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Stimulation of the Production of Neuraminidase in *Vibrio cholerae* Cultures by *N*-Acetylmannosamine

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SUMMARY: Addition of *N*-acetylmannosamine to a basal culture medium inoculated with *Vibrio cholerae* strain 4Z stimulated the production of neuraminidase. *N*-Acetylglucosamine and *N*-acetylgalactosamine under similar circumstances were inactive. *N*-Acetylmannosamine, added to medium inoculated with *V. cholerae*, persisted for at least 12 hr; under similar conditions *N*-acetylglucosamine was rapidly metabolized. Both D-mannosamine HCl and D-glucosamine HCl resisted enzymic attack by *V. cholerae*. The former compound severely inhibited growth of the organisms; the latter had no such effect. These findings are discussed, particularly in their relationship to the discovery that *N*-acetylneuraminic acid, which is a condensation product between *N*-acetylmannosamine and pyruvate, also induces neuraminidase.

In the preceding paper (French & Ada, 1959) it was shown that addition of sialyl-*N*-acetyl-D-galactosamine, sialyl lactose or *N*-acetylneuraminic acid (*N*-ANA) to a simple medium stimulated the production of neuraminidase by *Vibrio cholerae* without affecting the rate of growth of the organism. With the two latter compounds at least, the sialic acid portion was metabolized by the bacteria. Free *N*-ANA was broken down rapidly, and during this period the production of a small amount of a compound, possibly an *N*-acetyl hexosamine, was detected in the medium. Gottschalk's proposed structure of *N*-ANA as an aldol condensation product of an *N*-acetylated hexosamine with pyruvic acid (Gottschalk, 1956) is now generally accepted. Based on this interpretation, Cornforth, Firth & Gottschalk (1958) were successful in synthesizing from *N*-acetylglucosamine and oxaloacetic acid a compound which, on physical and chemical grounds, was identical with *N*-ANA isolated from bovine submaxillary gland mucin. Comb & Roseman (1958) have since shown that an aldolase from *Clostridium perfringens* breaks down *N*-ANA, isolated from human plasma, to pyruvate and an *N*-acetylated hexosamine identified as *N*-acetylmannosamine. Furthermore, incubation of *N*-acetylmannosamine and pyruvate with this enzyme yielded a substance which gave the direct Ehrlich reaction and which behaved like *N*-ANA when chromatographed on paper. No such substance appeared to be formed when pyruvate and either *N*-acetylgalactosamine or *N*-acetylglucosamine were incubated with the enzyme.

Earlier work (Ada & French, 1957) had indicated that addition of equimolecular amounts of *N*-acetylglucosamine and pyruvate to a peptone medium

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did not result in an increase in the concentration of neuraminidase over that produced in the control peptone medium. As quite appreciable amounts of neuraminidase were produced by *Vibrio cholerae* grown on the control peptone medium, this might possibly have masked the increased production of small amounts of enzyme by the added compounds. It seemed therefore appropriate to repeat this work with a basal medium which gave rise to only low titres of neuraminidase, but supported adequate growth of the organism. Furthermore, the results of Comb & Roseman (1958) made it desirable to investigate also the effect of added *N*-acetylmannosamine and pyruvate. The present paper reports a study of the activities of the *N*-acetyl derivatives of D-glucosamine, D-mannosamine and D-galactosamine and of D-mannosamine HCl and D-glucosamine HCl. *N*-Acetylmannosamine was found to stimulate neuraminidase production—the other compounds being inactive. The mode of action of *N*-acetylmannosamine in this respect, however, remains obscure.

METHODS

Details of the basal medium used, the methods of growing *Vibrio cholerae*, counting viable organisms, estimating neuraminidase and *N*-ANA were described in the previous paper (French & Ada, 1959).

Chromatography. The solvents used were described previously (French & Ada, 1959). *N*-Acetylhexosamines were detected on paper using the spray described by Partridge (1948).

N-Acetylhexosamines. Crystalline *N*-acetylglucosamine was prepared from D-glucosamine HCl by the method of Breuer (1898). The product moved as a single component ($R_F = 0.45$) when chromatographed with butanol + pyridine + water (6:4:3) as solvent. Crystalline *N*-acetylgalactosamine was a gift from Dr A. Gottschalk. Crystalline and amorphous *N*-acetylmannosamine preparations were generously supplied by Dr S. Roseman. The crystalline material ran as a single component ($R_F = 0.50$) when chromatographed in butanol + pyridine + water (6:4:3). *N*-Acetylhexosamines were estimated by the Morgan & Elson method (1934) as modified by Reissig, Strominger & Leloir (1955).

