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SEPARATION, PURIFICATION AND SOME PROPERTIES OF PNEUMOCOCCAL NEURAMINIDASE ISOENZYMES

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SUMMARY

1. Neuraminidase from a rough strain of *Diplococcus pneumoniae* has been partially purified by a series of steps involving $(NH_4)_2SO_4$ fractionations, ultrafiltration through a Diaflo XM-50 filter, heat treatment at 55–56° for 4 min, and batchwise absorption and desorption from DEAE-cellulose. The most variable stages in this procedure involved the heat and DEAE-cellulose treatments.

2. Enzyme purified in this manner was resolved into two peaks of activity by chromatography on DEAE-cellulose and into three populations of neuraminidase activity upon CM-cellulose chromatography. Both discontinuous and linear gradient elutions with phosphate–citrate buffer (pH 6.4) range 0.01–0.10 M were used.

3. The initially eluted CM-cellulose enzyme fraction (Fraction I) was in turn resolved into two activity peaks upon sequential chromatography on CM-Sephadex with the same developing buffer system. Comparable fractions, modified in their origins only insofar as deletion of the heating and batchwise DEAE-cellulose steps, provided 5 isoenzyme peaks on CM-Sephadex chromatography. It is presumed that these results reflect association–dissociation phenomena due to temperature and monovalent cation exposure.

4. The second CM-cellulose peak of enzyme activity (Fraction II) was additionally resolved into three isoenzymes by serial CM-Sephadex chromatography. Two of these neuraminidase forms appeared at elution volumes sufficiently different from those which were derived from CM-cellulose Fraction I or modified CM-cellulose Fraction I so as to indicate their non-identity. Furthermore, the CM-cellulose Fraction II isoenzymes possessed much lower specific activities than those which were similarly obtained from CM-cellulose Fractions I.

5. Confirmation of the existence of multiple forms of pneumococcal neuraminidase came from polyacrylamide gel electrophoresis experiments. The appearance of at least five zones of enzyme activity was found on electrophoretic zymograms of the partially purified material. CM-cellulose Fraction I preparations exhibited a minimum of three zones of activity, but each zone gave indications of heterogeneity. Correlations between isoenzyme zones as found above and activity peaks resultant from ion-exchange chromatography have not yet been carried out.

6. Further enrichment of pooled CM-cellulose Fractions II was effected by

precipitation with 0.65 saturated $(NH_4)_2SO_4$. This procedure led to a 400-fold purification, and an apparently crystalline enzyme. Polyacrylamide gel electrophoresis of the crystalline enzyme showed the presence of at least six protein staining bands. Zymograms of unstained replicate disc gels indicated that the enzymatic area, which encompassed only two of the protein species present, was now apparently somewhat more homogeneous. Whether the residual neuraminidase-negative proteins represent inactive forms of the enzyme, or impurities with almost identical salt solubility and ion-exchange properties, remains to be determined.

INTRODUCTION

Neuraminidase (mucopolysaccharide *N*-acetylneuramylhydrolase, EC 3.2.1.18) in culture filtrates from *Diplococcus pneumoniae* has been reported by several investigators[1–4] and an enzyme preparation from this source has been brought to a high degree of purity[5]. Despite the ubiquitous distribution of neuraminidase among the viruses, bacteria and animal cells[6], detailed observations concerning multiple forms (isoenzymes) of this enzyme have not been recorded. Chromatographic indications for more than one peak of neuraminidase activity can however be found in descriptions of purification of this enzyme from *Corynebacterium diphtheriae*[7], Streptococcus strains[8], and *Clostridium perfringens*[9]; as well as in a preliminary report[10] concerning ion-exchange separation of extracts from the protozoan *Trichomonas foetus*.

In attempting to modify and extend the fractionation procedure of HUGHES AND JEANLOZ[5] to the neuraminidase produced by a strain of Pneumococcus, we have found that partially purified preparations yield at least eight possibly different peaks of enzymatic activity on ion-exchange chromatography, and that five zones of enzyme appear following disc gel electrophoretic examination. In the present work, the separation of these distinct isoenzyme populations of pneumococcal neuraminidase, an initial survey of their electrophoretic and kinetic properties, and the crystallization of a heterogeneous enzyme mixture which includes several of these forms as well as inactive proteins, are described.

MATERIALS AND METHODS

Bacterial strain

A high-yield pneumococcal producer of neuraminidase (strain No. 70), first isolated by Dr. R. D. O'TOOLE, was kindly provided by Dr. C. HOWE. After repeated passage on blood agar, this isolate has undergone a transition to the rough form. Its capacity for forming neuraminidase remains unimpaired (*cf.* ref. 4).

