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THE INHIBITION OF NEURAMINIDASE AND ANTIVIRAL ACTION

BY

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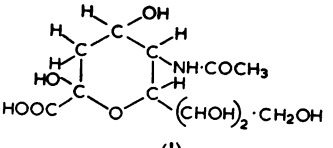
The number of compounds showing *in vitro* antiviral action increases daily. The mode of action of these compounds is mainly still uncertain. We have attempted to find an antiviral agent active because of its ability to inhibit a known component of a particular virus. Thus the enzyme neuraminidase appears to have an important role to play in the activity of the influenza virus; we have therefore tried to discover neuraminidase inhibitors, which, by virtue of this property, would be anti-(influenza) viral agents.

The neuraminidases are associated with the viruses of the myxo-group and with some bacteria. These enzymes have been excellently reviewed by Rafelson (1963); from this review it appeared that the most active inhibitor known was *N*-acetylneuraminic acid (I). Rafelson, in the same review, stated that of 85 compounds examined only glutathione, cysteine, ascorbic acid, thioglycollate and sodium cyanide inhibited the enzyme, but only at a concentration of 10^{-2}M. Our first approach, therefore, was to try and synthesize compounds structurally related to *N*-acetylneuraminic acid itself. However, chemical difficulties which were encountered made this approach appear unattractive. Accordingly we looked for other classes of compounds which might be inhibitors of the enzyme.



(I)

Among the most promising antiviral agents mentioned in the literature are the glyoxals (against influenza virus) and certain derivatives of isatin β-thiosemicarbazone (against vaccinia virus). The glyoxals are postulated as acting on the virus nucleic acid (de Bock, Brug & Walop, 1957), although at high concentrations there may be some effect on the neuraminidase system. The isatin derivatives are thought to chelate with a trace metal or to form intermolecular hydrogen bonds with some key molecule involved in the

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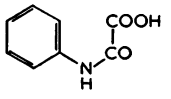
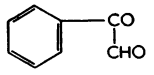
inclusion of deoxyribonucleic acid or perinuclear ribonucleic acid into the incomplete virus particle (O'Sullivan & Sadler, 1961).

We have carried out experiments on some of the known anti-influenza virus glyoxals and have found that at levels of 1:10,000 many showed marked neuraminidase inhibition; on the other hand the isatin derivatives investigated have shown no activity even at a concentration of 1:1,000.

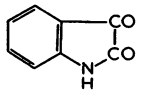
Structurally *N*-phenyloxamic acid (II) has some similarities to both phenylglyoxal (III) and isatin (IV).



(II)



(III)



(IV)

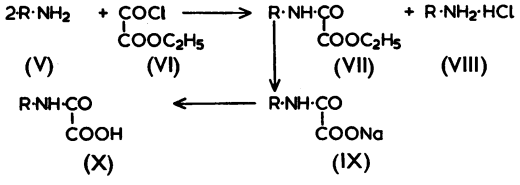
We have found that *N*-phenyloxamic acid, and subsequently many other *N*-substituted oxamic acids, inhibit the enzyme neuraminidase to a considerable extent at concentrations of 1:10,000.

This paper gives the results of this work along with the antiviral activity of those *N*-substituted oxamic acids which show the greatest inhibition of neuraminidase.

METHODS

Chemical. Starting materials for the syntheses and reagents for the estimations were obtained from commercial sources.

The N-*substituted Oxamic Acids.* These compounds were all prepared by a similar route (see annotated scheme below), *N*-phenyloxamic acid (1) being typical of the method.



