inhibitor ratio is highest) having the general form of a hyperbola:

$$y = \frac{x}{bx + a}$$

The constants, a and b, were evaluated for each of the inactivation curves by estimating the intercept on the ordinate for a and the slope of the line for b in their respective rectified plots. The resulting equations are shown in Table I. It can be seen in Figures 5 and 6 that the experimental points and the points calculated from the empirical equations fit very well. In order to determine the initial velocity, the equation of the earlier time period was differentiated to give

$$\frac{dy}{dx} = \frac{a}{(bx + a)^2}$$

and x (time) was then set equal to zero. Using this method the initial velocities were determined for each curve and these values were used to construct a reciprocal plot of $1/v$ versus $1/(S)$ (Lineweaver and Burk, 1934) shown in Figure 15. This plot clearly shows an initial competitive inhibition. The K_p of the inhibitor was graphically estimated. The K_i was calculated to be 2.5×10^{-3} M from the formula

$$K_i = \frac{i}{\frac{K_p}{K_m} - 1}$$

where i is equal to the inhibitor concentration (Dixon and Webb, 1958).

An important question regarding these inhibition studies is the manner in which the enzyme is inactivated. If the data from the preincubation experiments (Fig. 8) are replotted by taking the log of the velocity versus time, a straight line is obtained for the two lower concentrations of inhibitor (Fig. 16) and two straight lines for each of the higher inhibitor concentrations. This result suggests a complex mode of inactivation. Curves of this type can occur when a protection against inactivation develops during the course of the reaction. For instance, if the enzyme is "denatured" by the inhibitor, and the denatured enzyme can in some way combine with the undenatured enzyme in such a

way that combination protects the latter from inactivation, a plot of log velocity versus time would result in just such a biphasic curve (Reiner, 1959) where the early and late parts of the curve are nearly linear. Another mechanism which could result in this two-phase inactivation is a decrease in the effective concentration of the inhibitor. This might come about by a reaction of the inhibitor with one or several impurities in the enzyme preparation.

If one considers only the early linear phase of inactivation (Fig. 16), an equation of the form

$$\log v = -at + c$$

can be written for the decrease in velocity as a function of time at any specified inhibitor concentration, where c is the velocity in the absence of inhibitor (or the velocity at zero time), and a is the rate constant of inactivation under the specified conditions. In Figure 17 we plotted the values of a obtained in each experiment. The straight line which results has the slope

$$0.23\frac{\log(\Delta A_{340}/\text{min} \times 10^3)}{\text{mM inhibitor} \times \text{min}}$$

representing the apparent inactivation constant for the range of inhibitor concentrations studied.

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