Hexosamine. D-Glucosamine-HCl (British Drug Houses Ltd.) was used. Crystalline D-mannosamine-HCl was kindly supplied by Dr Roseman. Total hexosamine was determined after acid hydrolysis of the sample (2N-HCl, 100°, 6 hr.).

Influenza viruses. The following virus strains were used: Mel—Melbourne strain of influenza A isolated in 1935; WSE—classical strain WS of influenza A virus adapted to growth on the chorioallantoic membrane of chick embryos; PR8—classical strain of influenza virus; CAM—influenza A prime strain isolated in Melbourne in 1946; Swine—Shope's strain 15 of swine influenza virus; Lee—classical influenza B strain; Mil B—influenza B strain isolated in Melbourne in 1945.

The viruses were inoculated into the allantoic cavity of 11-day fertile hen eggs and after incubation for 2 days at 35° the allantoic fluid virus was

harvested and either mixed with equal parts of glycerol and stored at -20° before use or used within 24 hr. without glycerol and storage.

Calcium acetate saline and citrated saline; see French & Ada (1959).

Determination of neuraminidase receptor gradient. This was determined by incubating for 45 min. 0.1 ml. of washed packed red cells with dilutions of the enzyme under test in 4 ml. calcium acetate saline. After incubation, 0.4 ml. of 20% sodium citrate solution was added to each tube. The red cells were centrifuged down, washed once with citrated saline and finally resuspended in 10 ml. citrated saline to give a 1% (v/v) red cell suspension. To one volume of each suspension of treated cells, five agglutinating doses of the different viruses in one volume of normal saline were added and the red cells allowed to settle at room temperature.

Experimental procedure. The experiments to be reported were carried out as described previously (French & Ada, 1959). Media were inoculated with organisms subcultured in broth and incubated at 37° . In one type of experiment samples for enzyme estimation, etc., were taken after 16 hr. incubation; in the other experiments samples were taken from media at various times up to 12 hr. after inoculation.

RESULTS

Effect of added pyruvate and N-acetylhexosamine on neuraminidase production

Equimolecular amounts of sodium pyruvate and an *N*-acetylhexosamine, (either *N*-acetylglucosamine, *N*-acetylgalactosamine or *N*-acetylmannosamine), were added to portions of the medium to give final concentrations ranging from 2400 to 80 μM . The effect on neuraminidase production is shown in Table 1.

Table 1. *Effect of added N-acetylhexosamine and pyruvate on the amount of neuraminidase produced by Vibrio cholerae*

Enzyme titres in media inoculated with *Vibrio cholerae* were determined after 16 hr. incubation at 37° .

Concentration of <i>N</i> -Acetyl- hexosamine and pyruvate (μM)	Media containing		
	<i>N</i> -acetylglu- cosamine + pyruvate	<i>N</i> -Acetylgalac- tosamine + pyruvate	<i>N</i> -acetyl mannosamine + pyruvate
	Enzyme titre		
2400	10	15	320
800	20	25	200
240	15	10	180
80	15	15	80
Nil	40	10	< 10

Stimulation of neuraminidase production by N-acetylmannosamine without added pyruvate

The presence of *N*-acetylmannosamine alone (no added pyruvate) in the basal medium was found to stimulate neuraminidase production. Details of such an experiment are given in Table 2. In an experiment similar to that

described previously (French & Ada, 1959), it was shown that addition of *N*-acetylmannosamine to the medium did not affect the growth of the organism (as estimated by counts of viable organisms).

Table 2. *Effect of adding N-acetylmannosamine, with and without an equimolecular amount of pyruvate, to the medium on the stimulation of neuraminidase production by Vibrio cholerae*

Enzyme titres in media inoculated with *Vibrio cholerae* were determined after 16 hr. incubation at 37°.

N-Acetyl- mannosamine concentration (μM)	Enzyme titre	
	With added pyruvate	No added pyruvate
2400	320	220
800	200	280
240	180	80
80	80	40
Nil	<10	<10

Effect of added pyruvate on the rate of neuraminidase production in medium containing N-acetylmannosamine

Addition of pyruvate (final concentration 600 or 3,000 μM) did not significantly affect the rate of production of neuraminidase in medium containing *N*-acetyl-mannosamine (600 μM). At the higher concentration, added pyruvate caused a noticeable increase in the growth rate of the organisms. The details of an experiment are plotted in Fig. 1.