N-*Phenyloxamic Acid* (*X*; *R*=*Phenyl*). Ethoxalyl chloride (VI: 6.82 g, 0.05 M) in dry chloroform (50 ml.) was added dropwise at 10 to 20° C with stirring to a solution of aniline (V; R=Phenyl; 9.3 g, 0.1 M) in dry chloroform (50 ml.). After the addition was complete the reaction mixture was stirred for a further hour at 10° C and then extracted with water (4×50 ml.) to remove aniline hydrochloride (VIII; R=Phenyl). The chloroform solution was dried over anhydrous sodium sulphate and the solvent then removed by distillation to yield ethyl *N*-phenyloxamate (VII; R=Phenyl) as pale yellow crystals. These crystals, without further purification, were dissolved in ethanol and a solution of sodium ethoxide in ethanol [sodium (0.5 g) in ethanol (25 ml.)] added; the sodium salt of *N*-phenyloxamic acid was precipitated almost immediately (IX; R=Phenyl). The salt was collected by filtration and dissolved in the minimum of hot water. On acidification of the resulting aqueous solution with dilute hydrochloric acid and chilling, white crystals of *N*-phenyloxamic acid were deposited. These crystals were recrystallized from benzene as shiny white plates, m.p. 148 to 150° C in 80% yield.

N-(*2,6-Dimethylphenyl*)-N^1(*3-diethylaminopropyl*)-*oxamide* (18) was prepared in 77% yield by reaction of ethyl 2,6-dimethyloxanilate (VII; R=2,6-dimethylphenyl; 2.21 g; 0.01 M), in 20 ml.

95% aqueous alcohol with *N,N*-diethylaminopropylamine (1.30 g ; 0.01 M). After thorough mixing, the reactants were kept in a stoppered flask for 48 hr at room temperature, after which the solvent was removed by distillation under reduced pressure to yield the crude base which had m.p. 74 to 75° C when recrystallized from 60/80° petroleum ether. Hydrochloride m.p. 198 to 200° C (ethanol).

N-*Formyl-2,6-xylidine* (16) was prepared according to the method of Johnston & Kidd (1964a).

Thio-oxanilic acid (17) was prepared according to the method of Reissert (1904).

4,4¹-Diglyoxalyldiphenyl ether monohydrate (20) was prepared according to the method of Cavallini (1964), whilst *dibenzofuran-2,8-bisglyoxal monohydrate* (21) and *dibenzofuran-2-glyoxal monohydrate* (22) were prepared according to the method of Anderson, Casey, Emas, Force, Jensen, Matz & Rivard (1963). p-*Nitrophenylglyoxal monohydrate* (23) and m-*nitrophenylglyoxal monohydrate* (24) were prepared by the method of Steinbach & Becker (1954).

N-*Ethylisatin β-thiosemicarbazone* (26) was prepared according to the method of Bauer & Sadler (1960), whilst *N-allylisatin* (27) and its *β-hydrazone* (28) were prepared by the same route as Johnston & Kidd (1964b).

N-*Allylisatin β-thiosemicarbazone* (25) was prepared in 65% yield by refluxing *N*-allylisatin (1.87 g, 0.01 M) with thiosemicarbazide (0.91 g, 0.01 M) in 40 ml. 50% aqueous alcohol for 40 min. On cooling, the crude product was collected by filtration and recrystallized from alcohol; melting point 185° C (found C, 55.4 ; H, 4.7 ; N, 21.5 ; S, 12.3%. $C_{12}H_{12}N_4$ OS requires C, 55.7 ; H, 4.6 ; N, 21.6 ; S, 12.3%).

Table 1 contains a list of new *N*-substituted oxamic acids which were prepared by the same route as for *N*-phenyloxamic acid (oxanilic acid), along with a few closely related analogues.

BIOLOGICAL

Neuraminidase Inhibition. Neuraminidase activity was determined using erythrocyte stroma, prepared by the method of Herbert (1956), as substrate. As enzyme, a suspension of formaldehyde treated influenza virus (strain A2/Eng./1/61) of concentration 25,000 haemagglutinating units/ml. was used. (This was kindly supplied by Glaxo Laboratories Ltd.)

