Brit. J. Pharmacol. (1957), **12**, 66.

THE ANTIVIRAL ACTION OF *THREO*-β-PHENYLSERINE

BY

LOÏS DICKINSON AND MILDRED J. THOMPSON

From the Biology Division, Research Department, Boots Pure Drug Co., Ltd., Nottingham

WITH AN APPENDIX BY J. S. NICHOLSON

(RECEIVED SEPTEMBER 28, 1956)

L-*threo*-phenylserine and esters of *threo*-phenylserine were the most active of a series of compounds tested against influenza A virus in tissue culture. Substitution of the β-OH or α-NH_2 group abolished activity. The activity of phenylserine was reversed competitively by phenylalanine. Phenylserine did not act on free virus or on the adsorption of virus to host cells. It prevented virus growth if added during the first half of the latent period.

Phenylalanine appears to be necessary for virus synthesis and can be supplied by phenylalanylglycine or glycylphenylalanine.

Phenylserine had no significant activity against ectromelia infections in mice, even when the amino acid content of the livers had been depleted by starvation.

During studies of possible inhibitors of protein synthesis for virus chemotherapy trials, many amino acid derivatives have been tested against influenza virus in eggs (Dickinson, 1955). Tissue culture tests have been done in parallel and β-phenylserine was found to be active. This compound inhibits the growth of some bacteria, its action being reversed by phenylalanine (Bergmann, Sicher, and Volcani, 1953; Simmonds, Dowling, and Stone, 1954; Fox and Warner, 1954); recently the antibacterial action of *p*-fluorophenylserine has been reported (Edmonds, Volkman and Beerstecher, 1956). There have been no reports of its antiviral activity, but certain other amino acid analogues have shown antiviral action in tissue culture (Ackerman, 1951; Ackerman and Maassab, 1954; Ackerman, Rabson, and Kurtz, 1954). Matthews and Smith (1955) and Hurst and Hull (1956) include amino acid antagonists in their reviews on "virus chemotherapy."

Phenylserine derivatives are of particular interest in view of the structure of chloramphenicol, which is active against the large viruses of the psittacosis group. This paper reports the inhibition of influenza virus by *threo*-β-phenylserine and various related compounds. The mode of action of phenylserine and its use as a tool in studies of phenylalanine metabolism of virus-infected cells are also described.

METHODS

Virus Strains.—The following influenza A virus strains were kept at −65° C. as allantoic fluids: PR8, NWS, and the Francis and Moore (1940) neurotropic variant of WS (here called WSNF), supplied by Dr. D. A. J. Tyrrell as more suitable for cytopathogenicity tests than NWS. Mumps (Hampstead), vaccinia (Salaman), and ectromelia (Hampstead) were also used. For work *in vivo* ectromelia (Hampstead), mouse-adapted influenza A/PR8 and encephalomyocarditis (EMC) were used. These were kept as freeze-dried, crude suspensions, of known high titre, in 0.5 ml. or 1 ml. volumes. Broth was a satisfactory medium for drying influenza or ectromelia, but EMC was dried as a mouse-brain homogenate suspended in homogenized yolk sacs from chick embryos: this suspending agent was much superior to broth. All three freeze-dried viruses were reconstituted and diluted in broth for testing.

Titration of Viruses.—Serial ten-fold dilutions were titrated in mice or eggs; the inoculum was 0.2 ml. except for intranasal and intracerebral titrations, when it was 0.05 ml. Results were expressed as log LD50 or, for influenza virus titrations in eggs, as EID50 (egg infective dose 50), using the method of Reed and Muench (1938). For influenza virus 9-day chick embryos were infected via the allantoic sac, and the presence of haemagglutinin at 48 hr. was determined by a modified Salk (1944) method. Haemagglutinin titrations were done in plastic blocks, using two-fold dilutions in saline and a final fowl red blood cell concentration of 0.25%; results were expressed as reciprocals of the end-point dilution.

Suspended Fragment Culture and Testing of Drugs against Influenza A/PR8 Virus.—Finely chopped chorioallantoic membrane (CAM) fragments of 9 to 10 day chick embryos were suspended in the maintenance medium, consisting of Earle's saline (Earle, 1943) 2 parts, embryo extract 1 part. The propor-

tions were 0.25 ml. tissue suspension to 2.0 ml. medium, in tightly bunged 25 ml. flasks; one membrane provided tissue for 6 flasks. Then 0.25 ml. virus suspension containing 10^4 EID50 was added, and, 1 hr. later, 0.5 ml. of the drug (serial five-fold dilutions); controls were made up to the same volume. After 48 hr. stationary incubation at 37° C. the haemagglutination titres of the supernatants were found, and the possibility of false haemagglutination or haemagglutination inhibition was checked. Three flasks for each drug dilution and three controls were used throughout; haemagglutinin titres of controls were always 64–256. To find the effect of the drug on the cells, tissue fragments were removed after 24 hr., washed and embedded in plasma, then rotated in growth medium (2 parts horse serum, 3 parts maintenance medium) for 48 hr.; outgrowths of cells were observed microscopically from 24 hr. Results were expressed both as the drug concentration inhibiting the growth of virus and also as a ratio of the cytocidal concentration to the virus inhibitory concentration. To determine the cytostatic concentration a similar method was used, the drug being present during rotation of the embedded fragments.