Culture medium

The glucose–phosphate supplemented neopeptone–beef heart medium of LEE AND HOWE[4] was routinely employed. Todd–Hewitt medium (Difco) was also effective for growth of bulk quantities of pneumococci and for enzyme production.

Enzyme assays

Neuraminidase activity was assayed by incubating 0.20 ml of enzyme solution

with 0.80 ml Collocalia[11] mucoid (2.5 mg/ml in 0.1 M phosphate–citrate buffer) at pH 6.4 for 60 min at 37°. Aliquots of 0.20 ml were withdrawn at appropriate times from 0 to 60 min and were assayed by the method of WARREN[12]. Highly purified preparations or isoenzyme fractions were also tested for activity with *N*-acetylneuraminyl lactose (Calbiochem; 0.20 μmole per assay). A unit of enzyme activity is defined as that amount which releases 1.0 nmole of *N*-acetylneuraminic acid per min from the mucoid preparation, under conditions of the standard assay given above. β-D-Galactosidase determinations[13] and *N*-acetylneuraminic acid aldolase assays[14] were carried out by standardized procedures.

Protein determinations

These were carried out either by the biuret method[15] following initial precipitation of protein from solutions with 7.5% trichloroacetic acid (final concentration), or on solutions by the Folin procedure[16].

Polyacrylamide gel electrophoresis

This technic was carried out by the method of Ornstein and Davis as modified by NARAYAN *et al.*[17]. After electrophoresis, the gels were stained in 7.5% acetic acid containing 1% amido black. For determination of relative isoenzyme activities, unstained replicate disc gels were frozen and were cut into some thirty sections, each 1.5 mm thick, using the apparatus described by HOWE *et al.*[18]. These individual polyacrylamide wafers were then embedded, with suitable spacing, into 0.85% Ionagar held on microscope slides. Electrophoresis of the proteins into the agar across the narrow slide dimension, was allowed to proceed for 50–60 min (145 V, 10 mA) in the cold. After slicing the agar, the resultant migrated proteins were assayed for neuraminidase activity by incubating each of these strips, including their parental gel wafers, with Collocalia mucoid at 22° for 18 h. The number of μmoles of *N*-acetylneuraminic acid released was calculated from absorbance readings at 549 and 532 nm after color development with thiobarbituric acid[12]. Suitable corrections were made for the color contribution of agar blanks *plus* polyacrylamide wafers treated under identical conditions.

Preliminary enzyme fractionations

After overnight growth, the pneumococci were removed from nutrient broths by centrifugation in the cold (Step 1). The growth filtrates were concentrated by being brought to 0.70 saturation with $(NH_4)_2SO_4$ at 5°. After dialysis against 0.01 M phosphate–citrate buffer (pH 6.4) further removal of low molecular weight material was effected by pressure filtration (nitrogen) through a Diaflo XM-50 filter (Amicon Apparatus Co.; Step 2). An additional optional stage of purification involved heat treatment at 55–56° for 4 min (Step 2'), with subsequent removal of insoluble material by centrifugation in the cold. The protein which was precipitated in the range 0.30–0.70 $(NH_4)_2SO_4$ saturation was then collected and dialysed (Step 3). Activated, washed DEAE-cellulose (11 g per 150 ml volume) was added to this enzyme solution, and the suspension was agitated with a magnetic stirrer in the cold for 1 h. The ion-exchange cellulose was removed by centrifugation at 5°, and was then suspended in twice the original volume of 0.1 M phosphate–citrate buffer (Step 4). The desorbed protein was further salt fractionated to give an $(NH_4)_2SO_4$ precipitate at 0.4–0.65

saturation at 5° (Step 5). The resultant enzyme mixture was dialyzed, and was re-concentrated (Diaflo XM-50) as necessary. These preparations were then further resolved by chromatographic procedures (Step 6).

Ion-exchange chromatography

Step 5 enzyme mixtures were applied to DEAE-cellulose (Whatman DE-23) or to CM-cellulose (Whatman CM-23) ion exchangers, which had been acid–base washed and thoroughly equilibrated with 0.01 M phosphate–citrate buffer (pH 6.4). Glass columns 2 cm × 30 cm were employed. All operations were carried out in the cold. The eluting medium was usually a linear gradient of buffer, from 0.01 to 0.10 M. Chromatography with CM-Sephadex (Pharmacia, C-50) was carried out under the identical operating conditions. Where indicated under the appropriate figure, discontinuous chromatography was occasionally performed by step-wise increases in molarity of the eluting phosphate–citrate buffer.