Erythrocyte stroma (net weight 53 g), from group O Rh +ve human blood, was diluted to 100 ml. with potassium dihydrogen phosphate–sodium hydroxide buffer of *p*H 6.5 ; 7 ml. this solution, 0.37 ml. virus suspension and 4.3 ml. water were incubated at 37° C and 2 ml. samples were withdrawn at 0, 5, 10, 15 and 30 min. These samples were added to 2 ml. 5% phosphotungstic acid, allowed to stand for 10 min and centrifuged to remove protein (3,000 *g* for 15 min). To 0.5 ml. supernatant was added 0.1 ml. periodate solution (0.2 M in 9 M phosphoric acid) and the procedure thenceforth was exactly that of Warren (1959).

When inhibitors were added, these were dissolved in the water to give the final concentration specified. In most cases warming was necessary to dissolve the compound, the solution being cooled to 37° C before adding to the substrate and virus suspension.

The estimates of enzyme concentration were then taken as the initial velocities (tangents to the progress curves at zero time) obtained from the plot of extinction against time. The percentage inhibition achieved with a compound was taken as $(1=\frac{Vi}{V})$ where Vi = initial velocity in the presence of inhibitor and V = initial velocity without inhibitor with same substrate concentration, etc., and the experiments carried out side by side.

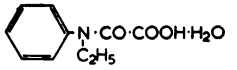
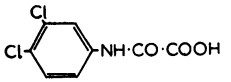
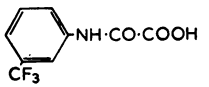
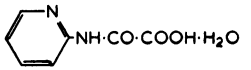
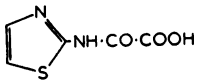
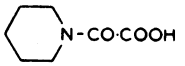
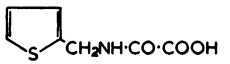
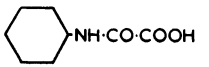
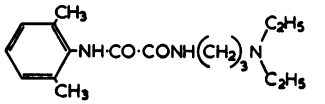
Antiviral Assessment. This was determined using the egg membrane method of Tamm, Bablanian, Nemes, Shunk, Robinson & Folkers (1961) and also, using the whole embryonated egg, by the method of Weinstein, Chang & Hudson (1957). In each case PR 8 influenza virus was used.

The activity factor was taken as the reciprocal of the haemagglutination titre, generally 32, of the control virus propagation divided by the reciprocal of the haemagglutination titre of the virus propagation in presence of the compound. The haemagglutination test was run for 1 hr in each instance, using human group O red blood cells and the temperature of incubation was 4° C.

TABLE 1

N-SUBSTITUTED OXAMIC ACIDS

Numbers refer to Table 2, where the systematic chemical names are given

No.	Compound	M.p. (°C)	Recrystallization solvent	% Yield	Analyses							
					Calculated				Found			
					C	H	N	Other	C	H	N	Other
4		71–72	Water	25	56·9	6·2	6·6	—	56·5	6·2	6·3	—
8		240–242	Acetic acid/ether (1 : 1)	25	41·0	2·2	6·0	(Cl) 30·3	40·9	2·1	6·1	(Cl) 29·1
9		167–168	Water	27	46·3	2·6	6·0	(F) 24·5	46·8	2·5	6·0	(F) 23·3
11		231–232	Water	10	45·7	4·3	15·2	—	45·9	4·2	15·0	—
12		230–231	Water	10	34·9	2·3	16·3	(S) 18·6	34·4	2·4	16·6	(S) 18·5
13		128–130	Water	5	53·3	7·0	8·9	—	53·1	6·9	8·5	—
14		120–122	80/100° Pet. ether	32	45·4	3·8	7·6	(S) 17·3	45·4	3·8	7·6	(S) 17·3
15		165–166	Water	40	56·1	7·6	8·2	—	56·2	7·8	8·1	—
18		74–75	60/80° Pet. ether	77	66·9	8·9	13·7	—	66·8	8·9	13·8	—
19		112–114 160–162*	Water	5	44·3	4·9	11·5	—	44·8	5·0	12·0	—

*After resolidifying.