Compounds.—Many of the compounds tested occur as optical isomers and also as *threo* and *erythro* forms. Except where otherwise stated, the racemic form is implied. Phenylserine is the DL-*threo* form unless otherwise stated. All the compounds tested, with the exception of 4550, 4923, and 4385 (Nicholson, see appendix), and the mandelic acid amidinium chlorides (Bristow, personal communication), have been described in the literature.

Solutions were prepared at *p*H 7.4 and kept frozen at −20° C. between experiments. Several, such as phenylserine itself, decomposed in solution, especially on heating. Solutions were not sterilized, but 100 i.u. each of penicillin and streptomycin were added per ml. tissue culture medium to inhibit bacterial growth.

Growing Cell Cultures.—The method of Tyrrell (1955) was used to "trypsinize" lungs of 9 to 14 day chick embryos. One ml. cell suspension, containing approximately 10^7 cells/ml. in growth medium, was allowed to settle overnight in horizontal, tightly stoppered, 150 by 13 mm. tubes. In some experiments 40 by 14 mm. vials were used; 0.5 ml. cell suspension was used in these. After removal of the supernatant, virus and drug dilutions were added and the tubes rotated at 12 r.p.h. The cells were examined microscopically daily and haemagglutination titres determined at 48 and 72 hr. The fluid added before rotation was either maintenance medium, BAF medium (2 parts 1% Armour's bovine serum albumin fraction V, 3 parts maintenance medium) or CWB medium, a synthetic medium (Waymouth, 1955) used here for the studies on phenylalanine metabolism.

Tests in Eggs.—The method of Dickinson (1955) was used, both drug and virus being given by the allantoic sac. Four- or five-fold dilutions of the drug were tested, up to and including the toxic dose (or 40 mg./egg if not toxic at this dose).

Tests in Mice.—White mice (20 g.) in groups of 10 to 30 were used. The dose of virus was calculated to infect all and to kill at least half of the mice of the control groups; controls were always infected last. Subcutaneous treatment commenced the day before infection and was continued at the maximum tolerated dose until the experiment ended. Influenza A/PR8 was given intranasally, influenza A/NWS intracerebrally, ectromelia and EMC intraperitoneally. Results were assessed on average survival times, and, for influenza A/PR8, on the extent of lung lesions; they were assessed for significance by the "t" test.

Phenylalanine Assay.—*Escherichia coli* K12/58–278 was kindly supplied by Dr. B. D. Davis. This mutant was inhibited by valine, but a valine-resistant mutant was readily obtained and was much more satisfactory for assay purposes than *Leuconostoc mesenteroides* previously used. The medium consisted of lactic acid 2.4 g., NaCl 5 g., $(NH_4)_2HPO_4$ 1 g., KH_2PO_4 1 g., $MgSO_4.7H_2O$ 0.2 g., and distilled water to 1 litre. The *p*H was adjusted to 7.0 and the medium was autoclaved at 15 lb./15 min. Biotin (0.25 μg./l.) was added and 3 ml. volumes were distributed. One ml. of dilutions of standards (range 0.01 to 10 μg./ml. phenylalanine) and unknowns were then added; tubes were prepared in triplicate and all dilutions made in the medium. The inoculum was prepared from a 24 hr. culture in the above medium containing 100 μg./ml. phenylalanine. After three washings in saline the final deposit was made up to the original volume in the medium and then diluted 1 in 10; one drop of this dilution was added to each tube, and the cultures were incubated at 37° C. for 48 to 72 hr. Growth could be detected at a concentration of 0.1 μg. phenylalanine/tube.

RESULTS

Activity of Phenylserine.—Phenylserine, at 0.1 to 0.2 mg./ml., inhibited the production of influenza virus, as judged by haemagglutination, in the suspended fragment test. It was not cytocidal to CAM fragments at 10 mg./ml. The haemagglutinin titres at 48 hr. for treated cultures were all <2, the controls being 64–256. These results were checked by infectivity determinations in eggs; the control titres at 48 hr. were 7.27, the initial titre being 1.75. The titres at 48 hr. for phenylserine-treated cultures were 1.75 (1.0 mg./ml.) and 5.75 (0.25 mg./ml.). The methyl ester of phenylserine was more active, virus increase being completely inhibited at 0.2 mg./ml., judged by both haemagglutination and infectivity determinations.