RESULTS

Chromatographic evidence for isoenzymes

Fractionation of pneumococcal growth filtrates following the initial procedures of HUGHES AND JEANLOZ[5] for absorbing β-galactosidase activity, led to the finding that the neuraminidase was almost quantitatively absorbed by the stage involving

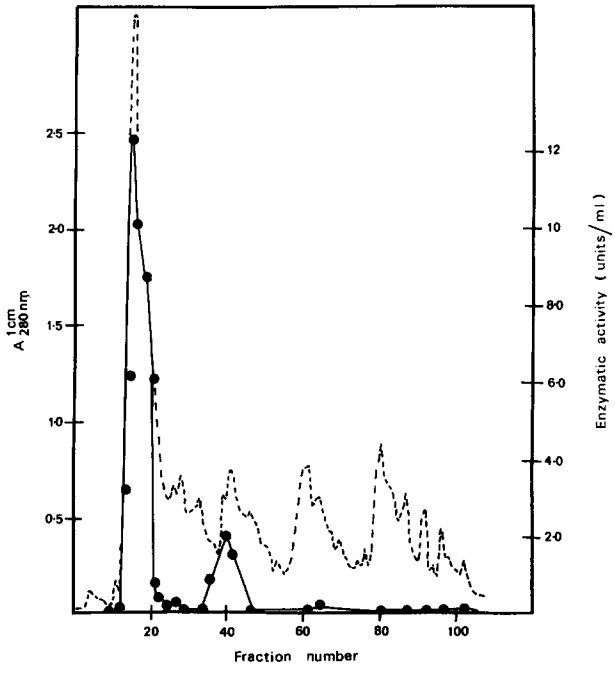

Fig. 1. Elution profile of pneumococcal neuraminidase on DEAE-cellulose after purification through Step 5. Discontinuous elution with 120-ml volumes each of phosphate–citrate buffer (pH 6.0) as follows: 0.01 M, 0.025 M, 0.05 M and 0.10 M. ●—●, enzymatic activity with Collocalia mucoid; — — —, absorbance at 280 nm. Fractions each contained 6–7-ml volumes.

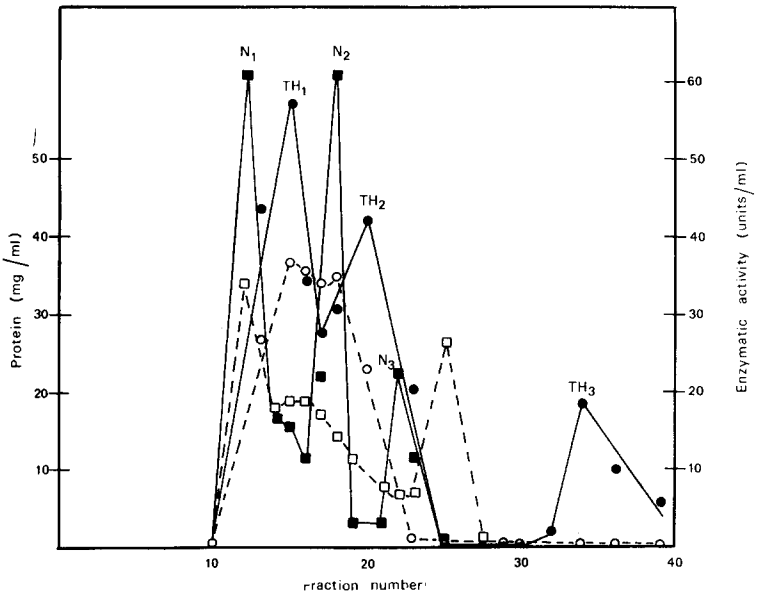

Fig. 2. Chromatography on CM-cellulose of partially purified (Step 5) neuraminidase obtained after pneumococcal growth on neopeptone medium (N), and on Todd–Hewitt broth (TH). ●—●, activity on Collocalia mucoid of Todd–Hewitt isoenzymes; ○- - - -○, absorbance of latter at 280 nm; ■—■, activity on Collocalia mucoid of neopeptone-derived isoenzymes; □- - - -□, absorbance of latter at 280 nm. Gradient elution with phosphate–citrate buffer as described under MATERIALS AND METHODS. Fraction volumes, 6–7 ml.

batch-wise treatment with DEAE-cellulose. These results were reproduced in several experiments with thoroughly dialyzed $(NH_4)_2SO_4$-concentrated growth filtrates, using DEAE-cellulose from two different commercial sources. The neuraminidase activity was readily recovered from the ion-exchange cellulose by batch elution with 0.1 M phosphate–citrate buffer at pH 6.0–6.4. Further investigation of the chromatographic behavior of partly purified neuraminidase (Step 5) on DEAE-cellulose was carried out. This chromatographic profile is given in Fig. 1. Although the bulk of the neuraminidase activity was eluted almost at the void volume, the appearance of a second, distinct peak, centered at fraction No. 40, demonstrated the existence of yet another population of enzymes.

