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SOME POTENTIAL ANTIVIRAL AGENTS*

BY

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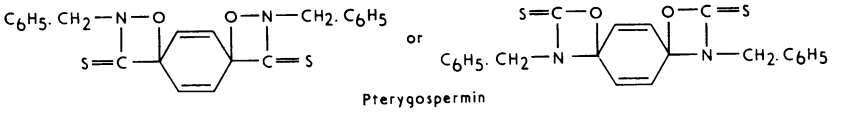
Following the spectacular achievements during the past two decades in the treatment of bacterial infections by antibiotics and other chemotherapeutic agents, concerted efforts towards control of viral infections are being made, though without notable success. The voluminous literature on potential antiviral drugs has been discussed by Matthews & Smith (1955), and more recently by Thompson (1964) and Pienta & Groupé (1964). Antiviral activities of a variety of compounds were evaluated against one or more dissimilar viruses, which generally included vaccinia and influenza viruses because of their representative character and the quantity of information available about them. The extensive efforts alluded to are remarkable for their persistency as much as for their failure to uncover any therapeutically useful agent.

The demonstration of the antivaccinia activity of pterygospermin (AB-1) (Kurup, 1953 ; Kurup & Narasimha Rao, 1954 ; Das, Kurup, Narasimha Rao & Ramaswamy, 1957 ; Krishnamurthy, 1958), the antibiotic principle occurring in the roots of *Moringa pterygosperma*, as well as of its fission product, benzyl isothiocyanate (AB-2) and the



Pterygospermin

less toxic potassium bisulphite addition compound of the latter, potassium benzylaminothiomethane sulphonate (AB-3), stimulated considerable interest in the study of compounds containing the N-C-S grouping particularly with an attached benzyl residue (Narasimha Rao, 1965). Earlier work in this laboratory demonstrated that among the aliphatic as well as the unsubstituted members of the homologous series, highest antibacterial as well as antivaccinia titres are reached in AB-2 and AB-3. The data now presented constitute an extension of the above studies and describe screening of several compounds containing the N-C-S group. In addition, a few chelate forming and other substances were included in the tests to explore the possibility of obtaining active agents on the basis of the metal-chelating hypothesis of antiviral action.

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METHODS

Excepting the commercially-available isonicotinic acid hydrazide (AB-41), all the other compounds screened were prepared by standard methods given in literature. In general, soluble compounds were dissolved in saline or buffer solutions and sterilized by steam at 15 lb for 10 min or by Seitz filtration before use. Sparingly soluble and insoluble compounds and oils were suspended (0.5–1.0%) in 2% sterile carboxymethyl-cellulose gel and sterilized.

Viruses: vaccinia virus (Bangalore strain) and influenza PR_8 strain were used.

Chick embryos: groups of 6 to 8 embryonated white Leghorn hen egg 10 or 11 days old were used in each experiment.

Screening compounds for anti-influenza and anti-vaccinia activities was carried out *in ovo*. No attempts have been made to use tissue culture techniques, for several reasons, not the least being the uncertainty of comparison with our own earlier unpublished records.

Vaccinia virus (egg-adapted strain)

The virus, from vaccine lymph produced locally, was isolated after several passages through the chlorioallantoic membrane of embryonated eggs 11 to 12 days old. This adaptation to eggs was necessary, since the virus from the fresh vaccine lymph did not produce any mortality in embryos. Growth of the virus on the membrane increased with each passage and the virus became quite virulent after seven passages, 0.1 ml. inoculum of thousand times diluted medium killing all the 11-day embryonated eggs by 72 hr. The virus isolated after seven passages was maintained at −20° C in 50% glycerine. Monthly examinations were made for virulence and when found attenuated, fresh passages were carried out as described, from fresh lymph.