Toxicity of Phenylserine to Growing Chick Embryo Cells.—Explants of CAM tissue were grown in growth medium in roller tubes. Cell growth occurred at 1.0 mg./ml. phenylserine. Similar results were found for lung tissue explants

and trypsinized lung cells. When the cells were infected with influenza virus in the same medium, phenylserine prevented virus growth (haemagglutination test) at 0.2 mg./ml. Thus phenylserine prevented virus growth in both suspended fragments and in growing cells, but the cytostatic concentration was lower than the cytocidal concentration, as might be expected.

Mode of Action of Phenylserine

To determine whether phenylserine was acting on extracellular virus, on adsorption of virus or during the multiplication of virus within the cell, the following experiments were done. Influenza A/PR8 in maintenance medium and 0.2 mg./ml. phenylserine were used, unless otherwise stated.

Inactivation of Extracellular Virus.—The rates of inactivation of influenza virus in the absence of cells with and without phenylserine (0.2 mg./ml.) were found by finding the EID50 at 0, 6, 24 and 48 hr. There was no significant difference between the two inactivation rates. The initial titre was 5.75 ; titres at 48 hr. were 3.5 (controls) and 3.75 (phenylserine treated).

Action on Adsorption of Virus and on Various Stages in the Growth Cycle.—To find the mode of action of an antiviral drug, the best system has uniform single cells and a virus which can be assayed accurately. This is easy with bacterial viruses and would be possible with the cytopathogenic western equine encephalomyelitis virus and HeLa cells (Dulbecco and Vogt, 1954). A simpler, if less accurate, method for influenza virus is to use the suspended fragment technique, infect the cells very heavily and remove the unadsorbed virus by washing and antiserum treatment ; virus release can be followed by infectivity or haemagglutinin titrations. In this system the cell population is not uniform and virus release is spread over a long period of time. However, the main features of the growth cycle are clear—a latent period followed by exponential release of virus, rising to a constant value after several hours.

In these experiments a total time of 1 hr. was allowed for adsorption and washing ; quickly adsorbed virus had, therefore, possibly an hour's start on that adsorbed towards the end of the period. Cultures were prepared as in the standard CAM fragment test, but a heavier inoculum (0.25 ml. of a 1/25 dilution of stock allantoic fluid) was used. After a 50 min. adsorption period the excess virus was removed by three washings, the second one incorporating specific antiserum. The washing took 10 min., so that zero time was at 60 min. Phenylserine either was present during the adsorption period (and removed during the washing procedure), or was added at intervals after zero time. Infectivity determinations were made on the supernatants at zero time and at 8½ hr., by which time virus was known to be released from control cultures. Table I shows the results of these tests. Phenylserine had no effect when present only during the adsorption period. If added up to 4 hr. after the end of the adsorption period it prevented virus growth. Tests where the drug was added at more frequent intervals, but using haemagglutination as the index of virus production, confirmed that phenylserine acted during the first half of the latent period. In these tests the inoculum was 0.25 ml. of a 1/2.5 dilution of allantoic fluid and the phenylserine concentration was 0.5 mg./ml. The tests were carried out in rotating flasks and

TABLE I

ACTION OF PHENYLSERINE (0·2 MG./ML.) ADDED AT DIFFERENT TIMES DURING THE GROWTH OF INFLUENZA A VIRUS IN TISSUE CULTURE

Virus titre at time 0 (end of adsorption period of 1 hr.) = 1.5.

Phenylserine Treatment	Log Infectivity Titre (EID50)	
	8½ hr.	24 hr.
Present during adsorption period (−1 to 0 hr.) ..	3·5	.
Added at 0 hr.	1·5	<1·0
1 ,, ...	1·5	.
2 ,, ...	1·25	.
4 ,, ...	1·5	.
7½ ,, ...	3·5	.
Control (no phenylserine) ..	3·25	>4·0

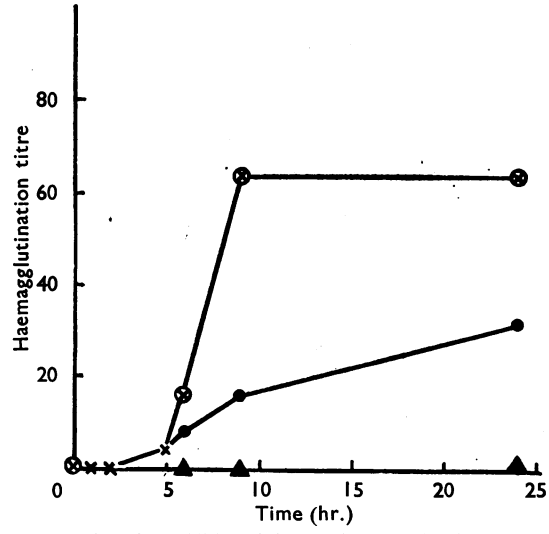


FIG. 1.—Effect of the addition of phenylserine at varying times during the growth cycle of influenza virus. ▲——▲ added at 0, 1, or 2 hr. ●——● added at 4 hr. O——O added at 5 hr. X——X Control.