TABLE 2
INHIBITION OF NEURAMINIDASE

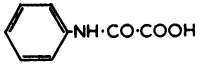
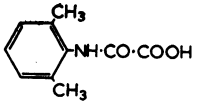
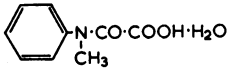
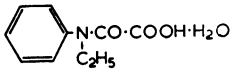
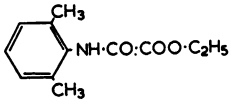
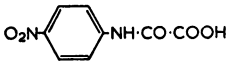
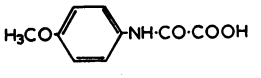
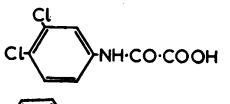
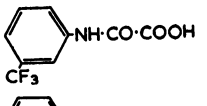
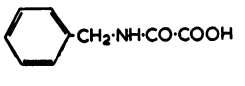
No.	Name	Formula		% Neuraminidase inhibition by different concentrations			
				1 : 1,000	1 : 10,000	1 : 25,000	1 : 100,000
1	*N*-phenyloxamic acid		(1)	s.i.	85	34	0
2	*N*-(2,6-dimethylphenyl)oxamic acid		(2)	—	54	—	—
3	*N*-methyl-*N*-phenyloxamic acid monohydrate		(3)	s.i.	30	—	—
4	*N*-ethyl-*N*-phenyloxamic acid monohydrate			—	10	—	—
5	ethyl *N*-(2,6-dimethylphenyl)-oxamate		(2)	0	—	—	—
6	*N*-(*p*-nitrophenyl)oxamic acid		(4)	—	45	—	—
7	*N*-(*p*-acetophenyl)oxamic acid		(5)	s.i.	30	—	—
8	*N*-(3,4-dichlorophenyl)oxamic acid			s.i.	20	—	—
9	*N*-(*m*-trifluoromethylphenyl)-oxamic acid			s.i.	83	0	0
10	*N*-benzyloxamic acid		(6)	—	0	—	—

TABLE 2—*Continued*

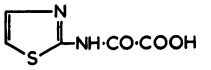
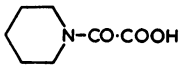
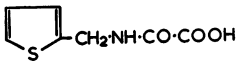
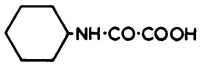
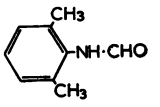
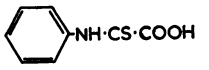
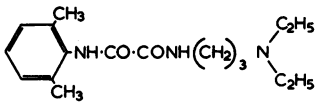
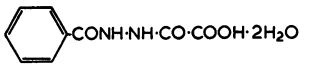
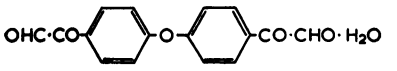
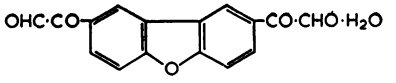
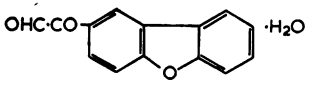
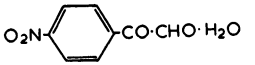
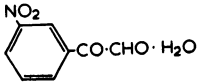
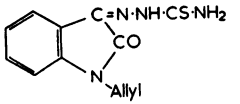
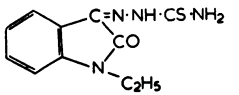
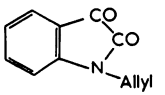
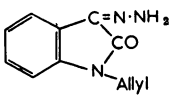
No.	Name	Formula	% Neuraminidase inhibition by different concentrations			
			1 : 1,000	1 : 10,000	1 : 25,000	1 : 100,000
11	*N*-(2-pyridyl)oxamic acid monohydrate		s.i.	s.i.	35	0
12	*N*-(2-thiazolyl)oxamic acid		s.i.	65	35	0
13	piperidinoglyoxalic acid		s.i.	70	0	—
14	*N*-(2-thienylmethyl)oxamic acid		s.i.	24	0	—
15	*N*-cyclohexyloxamic acid		s.i.	38	0	—
16	*N*-formyl-2,6-xylidine		0	—	—	—
17	thio-oxanilic acid		s.i.	68	32	—
18	*N*-(2,6-dimethylphenyl)-*N'*-(3-diethylaminopropyl)oxamide		0	—	—	—
19	*N*-benzamido-oxamic acid dihydrate		s.i.	75	0	—
20	4,4'-diglyoxalyldiphenyl ether monohydrate		—	—	—	33*