In ovo *assays*

The 11-day-old embryonated eggs were inoculated with 0.1 ml. stock virus equivalent to approximately 10 LD_{100} on the dropped chorioallantoic membrane. Doses less than the maximum tolerated of the test compound were administered, unless otherwise stated, after inoculation through the allantoic route into groups of 6–8 eggs. The eggs were candled daily to record the mortality and the embryos were cut along their elliptical axis, at the end of the test period—72 hr—and the virus growth on the membranes of both the treated and untreated groups were compared. A substance was considered active if it afforded protection to 25% or more of the embryos with or without reduction of the virus growth on the membranes.

Influenza virus

PR_8 strain of the virus was made egg-adapted by cultivation in allantoic fluid of the 10-day-old embryonated eggs. The strain isolated after the second passage was used in the tests. Freedom from contamination was ascertained by streaking on blood-glucose-agar plates and the virus was stored at −20° C and replaced monthly with fresh preparations. Haemagglutination titrations were carried out using a 1% suspension of fowl erythrocytes (Anderson, Burnet & Stone, 1946; Burnet & Lind, 1956).

0.1 millilitres of the suitably diluted stock virus containing 0.1 haemagglutination unit representing approximately 10^5 ID50 (Takemoto, Robbins & Smith, 1954) was used as the virus inoculum. Test substances, at doses less than the maximum tolerated dose, were administered, after inoculation, through the allantoic route (Burnet & Beveridge, 1943). The embryos were incubated at 36° C for 42 hr and they were candled daily and the dead ones discarded. Those alive at the end of the experiment were chilled for 6 hr at 4° C; the allantoic fluids harvested from the same groups of embryos were pooled and the haemagglutination titres of each of these pools including those of the virus controls were then determined. A reduction in the titre of the treated, to one-fourth and less, as compared with appropriate virus controls was taken as an index of pronounced antiviral effect of the test compound.

In vivo *experiments*

Rabbits, weighing approximately 1.2 to 2 kg, and mice, 20 to 25 g, were used for vaccinia virus and influenza virus respectively.

Rabbits were infected on the flanks intradermally by scarification with a 1:10 dilution of the stock virus suspension. Compounds which exhibited high activity in embryos were administered intravenously at varying time intervals after infection. Although absence of formation of well-developed vesicles between the 3rd and 5th days following inoculation was considered as indicative of definite antiviral effect, the animals were kept under observation for 2 weeks.

Mice were infected intranasally under light ether anaesthesia with 0.2 ml. (100 haemagglutination units) of a suitably diluted stock virus. The stock virus used in this case is the original lyophilized virus, after one passage in eggs. The virus produced 100% mortality in untreated animals in 6 to 10 days after infection and necropsy in all cases showed consolidation of lungs. The test compound was administered intraperitoneally, to groups of 8 infected mice immediately after infection and then daily for 3 days. The percentage of survivors at the end of 10 days was taken as an index of activity. Compounds showing high activity *in ovo* were tested in mice.

In vitro *experiments*

The possibility that absence of haemagglutination might be due to a direct antihaemagglutinating effect of the drug, rather than to absence of the influenza virus, was eliminated by keeping the virus (0.2 ml.; 10 haemagglutination units) in contact with the minimum effective dose of the drugs at 4° C and periodically testing the samples for (a) haemagglutination, by adding 0.2 ml. fowl RBC suspension, and (b) infectivity, by inoculating 0.2 ml. into groups of 10-day-old eggs through the allantoic route. The allantoic fluids were individually tested for haemagglutinating activity after incubation at 36° C for 42 hr as described earlier. In the case of vaccinia virus, a mixture of the virus (0.1 ml., 10 LD_{100}) and drugs were treated as described above and transferred to the surface of dropped chorioallantoic membranes at varying time intervals. Mortality counts were taken after incubation at 36° C for 72 hr.