haemagglutinin production was detectable at 5 hr. (somewhat earlier than in stationary cultures); Fig. 1 shows the result of a typical experiment. It was not possible to give a critical "time of action" from this type of test, especially as there was some, but not complete, inhibition of haemagglutinin production when phenylserine was added, even after halfway through the latent period. The possibility that phenylserine interfered with the release of virus from the cells was checked by grinding up the cells; treated cells contained no virus, whereas controls contained large amounts.

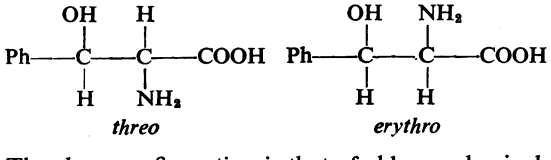
Action on Cytopathogenicity of Influenza A/WSNF on Lung Tissue.—Since phenylserine prevented virus growth even from large inocula of strain PR8, its action against large inocula of strain WSNF, which destroys chick embryo lung cells (Tyrrell, 1955), was investigated. At 1.0 mg./ml. it prevented both the development of haemagglutinin and the destruction of the cells by the virus; when 1 mg./ml. phenylalanine was added to antagonize the action of phenylserine, haemagglutinin production and the cytopathogenic effect occurred as in controls.

Use of Phenylserine in Virus Studies

The antiviral action of phenylserine was used in two ways: (1) As a starting point to find compounds of greater activity and to relate activity to structure; (2) as a tool to investigate the metabolism of virus infected cells, in the first place by antagonism studies.

Structure and Activity.—The relevant compounds prepared and tested, and their activities, appear in Table II.

There are four isomers of phenylserine—i.e., the D and L forms of the *threo* and *erythro* configurations:



The *threo* configuration is that of chloramphenicol, which has the structure indicated in Table II and which is, of course, only active against the largest viruses. Against influenza in these experiments, it was only active at 1.6 mg./ml., whereas DL-phenylserine was active at 0.1 mg./ml. The activity of phenylserine was confined to the L form of the *threo* isomer. The D form was inactive, but did not interfere with the action of the L form, and all work on further variation of the molecule refers

TABLE II

ACTIVITY OF PHENYLSERINE AND RELATED COMPOUNDS AGAINST INFLUENZA A IN TISSUE CULTURE

Bz=Benzyl. p=Partial activity. ..=Not found if compound was inactive at 1 mg./ml., insoluble, or not available in adequate amount. * Configuration unknown.

Ref. No.	Structure	Conc. Inhibiting Virus (mg./ml.)	Therap. Ratio
1412	DL-*threo*-phenylserine	0·1	100
5012	D- ,, ,,	>1·0	..
5223	L- ,, ,,	0·04p	..
4233	DL-*threo*-(*p*-hydroxyphenyl)serine	>1·6	..
4058	DL-*erythro*-phenylserine	>1·6	..
	DL-threo-*isomers of* Ph.CHOR¹.CHNHR².COR³		
	R¹ R² R³		
4386	H H OMe	0·04	>250
4536	H H OEt	0·04p	≈250
4549	H H OBu	0·04	>250
5024	H H OC_6H_{11}	0·04p	≈25
4550	H H $NHNH_2$	0·2p	..
4923	H H NHOH	0·2	5
4599	Me H OH	>1·0	..
4387	CO.Me =CHPh OH	1·0p	..
4433	H COMe OH	>1·0	..
5082	H $COCHCl_2$ OH	>1·0	..
5079	H $COCH_2Cl$ OH	1·0	1
Peptide	H $COCH_2NH_2$ OH	0·2p	..
	Threo-*isomers* (*except* 4540) *of* Ph.CHOH.CHNHR.CH_2OH		
4540	*Erythro*-phenylserinol; R=H	0·2p	..
4539	*Threo*-phenylserinol R=H	1·0	..
1796	*p*-NO_2 ,, ,, R=H	1·0	1
1272	*m*-NO_2 ,, ,, R=H	1·0	5
1237	*p*-Cl ,, ,, R=H	1·0	1
1273	*m*-NO_2 ,, ,, R=$COCHCl_2$	>1·0	..
1155	*m*-NO_2 ,, ,, R=COMe	>1·0	..
1161	,, ,, R=COMe	>1·0	..
	p-NO_2 ,, ,, R=$COCHCl_2$ (Chloramphenicol)	1·6	..
	Mandelamidine hydrochlorides Ph.CHOH.C(=NH)(NHR),HCl		
1053	R=H	1·0	..
1102	*m*-OH R=Me	>1·0	..
1055	*m*-OMe R=H	1·0p	..
1068	*m*-OH R=H	>1·0	..
1071	3:4($OH)_2$ R=H	1·0p	..
1175	*m*-Cl R=H	1·0	1
1176	*p*-Cl R=H	1·0	1
1180	*m*-OBz R=H	0·2	5
1177	*m*-OBz R=$CHMe_2$	0·2	5
1178	*m*-OBz R=C_7H_{15}	0·04	1
1179	*m*-OBz R=Cyclohexyl	0·04p	≈5
1282	*m*-OBz R=$CH_2.CH_2.NEt_2$	1·0p	..
1128	PhCHOH.C(=N—CH_2)(NH—CH_2)	1·0p	..
1205	*m*-OBz derivative of 1128	0·2	5
1206	*p*-Cl derivative of 1128 (double compound with ethylenediamine)	1·0p	..
	Phenylalanyl derivatives		
.	DL-Phenylalanine	>5·0	..
.	D-Phenylalanine	>1·0	..
.	L-Phenylalanine	>1·0	..
1435	DL-N-Acetylphenylalanine	>1·0	..
5078	DL-Phenylalanine ethyl ester	1·0p	..
.	D(+)-3:4-Dihydroxyphenylalanine	>1·0	..
.	L(−)-3:4-Dihydroxyphenylalanine	>1·0	..
4967	DL-Phenylalanylglycine	>1·0	..
5023	Glycyl-DL-phenylalanine	>1·0	..
4474	Poly-DL-phenylalanine	>0·2	..