Fig. 1

Fig. 2

Fig. 1. Effect of the addition of excess pyruvate on the rate of neuraminidase production in medium containing *N*-acetylmannosamine. Basal medium alone = x—x; basal medium + *N*-acetylmannosamine (600 μM) = O—O; basal medium + *N*-acetylmannosamine (600 μM) + pyruvate (3,000 μM) = ●—●.

Fig. 2. Rate of production of neuraminidase and the rate of disappearance of *N*-acetylmannosamine or *N*-acetylglucosamine added to media. Enzyme production in the presence of *N*-acetylmannosamine = O—O; amount of *N*-acetylmannosamine present = ●—●. Enzyme production in the presence of *N*-acetylglucosamine = □—□; amount of *N*-acetylglucosamine present = ■—■. Enzyme production in basal medium = x—x.

Metabolism of added N-acetylmannosamine and N-acetylglucosamine during growth of the organisms

N-Acetylmannosamine, when present in inoculated medium at 600 μ M, persisted for at least 12 hr. incubation. On the other hand, *N*-acetylglucosamine in a similar concentration was rapidly metabolized. Details of an experiment are presented in Fig. 2. In a separate experiment, samples were removed at similar intervals, hydrolysed with acid and the total hexosamine content estimated. After correcting for the amount of hexosamine in the samples from the control medium, the following results were obtained. In the medium containing added *N*-acetylglucosamine, the value of the hexosamine reaction fell to the level of the control medium within 6 hr. incubation following inoculation. In the medium containing added *N*-acetylmannosamine, the value of the hexosamine reaction remained constant during 12 hr. incubation after inoculation.

Difference in the behaviour of N-ANA and N-acetylmannosamine as a neuraminidase inducer

The rates of production of neuraminidase by *Vibrio cholerae* in medium containing *N*-ANA (600 μ M) and in medium containing *N*-acetylmannosamine (600 μ M.) were found to differ. Details of an experiment which shows this difference in behaviour are plotted in Fig. 3.

Fig. 3. Rate of production of neuraminidase in the presence of *N*-acetylmannosamine or *N*-acetylneuraminic acid. Basal medium + *N*-acetylmannosamine = O—O; basal medium + *N*-acetylneuraminic acid = Δ—Δ.

Effect of added D-mannosamine HCl on neuraminidase production

Addition of D-mannosamine HCl (to 600 μ M) to the basal medium severely inhibited bacterial growth as noticed visually and by counts of viable organisms. The production of neuraminidase by organisms grown in basal medium containing added D-mannosamine HCl was likewise profoundly affected. In a typical experiment, two portions of basal medium, one containing D-mannosamine HCl (600 μ M), were inoculated to give a final count of 5.4×10^6

organisms/ml. In the control tube the enzyme titre at 11 hr. was 100 and the viable count 9.2×10^8 organisms/ml. In the tube containing D-mannosamine HCl, the enzyme titre at 12 hr. was 10 and the viable count 0.85×10^8 organisms/ml. Estimation of the hexosamine content (no prior hydrolysis) of samples removed at different time intervals after inoculation (up to 12 hr.) showed that the added D-mannosamine persisted in the culture medium.

Effect of added glucosamine HCl on neuraminidase production

Addition of D-glucosamine HCl (to 600 μ M) to the basal medium did not stimulate neuraminidase production. Estimation of the hexosamine content (no prior hydrolysis) of samples removed at different time intervals after inoculation (up to 12 hr.) indicated that the added D-glucosamine persisted in the culture medium.

Receptor gradient of neuraminidase produced in medium containing either N-ANA or N-acetylmannosamine

Burnet, McCrea & Stone (1946), and later Stone & Ada (1952), observed that when red cells, which are normally agglutinated by the strains of influenza virus used, were fractionally treated with neuraminidase, the agglutinability of these red cells by the viruses was progressively lost in an order which defines the so-called receptor gradient. It was found that treatment of red cells with neuraminidase, produced in our basal medium containing either N-ANA or N-acetylmannosamine or in soft nutrient agar, yielded red cells which exhibited the same receptor gradient. With fowl red cells the neuraminidase gradient obtained was: PR8, WSE, Mel and CAM, Swine, Lee, finally Mil. B. With human red cells the order was: WSE, PR8, Mel, CAM, Swine and Lee, Mil B.