RESULTS

I. Primary screening in eggs

(a) Compounds showing pronounced inhibitory activity against influenza PR₈ and vaccinia viruses are given in Table 1. Of the eight active substances showing different types of inhibitory effect against influenza and vaccinia viruses, the following were chosen for detailed investigations of their antiviral properties in eggs and in animals: AB-3 and AB-41 were studied for their antiviral action against influenza infections; and AB-10, AB-41, AB-42 and AB-44 were further evaluated for their antivaccinia properties. The α- and β-guttiferins (AB-11 and AB-12) (Nageswara Rao & Narasimha Rao, 1961) and the metal-binding compound resacetophenone oxime (AB-14) (Appala Raju & Neelakantam, 1950), which have little or no *in ovo* activity, were also studied for their effects on influenza infection in mice.

II. Influenza infections in eggs and in mice

(a) Eggs

(i) Minimum effective doses of AB-3 and AB-41. The effect of administration of the compounds in graded doses immediately after infection on the production of virus haemagglutinin is shown in Table 2. AB-3 and AB-41 exert maximum inhibitory effect at dosage levels of 0.5 mg/egg and 2 mg/egg respectively. Higher doses could not be

TABLE 1

COMPOUNDS SHOWING ACTIVITY IN PRIMARY SCREENING TESTS AGAINST INFLUENZA PR_8 AND VACCINIA VIRUSES IN EGGS

AB No.	Compound	Dose* (mg/kg)	Influenza PR_8 virus: Mean haemagglutination titre Treated	Influenza PR_8 virus: Mean haemagglutination titre Control	Vaccinia virus: No. of eggs living/No. treated	Vaccinia virus: Growth on membranes of treated eggs
2	Benzyl isothiocyanate	0·2	4	256	7/8	Good growth
3	Potassium benzylamino-thiomethanesulphonate (Backer, Mulder & Froentjes, 1935)	0·5	4	256	7/8	Good growth
10	(*p*-Aminophenyl) methane-sulphonamide (Narasimha Rao, 1940)	2·0	256	256	0/8	Marked reduction in growth
41	Isoniazid	2·0	6	256	0/6	Good growth
42	Cyanacetic acid hydrazide (Saha, 1956)	2·0	128	256	2/7	Reduced growth
44	"Amine-X" (impure 5-amino-1,2,3,4-thiatriazole) (Freund & Schander, 1896)	4·0	128	256	8/8	Little or no growth
45	4-Cyanacetyl-1-benzyl-thiosemicarbazide	2·0	256	256	2/8	Reduced growth
48	4-Amino-2-thiouracil (Ganapathi & Palande, 1953)	2·0	128	256	0/8	Good growth (50% survival was observed by *in vitro* method when given immediately after mixing with the virus)

* Less than the maximum tolerated dose.

TABLE 2

MINIMAL INHIBITORY CONCENTRATIONS OF POTASSIUM BENZYLAMINOTHIO METH ANESULPHONATE (AB-3) AND ISONIAZID (AB-41) AGAINST INFLUENZA PR_8 VIRUS IN EGGS

AB-3 (mg/egg)	Mean haemagglutination titre	AB-41 (mg/egg)	Mean haemagglutination titre
0·05	64	0·1	64
0·1	64	0·2	64
0·2	64	1·0	8
0·5	8	2·0	6
Virus controls	512	Virus controls	256

administered as mortality was observed in some of the uninfected drug controls treated at these levels.

(ii) Effect of timing of administration of the drugs with respect to virus inoculation. The effect of administration of AB-3 (0.5 mg/egg) or AB-41 (2 mg/egg) at specific time intervals before or after inoculation with the virus on the haemagglutination titre is shown in Table 3. The results indicate that AB-3 inhibits haemagglutinin when administered as early as 24 hr before infection or during the latent period. A similar effect is, however, shown by isoniazid only when administered simultaneously with the infection.