TABLE II—*continued*

Ref. No.	Structure	Conc. Inhibiting Virus (mg./ml.)	Therap. Ratio
Miscellaneous compounds			
.	DL-*threo*-Threonine	2·0p	..
.	DL-Serine	5·0	..
.	β-Hydroxyglutamic acid*	1·0p	..
4966	(2-Furyl)-CHOH.CHNH$_2$.COOH*	>1·0	..
4385	Ph.CH — CH COOH O NH CO CH$_2$OH	>1·0	..
1209	Ph—C—NO$_2$ CH$_2$OH	0·04	5

to the DL form. In assessing results, activity in the tissue culture tests was only considered of interest if below 1 mg./ml. and egg tests were done where supplies permitted.

For activity in tissue culture, Table II shows that the NH_2- and OH-groups had to be free but that the COOH-group could be modified, though with no gain in activity except in the case of the esters. Omission of the ring or ring substitution with *p*-OH also prevented activity. The fact that glycylphenylserine was active, in spite of the N-substitution of phenylserine, suggests that it was broken down to phenylserine. Unfortunately phenylserylglycine was not available. Since both the β-OH and α-NH_2 appeared to be necessary for activity, it was not surprising that the phenylalanyl derivatives tested were inactive.

Nothing more active, or with a better therapeutic ratio, than L-phenylserine or the DL esters was found, and, of the series of esters tested, the methyl was considered the best because of the results of the egg tests. Although all the esters had therapeutic ratios of about 250 in tissue culture, the methyl ester was active at 40 mg./egg whereas the other esters were toxic at this dose and inactive at lower doses. The relative inactivity in eggs was probably due to the presence of phenylalanine which antagonized the action of both phenylserine and its ester (see later). Because of the ease of preparation compared to L-phenylserine itself, DL-phenylserine methyl ester was used for *in vivo* tests.

Various mandelic acid amidinium chlorides were also tested, but, though several were active, the activity was associated with toxicity and the therapeutic ratios were very poor. The action of compounds 1177 and 1180 (Table II), unlike that of phenylserine and phenylserine methyl ester, was not reversed by phenylalanine.

The Use of Phenylserine in Studying Phenylalanine Utilization

Antagonism Experiments. — The following amino acids: DL-serine, glycine, L-tyrosine, L-histidine, L-glutamic acid, DL-valine, DL-alanine, DL-lysine, DL-tryptophane, and L-, D-, and DL-phenylalanine, were tested for their effect on the antiviral action of phenylserine in the suspended fragment test. At concentrations of 1 mg./ml. of both phenylserine and amino acid, only L- and DL-phenylalanine had any antagonistic effect; D-phenylalanine had none. In further work, when the concentrations of phenylserine and phenylalanine were varied, the DL-isomer was used. Phenylalanine itself did not inhibit the growth of virus at 5 mg./ml. and its action in antagonizing the action of phenylserine was competitive in nature. Phenylserine was antagonized by an equal amount of phenylalanine over a range of concentrations of both from 0.2–5.0 mg./ml. Phenylalanine also antagonized competitively the action of phenylserine methyl ester and glycylphenylserine.