TABLE 2—*Continued*

No.	Name	Formula	% Nuraminidase inhibition by different concentrations			
			1:1,000	1:10,000	1:25,000	1:100,000
21	dibenzofuran-2,8-bisglyoxal monohydrate		—	50	—	—
22	dibenzofuran-2-glyoxal monohydrate		—	0	—	—
23	*p*-nitrophenylglyoxal monohydrate		46	0	—	—
24	*m*-nitrophenylglyoxal monohydrate		73	15	—	—
25	*N*-allylisatin-β-thiosemicarbazone		0	—	—	—
26	*N*-ethylisatin-β-thiosemicarbazone		0	—	—	—
27	*N*-allylisatin		0	—	—	—
28	*N*-allylisatin-β-hydrazone		0	—	—	—

* Maximum solubility possible.
s.i. = The compounds interfere with the stroma at these concentrations, causing coagulation and precipitation.
0 = No inhibition.
— = Not determined at that concentration.

Literature references to the preparation of compounds not given in the text are: (1) Aschan (1890). (2) Johnston & Kidd (1964a). (3) Usherwood & Whitely (1923). (4) Aschan (1885). (5) Castellaneta (1895). (6) Curtius & Raschig (1930).

RESULTS

A2 viruses have high neuraminidase activity (see for example Howe, Lee & Rose, 1960) and preliminary work indicated that the GLAXO virus suspension, although formalin treated and hence non-infective, had high haemagglutinating activity and high neuraminidase activity. This agrees with the observations of Chu (1948) who found, using influenza type B virus, that formalin can be used to make the virus non-effective without appreciably impairing its haemagglutinating or enzymic activities. *Some* enzymic activity may be lost on treatment with formalin but this in no way invalidates our results.

Most of the compounds tested caused, at the highest concentrations, visual changes in the stroma solution. When this was noticed the estimations were not completed ; this is indicated in the table by s.i. The results of the neuraminidase inhibition assay are given in Table 2.

The isatin derivatives, as expected, are inactive in this test. The oxamic acids show activity to varying degrees, as do the glyoxals (see Table 2 and Discussion).

The results of the anti-viral assessment on selected compounds are given in Table 3. In the whole embryonated egg virtually all activity is lost.

TABLE 3
ANTI-VIRAL ACTIVITY

No.	Concentration	Activity factor	
		Whole egg	Egg membrane
1	1 : 1,000	1	1
2	1 : 1,000	1	—
9	1 : 1,000	2	8
12	1 : 1,000	1	2
13	1 : 10,000	1	16
17	1 : 10,000	1	1
19	1 : 1,000	2	4

DISCUSSION

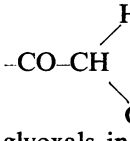
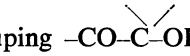
The role of the neuraminic acids and neuraminidase in relation to the influenza virus can be postulated as follows. The influenza virus attaches itself to the host cell membrane, this allows invasion of the cell (injection of nucleic acid into the " mechanism " of the cell and synthesis by it of further virus particles) but if agglutination was still continued no new virus could emerge. The neuraminidase in the virus allows removal of neuraminic acid from the receptors and release of the influenza virus.