This observation stimulated a study of the chromatographic pattern of partially purified enzyme on CM-cellulose[5]. Enzyme fractions (Step 5) from pneumococci which had been grown on neopeptone–glucose–phosphate medium, or on Todd–Hewitt broth, were each subjected to such chromatographic resolution (Fig. 2). In both preparations, three peaks of neuraminidase activity were found. The first two peaks (N_1, N_2) and (TH_1, TH_2), respectively, were of relatively low specific activities in comparison to their third peaks, which were eluted in later fractions coincident with lower protein concentrations. It is concluded from these experiments, that either nutrient medium provided almost comparable populations of neuraminidase isoenzymes. When enzyme-containing fractions corresponding to the foregoing peaks N_1 and N_2 were each concentrated by ultrafiltration, and were then individually

subjected to chromatography on CM-Sephadex, N_1 was resolved into two peaks of activity (Fig. 3). The second CM-cellulose peak (N_2) exhibited only one area of enzyme activity under an identical elution schedule. These findings further reinforced the supposition of the existence of multiple molecular forms of pneumococcal neuraminidase.

To obviate the possibility that all or part of the observed multiple enzymatic activities were manifestations of the purification procedure, the heat treatment stage (Step 2′) and DEAE-cellulose batch process (Step 4) were next omitted from the preliminary fractionation procedure. A representative chromatographic pattern of this enzyme concentrate (Step 5) on CM-cellulose is depicted in Fig. 4. Once again,

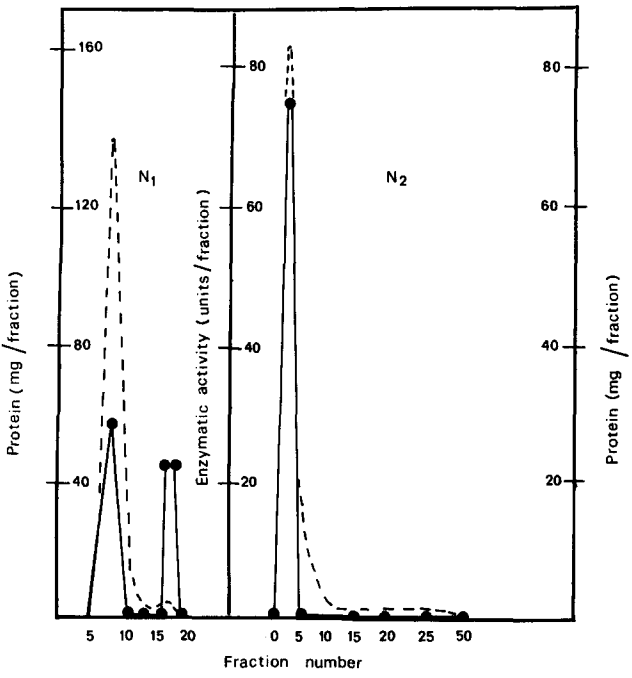

Fig. 3. Chromatography on CM-Sephadex (C-50) of pooled and concentrated peaks N_1 and N_2 from CM-cellulose fractions depicted in Fig. 2. Elution schedule and fraction volumes as given under Fig. 2. Symbols same as in Fig. 1.

the multiple nature of pneumococcal neuraminidase was apparent, since both CM-cellulose Fraction I and a higher specific activity peak CM-cellulose Fraction II were observed. Occassionally, the chromatographic profile CM-cellulose Fraction II appeared as an incompletely resolved overlap of two or more peaks, indicating the relationship of these multiple forms to the three peaks shown previously in Fig. 2.

CM-Sephadex chromatography of CM-cellulose Fraction I (collected in Fractions 20–25; Fig. 4) which had been concentrated by ultrafiltration, gave the results depicted in Fig. 5. When the resultant fractions were tested with Collocalia mucoid substrate, 5 peaks of moderate specific activity (range 8–39) were found. Each fraction pool corresponding to these 5 major peaks was then again individually tested with