(iii) In vitro *action of AB-3 and AB-41 on haemagglutination and infectivity.* Assays of mixtures containing AB-3 (0.5 mg) and virus (0.2 ml. ; 10 haemagglutinating units) and

TABLE 3

EFFECT OF POTASSIUM BENZYLAMINOTHIOMETHANESULPHONATE (AB-3) AND ISONIAZID (AB-41) ON HAEMAGGLUTININ PRODUCTION BY THE INFLUENZA PR_8 VIRUS IN EGGS WHEN ADMINISTERED BEFORE OR AFTER THE INFECTION

AB-3 dose : 0·5 mg/egg			AB-41 dose : 2 mg/egg	
	Mean haemagglutination titre: Drug given			Mean haemagglutination titre: Drug given
Time interval (hr.)	Before infection	After infection	Time interval (hr.)	After infection
0·5	64	8	0·5	64
1	64	8	1	128
2	64	64	2	256
6	24	48		
12	0	128		
24	0	128		
Virus controls	512	256	Virus controls	512

AB-41 (2 mg) and virus (0.2 ml. ; 10 haemagglutinating units) kept for varying periods are described below.

Both AB-3 and AB-41 in contact with the virus for 0–2 hr do not inhibit its haemagglutination properties. Table 4 records the production of haemagglutinin in eggs after inoculation with the drug-virus mixture at specific intervals of time after mixing. The

TABLE 4

INFECTIVITY OF INFLUENZA PR_8 VIRUS KEPT IN CONTACT WITH POTASSIUM BENZYLAMINOTHIOMETHANESULPHONATE (AB-3) AND ISONIAZID (AB-41) FOR VARYING PERIODS BEFORE INOCULATION

Period of contact of the virus with the drug (hr)	Mean haemagglutination titre	
	AB-3-treated	AB-41-treated
0	4	4
0·5	4	4
1	0	4
2	0	4
Virus controls	384	768

results indicate that both the substances affect the infectivity of the virus. AB-10 inhibits, under the same conditions, the haemagglutination of the virus.

(iv) The effects of aneurine, p-nitrosalicylaldehyde (Bavin, Rees, Robson, Seiler, Seymour & Suddabry, 1950) and riboflavin on the inhibitory activity of AB-3, given immediately or 1 hr before or after viral inoculation followed by AB-3, are summarized in Table 5. While AB-3 was given at the maximum tolerated inhibitory dose (0.5 mg/egg) for studies with aneurine and *p*-nitrosalicylaldehyde, a sub-lethal dose (0.25 mg/egg) was used in case of riboflavin to ascertain its synergistic action. Vitamin controls show no difference in haemagglutination titre as compared with that of virus controls. Under these conditions, glutamic acid (0.5 mg/egg) and *p*-aminobenzoic acid

(1 mg/egg) have also no effect on the inhibitory activity of AB-3 (dose: 0.5 mg/egg).

(b) Mice

(i) In vivo *action of AB-3 in experimental influenza infections in mice* is described in a subsequent communication.

(ii) α- and β-Guttiferins and resacetophenone oxime (AB-11, AB-12 and AB-14) in experimental influenza infections in mice. Results of their intraperitoneal administration

TABLE 5

EFFECT OF ANEURINE, *p*-NITROSALICYLALDEHYDE AND RIBOFLAVIN ON THE INHIBITORY ACTIVITY OF POTASSIUM BENZYLAMINOTHIOMETHANESULPHONATE (AB-3)

Time interval between administration of virus-AB-3 and the test compound	Mean haemagglutination titre of the virus recovered from eggs treated with		
	Aneurine (1 mg/egg)	*p*-Nitrosalicylaldehyde (1 mg/egg)	Riboflavin (1 mg/egg)
0	32	48	4
1 hr. before	512	128	8
1 hr. after	32	4	4
Virus-AB-3 controls	4	4	4
Virus controls (no drug)	256	256	512

TABLE 6

EFFECT OF α- AND β-GUTTIFERINS AND RESACETOPHENONE OXIME AGAINST INFLUENZA PR_8 INFECTION IN MICE

Compound	Dose (mg/kg/day for 2 days)	No. of survivors/ total no. treated
α-Guttiferin	10	0/8
	30	3/8
β-Guttiferin	10	1/8
	30	2/8
Resacetophenone oxime	25	2/8
Untreated infected controls	—	1/8