The reversal by phenylalanine of the phenylserine inhibition of virus growth was also tested in sheets of chick embryo lung tissue. Cells were grown in growth medium, infected by influenza A/PR8 and then maintained in either maintenance or CWB medium. Phenylalanine antagonized the action of phenylserine as in the suspended fragment test. These results possibly explain the inactivity of phenylserine in eggs, although the concentration of phenylalanine in the cells of the allantoic sac is not known.

Peptide Utilization.—Eagle (1955a) found that glycylphenylalanine could replace phenylalanine in tissue cultures of HeLa cells and strain L fibroblasts, but he did not determine whether dipeptides were used intact. This is, indeed, difficult to prove, but one approach to the problem is to see whether natural peptides antagonize an antagonist of one of the constituent amino acids.

Using the suspended fragment test, the following compounds were tested at 5:1 and 1:1 ratios against phenylserine: glycylphenylalanine, phenylalanylglycine, glycylglycine, N-acetylphenylalanine and phenylalanine ethyl ester; they were also tested by themselves to rule out any antiviral action of their own. Phenylalanylglycine, glycylphenylalanine and phenylalanine ethyl ester antagonized the action of phenylserine at an approximate ratio of 1:1. Glycylglycine and N-acetylphenylalanine, even at a 5:1 ratio, did not. Since blockage of the NH_2 of phenylalanine prevents its antagonistic effect, it appears unlikely that glycylphenylalanine

is utilized intact. It is possible that phenylalanine ethyl ester is hydrolysed to the free acid which would itself antagonize phenylserine. Solutions of this ester give strongly positive ninhydrin reactions, but one cannot, from the present evidence, eliminate the possibility that phenylalanine compounds (including phenylalanylglycine) with the carboxyl group substituted may antagonize phenylserine. As far as can be judged, since only small amounts of the peptides were available, both phenylalanylglycine and glycylphenylalanine antagonized phenylserine competitively; thus 5 mg. peptide antagonized 5 mg. drug, 1 mg. antagonized 1 mg. drug, 0.2 mg. antagonized 0.2 mg. drug.

Evidence for the utilization of peptides was also sought by using them as sole sources of phenylalanine in sheets of infected and uninfected lung tissue. Cell suspensions of lung tissue were grown in growth medium, which was then replaced by modified CWB media, in which DL-phenylalanine (0.1 mg./ml.) and glycine (0.2 mg./ml.) were replaced by 0.4 mg./ml. phenylalanylglycine or glycylphenylalanine. After 24 hr. rotation, the media were renewed and half the cultures infected with strain PR8. No gross degeneration occurred in either infected or uninfected tissue within 6 days, in either CWB or the modified media. Virus was produced in all media and even, in some experiments, when added free or combined phenylalanine was not present. Presumably sufficient phenylalanine was present within the cells to allow virus growth unless the utilization of phenylalanine was stopped by an antagonist. The amount of free phenylalanine was less than 0.1 μg./vial, as determined by the *E. coli* assay, but it could be detected if the contents of several vials were pooled. In these assays streptomycin was omitted from the medium and the penicillin inactivated during the extraction of the amino acids by boiling. It was not possible to use the *E. coli* assay culture to follow the postulated breakdown of peptides since it utilized the peptides as readily as free phenylalanine.

Apart from the evidence that glycylphenylalanine is broken down, there is also evidence that glycylphenylserine is broken down because the antiviral action of this peptide is antagonized by phenylalanine.

Effect of Phenylserine on Other Viruses

Other virus-cell systems were investigated to see if phenylserine prevented the growth of other viruses and also to form some basis for *in vivo* tests.

Using a similar technique to the influenza test, but with an incubation period of 8 days, it was found that mumps virus was inhibited by phenylserine. Vaccinia and ectromelia viruses (passaged in eggs to give good lesions on the CAM) were also grown in tissue culture, using whole chick embryo fragments and CAM fragments respectively. The viruses were titrated by plaque counts on the CAM of 10–13 day embryos. Phenylserine (1.0 mg./ml.) prevented the 10- to 100-fold rise in infectivity shown by control cultures.

The growth of EMC virus in suspended fragments of brain tissue from one-day-old mice was inhibited by 0.2 mg./ml. phenylserine in BAF medium; toxicity to cells was not determined. Growth of EMC virus was assessed by a haemagglutination test (modified from Gard and Heller, 1951): 0.25 ml. of 0.2% sheep cells in KCl solution (10.43 g./l.) was added to 0.25 ml. of dilutions of the tissue culture supernatants removed after incubation for 1, 2, and 3 days. The diluent was a 1:1 mixture of barbiturate buffer (*p*H 7.2) and KCl solution. Tests were read after 2 hr. at 4° C. Control titres were 8–32 at 2 to 3 days; titres of phenylserine-treated cultures were all less than 2.