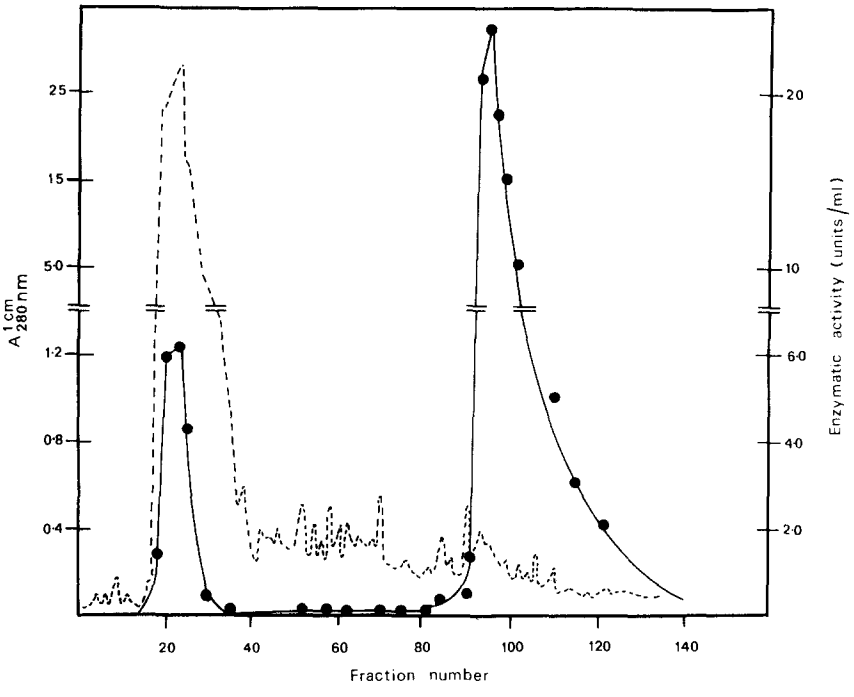

Fig. 4. Chromatography of Step 5 neuraminidase on CM-cellulose (CM-23). In this preparation, the heat and DEAE-batch stages of purification were omitted. Elution schedule carried out as given under Fig. 2. Symbols same as in Fig. 1.

the more defined substrate *N*-acetylneuraminyl lactose. These results confirmed that each isoenzyme indeed possessed neuraminidase activity. Control fractions from other portions of this chromatogram which exhibited no enzyme activity against Collocalia mucoid were also inactive with *N*-acetylneuraminyl lactose. When CM-cellulose Fraction II (Fig. 4) was further subjected to combined ion-exchange-gel exclusion chromatography on CM-Sephadex, three peaks of diminished specific activities (range 1–2) were observed (Fig. 6). The first of these, (Fraction 10), corresponds in elution volume to the forepeak of low neuraminidase activity noted in the preceding experiment (Fig. 5). The other two peaks however, do not correspond in their elution placement with any of the 5 major peaks described above. It is supposed that they represent additional multiple molecular enzyme forms. The relatively low specific activities of these latter peaks suggests that they might have been resolved from prosthetic groups by the serial chromatographic treatments.

Enzyme crystallization

The isolation of a crystalline pneumococcal neuraminidase preparation purified almost 400-fold followed from application of the above-described fractionation steps. These protocols are outlined in Table I. Selection and further salt fractionation of CM-cellulose Fraction II (Fig. 4; Step 6) facilitated a large increase in purification. The crystallized enzyme, which was readily precipitated from concentrates of CM-cellulose Fraction II with $(NH_4)_2SO_4$ at 0.65 or 0.70 saturation, flocculated as off-white

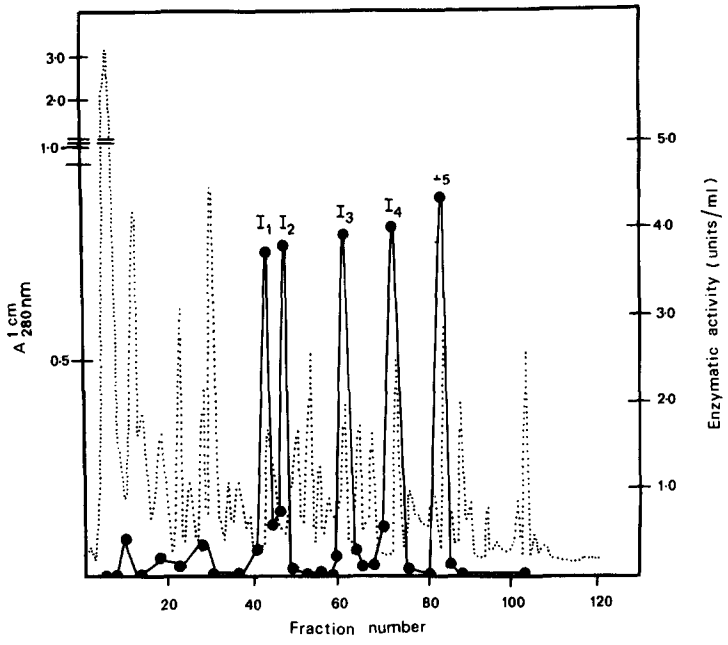

Fig. 5. Profile following chromatography of CM-cellulose Fraction I (Fig. 4) on CM-Sephadex. Enzyme activity was measured initially by assay with Collocalia mucoid. Results were verified using *N*-acetylneuraminyl lactose as the substrate. Symbols (Fig. 1) and elution schedules are the same as detailed earlier (Fig. 2).

needles. These were reminiscent in appearance of impure tyrosine at its isoelectric point. Twice-crystallized enzyme from 0.65 saturated $(NH_4)_2SO_4$ showed no particular increase in its specific activity (231) over precursor material which had been purified to Stage 6. In another series of purifications, crystalline enzyme purified to Stage 7 exhibited a slightly lower specific activity (210 units per mg protein). No β-D-galactosidase or aldolase activities, earlier recorded[5,9] to accompany bacterial neuraminidases, were detected in these preparations.