TABLE 7

COMPARATIVE ACTIVITIES OF AB-3, AB-44, AB-10, AB-42 AND AB-41 AGAINST VACCINIA VIRUS IN EGGS

Compound	Dose (mg/egg)	Maximum tolerated dose (mg/egg)	Activity
AB-3	0·5	10	Survival rate: 87·5% No effect on virus growth
AB-44	2	>6	Survival rate: 50% Marked reduction in virus growth Active *in vitro*
	4		Survival rate: 100% Little or no growth of the virus
AB-10	4	6	No effect on mortality Distinct reduction in viral growth Possesses no *in vitro* activity
AB-42	2	4	Survival rate: 25% Reduction in viral growth
AB-41	2	4	No effect on mortality Active *in vitro*

to infected mice (Table 6) seem to indicate that the compounds which chelate with copper have very little activity against influenza in mice.

III. Vaccinia infections in eggs and in rabbits

(a) Eggs

(i) Comparison of in ovo *activity of the active compounds.* Comparative activities of the compounds that showed pronounced antiviral effects in preliminary screening tests are indicated in Table 7.

(ii) In vitro *action of AB-41 and AB-44.* The effect of keeping compounds (2 mg) in contact with the virus for varying periods is given in Table 8.

(iii) Effect of time of administration of AB-44. The results of administration, before

TABLE 8

IN VITRO ACTIVITY OF AB-44 (2 mg) AND AB-41 (2 mg) ON THE INFECTIVITY OF VACCINIA VIRUS

Compound	Time interval (hr.)	No. of embryos alive/total no. treated	Virus growth on membranes
AB-44	0	6/6	No visible growth
	0·5	6/6	No visible growth
	1	5/6	No visible growth
	2	5/6	No visible growth
AB-41	0	5/6	Good growth
	0·5	4/6	Good growth
	1	3/6	Good growth
	2	3/6	Good growth
Virus controls (no drug)		0/6	Good growth

TABLE 9

EFFECT OF ADMINISTRATION OF AB-44 BEFORE OR AFTER INFECTION OF EGGS WITH VACCINIA VIRUS

	Embryos alive/total number treated			
Time interval (hr.)	Before infection	Virus growth on membranes	After infection	Virus growth on membranes
0·5	4/8	Very little growth	6/8	No visible growth
1	4/8	No visible growth	6/8	No visible growth
2	4/8	No visible growth	4/8	Very little growth
6	4/8	No visible growth	6/8	Very little growth
12	4/8	Very little growth	6/8	Very little growth
24	4/8	Very little growth	4/8	Very little growth

and after inoculation of eggs with the virus, at a dosage level of 2 mg/egg are shown in Table 9.

AB-44 exerts marked inhibitory action against vaccinia in eggs when administered

immediately or as late as 12 hr after the infection. The effect is less pronounced when the drug is given before or 24 hr after the infection.

(b) Experimental rabbit infections

The effect of intravenous administration of AB-10, AB-42 and AB-44 to rabbits following intradermal infection with vaccinia virus is shown in Table 10.

TABLF 10

EFFECT OF INTRAVENOUS ADMINISTRATION OF AB-44, AB-10 AND AB-42 TO RABBITS INFECTED INTRADERMALLY WITH VACCINIA VIRUS

Five animals were used for each experiment

Compound	Dose (mg/kg/day for 3 days)	Effect on vesicle formation
AB-44	20	No vesicle formation
AB-10	30	Vesicle formation was observed in two animals after 5 days
AB-42	20	Normal vesicle formation
Untreated animals (controls)		Normal vesicle formation

While more detailed experiments are under way, the effect of AB-10 indicates that it is mostly virustatic and the favourable results observable seem to depend upon the past history of the animals and the presence of antibodies. AB-44 does not seem to be influenced by these factors and further elucidation of its action is being sought.