Gottschalk (1958) has also this to say about the occurrence of the enzyme neuraminidase. " This is present in influenza virus, *Vibrio cholerae,* mumps virus, Newcastle disease virus, *Clostridium welchii* and *pneumococcus* type II and there is circumstantial evidence for its presence in the diphtheroid *bacillus.* All these organisms when infecting their hosts inhabit the respiratory or intestinal tracts, the lining cells of which are covered with haemagglutinin inhibitory mucins susceptible to neuraminidase action. It may be tempting to look on the enzyme as a powerful weapon of these parasitic organisms to be used when threatened with solitary confinement in a coating of host mucin."

The inhibition of this enzyme could therefore be a means of restricting the multiplication of the virus and aiding its elimination.

We chose a method for estimating neuraminidase which was relatively simple and used materials closely corresponding to those in the infective stage of the disease—influenza virus particles and the stroma of erythrocytes which in its mucopolysaccharide character appears to be the same or very similar to that on the surface of the epithelial cells of the respiratory tract. In the method the virus enzyme splits off a sialic acid (*N*-acyl neuraminic acid) which is estimated by the thiobarbituric acid method of Warren (1959). [We found, as did Mayron, Robert, Winzler & Rafelson (1961), that it was unnecessary to add calcium for maximum enzymic activity.]

With some compounds (9, 13, 19) it can be seen that there is a very marked loss of inhibitory power in going from one concentration to another. This could possibly suggest that these compounds are competitive inhibitors but might also suggest that the effective concentration of the inhibitor is being reduced by the presence of a trace component in the system which reacts with the inhibitor. Thus (at the lower concentration) the effective level of compound might be virtually zero.

All the glyoxals active in the test are hydrates and presumably exist in the form  . An obvious similarity then exists between the oxamic acids and the glyoxals in that both contain the grouping  . *N*-acetylneuraminic acid also possesses this grouping. The oxamic acid derivative does, however, appear to be more potent than the corresponding glyoxal (compare 6 and 23). The nitrogen atom therefore seems important.

If (using Dreiding Stereomodels) one makes a model of the *N*-acetylneuraminic acid molecule, in the conformation suggested by Gantt, Millner & Binkley (1964) in which all the more bulky groups are in the equatorial position, the distance between the nitrogen atom and the acidic hydrogen of the carboxyl group is found to be (in one possible position) approximately 4 Å. In oxanilic acid (measuring from models) the distance is again approximately 4 Å between the nitrogen atom and the carboxylic hydrogen (in one possible position). In this position the carbonyl oxygen is 2.3 Å from the nitrogen (at an angle) making a triangle of sides 4, 2.8 and 2.3 Å. We thus have a possible three-point attachment for the substrate or inhibitor (assuming competitive inhibition) to the receptor on the enzyme surface. With *N*-acetylneuraminic acid the third point of attachment could be the hydroxyl at C_4 (possibly the oxygen itself). This would give a triangle of sides 4, 3.6 and 2.9 Å.

Thus the corresponding points of the attachment in the two molecules would be the carboxyl groups, perhaps by formation of a hydrogen bond to a δ −ve site and the amide nitrogens, possibly by virtue of the lone pair of electrons attaching to a δ +ve centre. The third point of attachment is either the oxygen atoms of the C_4 hydroxyl (of *N*-acetylneuraminic acid) and of the α-carbonyl (of oxanilic acid) to a δ +ve site or the hydrogen of the hydroxyl and the hydrogen of a protonated carbonyl group (in this case by hydrogen bonding to a δ −ve site).

With the glyoxals the corresponding points of attachment are the acidic hydroxyl of the hydrated aldehyde group, the α-carbonyl and the high electron density of the aromatic ring which would allow some complexing with a δ +ve site on the receptor.