Tests in Mice

Mice tolerated a daily subcutaneous dose of 2 mg./g. of phenylserine or its methyl ester and the effective inhibitory concentrations in tissue culture were 0.2 mg./ml. and 0.04 mg./ml. respectively. Phenylalanine in the body would obviously reduce any activity, but small-scale *in vivo* tests were done against viruses which had been inhibited in tissue culture, such as influenza, ectromelia and EMC.

Both phenylserine and its ester, given subcutaneously, were inactive against influenza A given either intranasally (PR8 strain) or intracerebrally (NWS strain). Phenylserine was also inactive against the PR8 strain, given intranasally, when treatment was given as an aerosol (1 hr. daily using a 2.5% solution).

In the first test against EMC virus, results were: controls 0/20, phenylserine methyl ester 6/20 survivors, but in a second test the ester was inactive. The EMC-Col SK group of viruses is not very satisfactory for *in vivo* testing, since the response of control groups to a fixed dose of virus, given intraperitoneally, sometimes varies with different batches of the same strain of mice, even though these are the same weight. This means that *slight* activity may not be observed simply because in that particular experiment the infection is too severe. The work of Hurst, Melvin, and Peters (1952) on other neurotropic infections of mice showed that a weak therapeutic effect could be lost when the infection was more virulent than expected.

It was obvious that the chief hope of antiviral action *in vivo* lay in reducing the phenylalanine level. The brain is the last organ in the body to suffer amino acid depletion, but the liver protein level can be considerably reduced by a 48 hr. fasting period (Addis, Poo and Lew, 1936). It was hoped to time this depletion so that it occurred when a virus susceptible to phenylserine would normally be multiplying there. Ectromelia was a suitable virus and the mice were fasted at different times. Table III shows the results of these

TABLE III

PHENYLSERINE METHYL ESTER TESTED AGAINST ECTROMELIA VIRUS IN MICE

Treatment, indicated by PS ester, was 2 mg./g./daily subcutaneously, from the day before infection. The mice were infected on day 0. Fasting, with or without PS ester treatment, is indicated.

	Treatment (PS Ester and/or 48 Hr. Fast)	No. Survivors (At Time Indicated)	Average Survival Time (Days)
I	Controls	1/15 (10 days)	7·2
	PS ester	1/14 ,,	7·4
	Fasted −2, −1 days	1/13 ,,	7·6
	,, −1, 0 ,,	1/10 ,,	7·1
	,, −1, 0 (+PS ester)	2/14 ,,	8·1
	,, +3, +4 days	4/5 ,,	9·8
II	Controls	0/22 (9 days)	5·6
	Fasted 0, +1 days	1/22 ,,	6·2
	,, +1, +2 ,,	0/22 ,,	5·8
	,, +2, +3 ,,	1/22 ,,	5·8
	,, +3, +4 ,,	5/22 ,,	6·9
	,, +4, +5 ,,	1/22 ,,	6·3
III	Controls	6/28 (15 days)	9·25
	Fasted +3, +4 days	6/28 ,,	9·6
	,, +3, +4 (+PS ester)	5/28 ,,	9·3
	,, +4, +5 days	3/28 ,,	8·9
	,, +4, +5 (+PS ester)	10/28 ,,	10·5

experiments; in none was $P<0.05$ for the difference in survival times. In experiment III, virus was isolated from all control animal livers, but only from 3/10 fasted and treated animals.

DISCUSSION

The results indicate that *threo*-β-phenylserine exerts its effect against influenza virus during the first half of the latent period, presumably by interfering with the utilization of phenylalanine. It is relatively non-toxic to the host cells investigated compared with, for example, *p*-fluorophenylalanine which was stated to be active against poliomyelitis virus at 0.04–0.1 mg./ml. but cytostatic at 0.1 mg./ml. (Ackerman *et al.*, 1954). It is of interest that the active phenylserine has the same *threo* configuration as chloramphenicol, but, whereas chloramphenicol is the D-(−) isomer, the active phenylserine is the L-(−) isomer, the configuration of naturally occurring phenylalanine. The replacement of the amino-group by $NHCOCHCl_2$ abolishes activity against influenza virus, although this radical is present in chloramphenicol. Chloramphenicol is thought to act at a late stage in the protein synthesis of bacteria (Hahn, Wisseman and Hopps, 1954), but its mode of action on the larger viruses has not been determined.