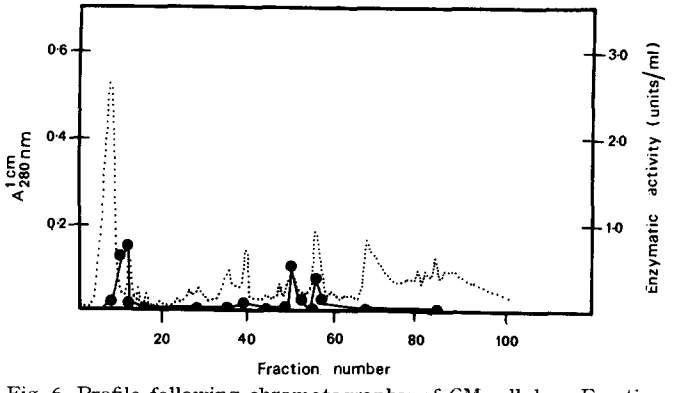

Fig. 6. Profile following chromatography of CM-cellulose Fraction II (Fig. 4) on CM-Sephadex. All symbols and elution details are identical with those in Fig. 5.

TABLE I

PURIFICATION OF PNEUMOCOCCAL NEURAMINIDASE

Fraction	*Final volume (ml)*	*Protein (mg)*	*Total activity (units)*	*Specific activity*	*Recovery (%)*
1. Growth broth	1620	19050	11350	0.594	100
2. Heat-treated; XM-50 concentrated	140	7300	10710	1.47	95
3. 0.30–0.70 $(NH_4)_2SO_4$	150	2940	9550	2.59	84
4. DEAE-cellulose batch eluate	342	1535	6658	4.33	59
5. 0.40–0.65 $(NH_4)_2SO_4$	40	540	8650	16.1	(76)
6. CM-cellulose Fraction II	98	23	5280	231	46
7. 0.70 $(NH_4)_2SO_4$	0.5	11.5	2640	231	23

General enzyme characteristics

We have confirmed the prior observation[5] that purified pneumococcal neuraminidase activity is not stimulated by Ca^{2+} ions. Some 40% of enzymatic activity was removed from solution at pH 8.0 and 0° by treatment with 5 vol. % of packed red blood cell ghosts. About 65% of this absorbed activity in turn, was released from the cells with 0.1 M phosphate–citrate buffer in 0.9% NaCl at pH 6.0. Contrary to the recent report concerning viral neuraminidase[19], congo red ($1 \cdot 10^{-5}$ M) was not observed to inhibit the activity of the pneumococcal enzyme on Collocalia mucoid. Michaelis constants (K_m's) were estimated for the high specific activity peak N_3 (Fig. 2), and for the first of two sub-peaks from fraction N_1 as well as for the single peak of activity which was obtained from N_2 by CM-Sephadex chromatography (Fig. 3). Using Collocalia mucoid as substrate (assumed mol. wt. 45 000) and Lineweaver–Burke plots, the extrapolated values were approx. $1.4 \cdot 10^{-4}$ M for each of these three isoenzyme populations. This K_m is of the same order of magnitude as that earlier reported[5] for the activity of pneumococcal neuraminidase acting on α_1-acid glycoprotein.

Electrophoretic zymograms

Polyacrylamide-gel electrophoretic staining patterns from selected enzyme purification stages and from chromatographically resolved peaks are given in Fig. 7. Thus, disc gel 106 typifies the appearance of partially purified (Step 5) neuraminidase prior to ion-exchange chromatography. This preparation exhibited 14 major and 3 minor protein lines which stained with amido black. The CM-cellulose Fractions I, which were collected with the bulk of Step 5 protein almost at the void volume of the column (Fig. 4), displayed 10 major and 6 minor protein lines (gel 107). Gel 72 depicts one of the five isoenzyme peaks (I_2) obtained from CM-cellulose Fraction I. (Fig. 5). Because of limited quantities of material, these particular electrophoretic separations were carried out with only 20% of the total protein which was applied to the preceding samples. In light of the fact that replicate disc gels were devoid of detectable enzymatic activity (see below), the appearance of a single protein-staining band in this case is inconclusive. The twice-crystallized neuraminidase, in turn obtained from CM-cellulose Fraction II, showed at the same approximate protein