This theory is substantially proved by our results. Taking the points of attachment in order we find (*a*) the free carboxyl would appear essential for activity since an ester (5), an amide (18) and the *N*-formyl derivative (16) formed by decarboxylation of (2) are all inactive; (*b*) any group on the nitrogen liable sterically to interfere with a "fit" on a receptor site, e.g., the *N*-methyl compound (3) and the *N*-ethyl compound (4), are less active than the unsubstituted compound (1). The *N*-(2,6-dimethylphenyl)-oxamic acid (2) probably owes its lower activity to steric hindrance from the *ortho*-methyl groups. Since we based our series initially on the glyoxal structure and later on the oxamic acid molecule all our related compounds have the α-carbonyl group [except (17)]. This grouping is common to both glyoxals and oxamic acids and it seems reasonable to expect it to be one point of attachment to a receptor site. In (17) the α-carbonyl is replaced by the $-\underset{|}{C}=S$ group and as would be expected (since the two groups are very similar in character) activity is still retained.

As might be expected, in view of their planar and aromatic-like structure, the *N*-(2-pyridyl)-(11) and *N*-(2-thiazolyl)-(12) radicals are able to replace the *N*-phenyl radical without much change in activity. Para substitution in the phenyl ring (either by an electron donating or electron withdrawing group) reduces activity (6, 7 and 8). The presence of a $-CF_3$ group meta to the $-NH-$ (9) makes virtually no difference to the activity [comparing 1:10,000 concentration figures for (1) and (9)]. We feel that these differences are due to the steric factors and probably show that the phenyl group, when para substituted, is hindered, perhaps by some other portion of the virus molecular structure, from attaining a close "fit" at the receptor sites.

The importance of the stereochemistry adjacent to the nitrogen atom is also shown by 10, 14 and 15. In (10) and (14) the interposition of a $-CH_2$ group lowers activity considerably; this presumably is due to the extension of the chain and/or the free rotation of the benzyl grouping giving considerable interference to attachment at the receptor. In (15) the puckered cyclohexane ring takes up more space and this presumably is enough to lessen the binding to the receptor and hence lower its activity as an inhibitor.

With (13) attachment to the receptor by the nitrogen atom will allow little movement of the piperidine ring and hence a good "fit" and good activity should be expected—this is so. In (19), which also is fairly active, it could be assumed that hydrogen bonding between the δ-carbonyl and the β-NH group stabilizes this part of the molecule at approximately right angles to the plane of the α-β amide grouping, the phenyl ring is then removed from the possibility of obstructing the virus receptor surface.

$$\underset{\delta\ \ \gamma\ \ \beta\ \ \alpha}{C_6H_5\cdot CO\cdot NH\cdot NH\cdot CO\cdot COOH}$$

(19)

We feel from the above that some correlation between neuraminidase inhibition activity and structure has been demonstrated in our series. The other facet of the work was to see if neuraminidase inhibitors were active anti-virals.

The activity of seven active neuraminidase inhibitors as virus growth inhibitors is listed in Table 3. Four of these (9, 12, 13 and 19) show anti-influenza virus (PR 8) activity when incubated with the virus on a portion of egg membrane. However, in all cases the activity is almost completely abolished when the compounds are incubated with the virus in embryonated eggs. This loss of activity is presumably due to the compounds being metabolized by the chick embryo. It is possible that decarboxylation by enzymes in the embryonated egg may be the pathway by which the compounds are inactivated.

From the egg-membrane tests we can say that it is *possible* that the possession of neuraminidase inhibitory activity could give a compound an anti-viral action; however, our compounds appear to be too labile biologically to give any anti-influenza effect in an intact animal.

SUMMARY

1. Some *N*-substituted oxamic acids have been prepared and examined for neuraminidase inhibition with a view to being potential anti-influenza viral agents. Some known anti-viral glyoxals and isatins have been investigated in the same way.

2. Several *N*-substituted oxamic acids inhibit neuraminidase and prevent the growth of influenza A/PR-8 virus when incubated with the virus on portions of egg membrane.

3. This anti-influenza viral activity is almost completely abolished when the compounds are incubated with the virus in embryonated eggs, presumably due to breakdown of the compounds to inactive metabolites.

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