DISCUSSION

The primary *in ovo* screening followed by *in vitro* and *in vivo* experiments has brought out the pronounced antiviral properties of five compounds—viz., AB-3, AB-10, AB-41, AB-42 and AB-44 (Table 1). AB-11, AB-12 and AB-14 which bind copper readily showed no activity on the two viruses tested. Thus the prospects of evolving new antiviral agents on a metal binding hypothesis alone seem slender. On the other hand, AB-2 and its derivatives, which would conceivably liberate the parent compound *in vivo*, are found to exert antivaccinia and anti-influenza activity. The effect of AB-41, the only other compound found significantly active on both the viruses, is, however, less pronounced, its activity on vaccinia virus being detectable only *in vitro*. The drug has been reported to be ineffective against influenza viral infections in mice (American Trudeau Society, 1952). AB-44, an impurity associated with certain preparations of 5-amino-1,2,3,4-thiatriazole, appears to be highly specific in its action on the vaccinia virus. Not only does it reduce mortality of infected embryos but it effectively suppresses lesion formation and viral growth. These results are well reproduced in the rabbit infections. The antiviral activity and therapeutic implications of the thiatriazole derivatives including those of AB-44 will be described elsewhere.

The antiviral activities of the compounds referred to—particularly that of AB-3—seem to be influenced by added aneurine. Addition of *p*-nitrosalicylaldehyde, which simulates pyridoxal (Snell, 1958), alters the activity significantly. Aneurine-AB-3 antagonism has been previously observed in bacterial systems (Kurup & Narasimha Rao,

1954) and was attributed to their structural analogy. The available limited data on the mode of action of AB-3 seem to suggest interference with the altered metabolic regulation enforced by virus invasion. The aneurine-AB-3 antagonism raises several intriguing possibilities regarding the foci of the drug action. They may implicate alterations of lactic dehydrogenase activity, glycolysis and increased turnover of pentose cycle of the infected tissue metabolism (Kun, Ayling & Siegal, 1960). These conditions are reminiscent of those obtaining in neoplastic tissues (Magill, Wroblewski & LaDue, 1959). Indeed, the levels of lactic dehydrogenase activity were previously reported (Kelly & Grieff, 1961) as offering a better measure of influenza virus growth in chorioallantoic membrane than the customary haemagglutination titrations. Whatever the above possibilities, the data presented appear to be suggestive of a fruitful approach to antiviral chemotherapy.

SUMMARY

1. Representative compounds containing the N-C-S sequence as well as a few natural products and others have been screened *in ovo* for antivaccinia and anti-influenza activity using a dermal strain of vaccinia virus (Bangalore strain) and influenza virus PR_8. The active compounds have been further tested for their *in vivo* activity in rabbits and in mice respectively. The following compounds are found to exhibit antiviral activity *in ovo*. (a) Potassium benzylaminothiomethanesulphonate (AB-3) and free benzylisothiocyanate (AB-2) (earlier reported from this laboratory); (b) an impure preparation of 5-amino-1,2,3,4-thiatriazole (amine-X, AB-44); (c) isoniazid (AB-41); (d) cyanacetic acid hydrazide (AB-42) and (e) p-aminophenyl-methanesulphonamide (AB-10) (slight activity). In addition, 4-cyanacetyl-1-benzylthiosemicarbazide (AB-45) and 4-amino-2-thiouracil (AB-48) show slight activity.

2. AB-3 and AB-41 display antiviral activity on both the viruses. The activity of the latter compound on vaccinia virus is feeble, however, being detectable only in the *in vitro* system. Both the compounds seem to act best when administered before infection *in ovo*.

3. AB-44 is specifically active against vaccinia virus in all the three test systems—viz., *in vitro*, *in ovo* and *in vivo*. It suppresses lesion formation in rabbits infected intradermally with the virus.

4. The activity of the compounds, particularly of AB-3, is influenced by the addition of aneurine.

5. The metal-binding compounds screened exhibit little or no detectable antiviral activity *in ovo* and therefore the prospects of evolving new antiviral drugs based on metal-chelating hypothesis thus seem poor.

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