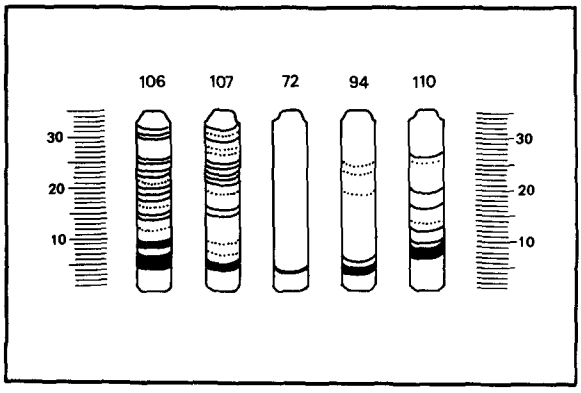

Fig. 7. Polyacrylamide disc gel patterns of neuraminidase fractions. Gel 106, Step 5 enzyme, 300 μg total protein; gel 107, CM-cellulose Fraction I, 310 μg protein; gel 72, Fraction I_2 (Fig. 5) at a concentration of 60 μg protein; gel 94, twice-crystallized enzyme, 60 μg protein; gel 110, same, but at 200 μg protein concentration. Ordinates at left and right orient the 1.5-mm segments into which replicate unstained disc gel electrophoretic separations were later sliced for enzyme assay.

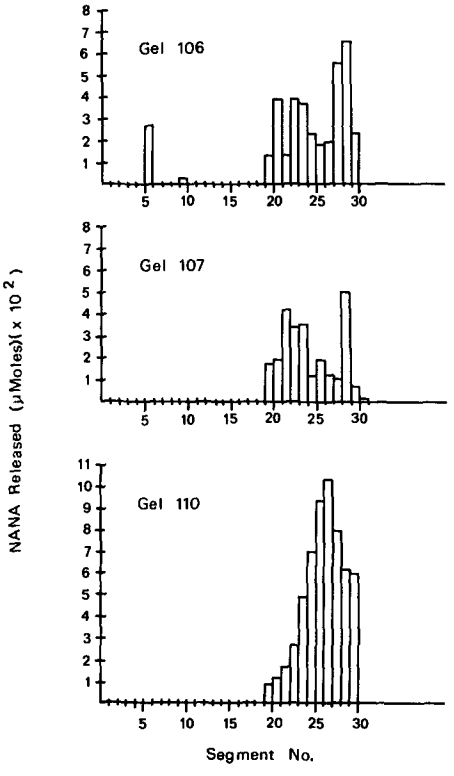

Fig. 8. Relative enzymatic assays (modified WARREN[12] TBA test; see MATERIALS AND METHODS) of gel segments sliced as numbered in Fig. 7. NANA, *N*-acetylneuraminic acid.

concentration applied to gels 106 and 107, 6 distinct amido black protein staining bands (gel 110).

Examination of the zymograms corresponding to gels 106, 107 and 110 (Fig.8), revealed that at least 3 clearly defined zones of neuraminidase activity can be found in the partly purified enzyme prior to ion-exchange chromatography. However, the CM-cellulose Fraction I material appeared to have been resolved from those isoenzymes with greatest anodal mobility (zones 5–6 and 9–10). In these particular fractions (gel 107), an obvious degree of heterogeneity in the main enzyme area encompassing zones 19–31 is indicative of several electrophoretic forms of the enzyme. This result accords with the appearance (Fig. 5) of 5 major peaks of neuraminidase following CM-Sephadex chromatography of CM-cellulose Fraction I. The zymogram pattern of the crystalline material (gel 110, Fig. 8) is quite symmetrical. This result suggests the occurrence of a set of electrophoretically homogeneous isoenzymes at Stage 7 of purification. This surmise was reinforced by a preliminary immunoelectrophoresis experiment. Here, sections from an unstained replicate gel corresponding to No. 110 were each diffused against a γ-globulin fraction from rabbit antibodies to pneumococcal growth filtrate. It was found in these slices corresponding to maximum enzyme activity (zones 23–28; Fig. 8) that only two, identical precipitin bands were formed throughout.

DISCUSSION

The occurence of multiple molecular forms of neuraminidase in the extracellular culture fluid of gram-positive microorganisms following ion-exchange chromatography of partly purified preparations has been observed in the past. MORIYAMA AND BARKSDALE[7] depicted 2 peaks of enzyme activity following CM-cellulose chromatography of a crude enzyme preparation from *C. diphtheriae* and HAYANO AND TANAKA[8] noted 2 peaks of neuraminidase with high salt elution of streptococcal extracts on a DEAE-cellulose column. The apparent dissociation of highly purified *Cl. perfringens* enzyme into several forms upon storage, has also been commented upon[9]. Although the majority of pneumococcal strains which have been examined produce neuraminidase[3,4,20], the phenomenon of isoenzyme formation from this source has, however, not heretofore been described. This appearance of pneumoccocal neuraminidase in a number of chromatographically separable forms was found to be reproducible from one bulk cultivation to another and was observed following growth on two different nutritive media.