DISCUSSION

N-acetylmannosamine induced the production of neuraminidase in *Vibrio cholerae* cultures, the amount of enzyme produced being approximately proportional to the concentration of inducer added. Addition of pyruvate to a medium containing N-acetylmannosamine did not appreciably affect the rate of production of the enzyme. This effect of N-acetylmannosamine was specific; two isomers—N-acetylglucosamine and N-acetylgalactosamine—were inactive. This latter finding confirms an earlier observation (Ada & French, 1957) that addition of N-acetylglucosamine and pyruvate to a peptone medium did not stimulate neuraminidase production by *V. cholerae*. The further observation was made that whereas N-acetylglucosamine added to the basal medium was readily metabolized by the organisms, the evidence obtained suggests that N-acetylmannosamine was not broken down. Thus the concentration of N-acetylhexosamine (or of total hexosamine after acid hydrolysis of the samples) in medium to which N-acetylmannosamine had been added remained the same during a period of 12 hr. incubation at 37° following inoculation. The present evidence suggests therefore that N-acetylmannosamine is a gratuitous inducer.

What then is the mechanism of neuraminidase induction by *N*-acetylmannosamine? It is helpful to point out the relationship of *N*-acetylmannosamine to the structure of a substrate of neuraminidase. This is shown in the accompanying diagram (Fig. 4) where *N*-ANA is shown linked to galactose as in sialyl lactose (see Gottschalk, 1957). Carbon atom 2 of the *N*-acetylhexosamine portion corresponds to C₅ of *N*-ANA in the diagram and is thus 4 atoms distant from the enzyme susceptible bond. What may be of great importance is the spatial disposition of the *N*-acetyl group to C₂ of *N*-ANA. Comb & Roseman (1958) showed that the partner with pyruvate in *N*-ANA is *N*-acetylmannosamine, not *N*-acetylglucosamine as previously thought. Our first reaction to the discovery that *N*-acetylmannosamine stimulated neuraminidase production was to consider the possibility that the *N*-acetylmannosamine was converted by the organism to *N*-ANA which was known to induce neuraminidase (French & Ada, 1959). The lack of effect of pyruvate when added

Fig. 4. Diagram showing the structure of the *N*-acetylneuraminic acid (*N*-ANA) portion of the sialyl lactose molecule. The carbon atoms of the *N*-ANA portion are numbered from left to right, no. 1 being the carbon atom of the COOH group. The carbon skeleton of the hexosamine portion of the *N*-ANA molecule is outlined in heavier print. The arrow indicates the bond split by neuraminidase.

to the system and the apparent resistance to enzymic attack of the added *N*-acetylmannosamine argued against this possibility. Conversely, the possibility was entertained that *N*-ANA might act as inducer by virtue of its degradation, by de-aldolization, into *N*-acetylmannosamine. At present two observations seem to argue against a quantitative conversion of *N*-ANA to *N*-acetylmannosamine. First, the breakdown of *N*-ANA in *Vibrio cholerae* cultures does not result in the accumulation of large amounts of an *N*-acetylhexosamine (French & Ada, 1959). Secondly, the finding that neuraminidase production stops as soon as *N*-ANA disappears from the medium would not be expected if *N*-acetylmannosamine were the sole degradation product, as can be seen from Fig. 3. In view of the findings of Comb & Roseman (1958) the above results were unexpected; clearly, more work needs to be done with our system. If the production of neuraminidase in medium containing added *N*-ANA ceases as soon as the intact *N*-ANA molecule disappears, this suggests a difference in the mode of action as inducer of *N*-ANA and *N*-acetylmannosamine. If, on the other hand, a degradation product of *N*-ANA is active as inducer, it is necessary to find the relationship between such a compound and *N*-acetylmannosamine. A further point which arises concerns the specificity of the enzyme produced on medium containing added *N*-ANA or on medium containing added *N*-acetylmannosamine. This aspect has not been fully investigated, but it has been shown that the two enzyme preparations acted

on red cells in a similar fashion as evidenced by their identical receptor gradients.

Finally, the response of *Vibrio cholerae* to medium containing D-mannosamine or D-glucosamine is of interest. Both compounds appeared to resist enzymic attack by the organisms; D-mannosamine, but not D-glucosamine, severely interfered with the growth of the bacteria. No explanation is offered for these findings, but it is not unlikely that when better understood, the mode of action of N-acetylmannosamine as enzyme inducer may throw some light upon the mechanism of growth inhibition by D-mannosamine. It is quite clear that the 4Z strain of *V. cholerae* will be a useful tool in demonstrating some of the biochemical relationships of the amino sugars and their derivatives.

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