Insofar as purification and crystallization of the pneumococcal neuraminidase is concerned (Table I), several stages are variable and require additional comment. Perhaps the most delicate is the heat treatment at 55–56° (Step 2'). This procedure was effective when carried out on the crude culture broth, or at an early stage of concentration. When performed at later stages of purification, inactivation of enzymatic activity occurred. Likewise, the batch process adsorption of enzyme to DEAE-cellulose and its subsequent elution with 0.1 M phosphate–citrate buffer, while effecting an approx. 2-fold purification (Step 4), may in light of the experience of HUGHES AND JEANLOZ[5] be a stage which is difficult to reproduce. These steps may be omitted from the preliminary fractionation, still permitting subsequent isolation of high specific activity and indeed crystalline enzyme. In Step 5 of enzyme purification

(Table I), an apparent increase in total enzyme activity occurred. This phenomenon may be due to the observation that cold storage of partly purified neuraminidase at stages of fractionation varying from the first to the fifth, was often found to result in an increase in enzyme titer. Whether this effect was due to release from inhibition, to disaggregation, or to other conformational changes, has not yet been investigated.

That the final, twice-crystallized enzyme is far from homogeneous, aside from a possible proportion of isoenzyme forms, was indicated in the combined disc gel–zymogram studies. This follows from the fact that the predominating proteins, found nearer to the anode, appeared to be devoid of enzyme activity. However, several of these segments (Fig. 8) paralleled zones of protein in the partly purified enzyme which did exhibit isoenzyme activity. Whether these neuraminidase-negative proteins in crystalline preparations represent inactivated forms of the enzyme, or are adventitious proteins with chromatographic and salt solubility properties which resemble those of the neuraminidases, has not yet been ascertained. In this regard, cognizance should be taken of the data assembled by JACOBY[21], in which it was shown that a number of enzymes can be brought to crystallization at widely varying stages of purification.

The results delineated in this work have demonstrated a multiplicity of at least 5 neuraminidase isoenzymes on the basis of polyacrylamide disc gel electrophoresis; while some 8 or possibly more multiple forms of this enzyme were manifested by serial chromatography on either DEAE- or CM-cellulose, followed by a CM-Sephadex separation. These findings parallel the classic example described for 10 different forms of the proteolytic enzyme cathepsin D, which were observed upon gradient elution chromatography on DEAE-cellulose followed by rechromatography on CM-columns[22]. It can be assumed that the mol. wts. of these multiple forms of pneumococcal neuraminidase are approx. 35 000 or above, by virtue of the fact that they have all been retained by the Diaflo XM-50 filter[23,24]. In general, it was observed that deletion of the heat treatment and DEAE-cellulose batch absorption–desorption stages usually gave the foregoing distribution of neuraminidase isoenzymes; whereas their inclusion in the fractionation scheme frequently reduced the number of active peaks detected on serial CM-cellulose and CM-Sephadex chromatography. These observations may be related to the recent work of BOEKER AND SNELL[25], in which it was found that heat and exposure to monovalent cations promoted dissociation of arginine decarboxylase from *Escherichia coli* to less active or inactive forms. In this light, the above shift in the number of active isoenzymes of the neuraminidase system will require further study.

Several covalent as well as non-covalent differences[26,27] among these isoenzymes may account for the multiplicity which has been observed. First, resolution into broad isoenzyme groups by means of both cation and anion exchangers suggests in terms of net charge, that 2–3 charge populations of pneumococcal neuraminidase exist. Second, further resolution of the CM-cellulose Fractions I and II enzyme peaks into additional multiple forms by chromatography on CM-Sephadex implies, as mentioned above, that varying degrees of aggregation or disaggregation of enzyme subgroups may occur. Finally, the fact that CM-cellulose Fraction I under the foregoing procedure gave 5 isoenzymes each with relatively high specific activity (Fig. 5), whereas CM-cellulose Fraction II yielded a somewhat different set with much lower specific activities (Fig. 6), is indicative of possible active site heterogeneity insofar as metal or coenzyme binding is concerned. In preliminary experiments, low specific activity

pneumococcal neuraminidase isoenzymes did not appear to be stimulated by an artificial trace mixture of cations, or by yeast extract. However, a search for catalytic effects of inorganic or organic ligands has not yet been fully carried out.

The facile chromatographic resolution of neuraminidase isoenzymes from pneumococcal growth filtrates offers a convenient source for further study of the conformational, sequential or aggregative parameters involved in this multiplicity of activity. It is anticipated that these facets will be further investigated by biochemical, immunochemical and physical chemical means.

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