It is not possible to correlate the antibacterial and antiviral activities of the phenylserines. Early workers did not distinguish between the different isomers, and the test organism and medium varied. The phenylalanine-requiring mutant of *E. coli* K12, used in the assays, was found by us to be unaffected by amounts of phenylserine 100 times that of the phenylalanine present, the concentrations of the latter ranging from 1.0 to 100 μg./tube. Many bacteria synthesize their own phenylalanine, but this amino acid is essential for the growth of animal cells in tissue culture (Eagle, 1955b). Although cells remain sufficiently healthy to support virus growth in the absence of added L-phenylalanine, they do not multiply and in time they degenerate.

Conclusions based on the study of analogues must be assessed cautiously, but this work suggests that phenylalanine is necessary for virus production, at least in the system used here, and that it can be supplied as phenylalanylglycine or glycylphenylalanine. The evidence supports the view that glycylphenylalanine is broken down by virus-infected cells, but there is no conclusive evidence for or against the breakdown of phenylalanylglycine, though this seems likely. It is only possible, from the present work, to refer to virus-infected cells since all the metabolism work was interpreted in the light of virus production, as measured by haemagglutination; there is no information about corresponding events in uninfected cultures.

Although in some of the experiments with ectromelia treated and fasted mice survived longer than the controls, the *in vivo* tests were inconclusive and no result was statistically significant. The antagonism of the antiviral action of phenylserine by phenylalanine at a 1:1 ratio makes *in vivo* activity unlikely. Furthermore, an enzyme present in the liver destroys phenylserine, although the *erythro* form is attacked much more readily than the *threo* (Shaw and Armstrong, 1955).

APPENDIX

THE PREPARATION OF HYDRAZINE AND HYDROXAMIC ACID OF PHENYLSERINE

By J. S. NICHOLSON

Threo-*Phenylserylhydrazide*. — *Threo*-Phenylserine ethyl ester (2 g.) (Shaw and Fox, 1953) in 10 ml. absolute alcohol and 4 ml. of 90% hydra-

zine hydrate were refluxed for 1½ hr. On cooling to 0° C. the hydrazide crystallized ; it was collected and washed with a small volume of absolute alcohol. Recrystallization of the hydrazide (0.9 g., m.p. 135–137° C.) from absolute alcohol gave colourless needles, m.p. 135.5–137.5° C. (Found: C, 55.5 ; H, 6.7 ; $C_9H_{13}N_3O_2$ requires C, 55.4 ; H, 6.7%.)

Threo-*Phenylserylhydroxamic Acid.*—Hydroxylamine hydrochloride (0.695 g.), dissolved in 0.7 ml. water, was treated with a solution of sodium ethoxide from 0.23 g. sodium in 10 ml. absolute alcohol. The filtrate from sodium chloride was refluxed with 2.1 g. *threo*-phenylserine ethyl ester for 2 hr., cooled and diluted with an equal volume of ether. The solid (0.57 g.) which separated was collected, dried and recrystallized from water ; the yield was 0.2 g., m.p. 160° C. (decomp.). Recrystallization from water gave colourless prisms, m.p. 160° C. (decomp.). (Found: C, 55.3 ; H, 6.5: $C_9H_{12}N_2O_3$ requires C, 55.1 ; H, 6.1%.)

The reaction between *threo*-β-phenylserine and benzyl chloroformate in alkaline solution gave a compound, which is believed to be 4-carboxy-5-phenyloxazolid-2-one, crystallizing from ethyl acetate-chloroform in colourless rods, m.p. 202–203° C. (decomp.). (Found: C, 57.8 ; H, 4.3 ; N, 7.0 ; neutralization equivalent 210: $C_{10}H_9NO_4$ requires C, 58.0 ; H, 4.3 ; N, 6.8% ; neutralization equivalent 207.)

The authors wish to thank Dr. Carlo Alberti, of Farmitalia, Milan, for the D and L isomers of *threo*-phenylserine. Dr. N. W. Bristow, Dr. J. S. Nicholson, and Mr. P. Oxley, and members of the Chemistry Division, Research Department, Messrs. Boots Pure Drug Co., Ltd., prepared all the other compounds; Mr. S. S. Randall, of the Biochemistry Division, Research Department, supplied the β-hydroxyglutamic acid. The authors also wish to thank Miss Mary Stephenson for technical assistance.

References

Ackerman, W. W. (1951). *J. exp. Med.*, **93**, 333.
—— and Maassab, H. F. (1954). Ibid., **100**, 329.
—— Rabson, A., and Kurtz, H. (1954). Ibid., **100**, 437.
Addis, T., Poo, L. J., and Lew, W. (1936). *J. biol. Chem.*, **115**, 117.
Bergmann, E. D., Sicher, S., and Volcani, B. E. (1953). *Biochem. J.*, **54**, 1.
Dickinson, L. (1955). *Brit. J. Pharmacol.*, **10**, 56.
Dulbecco, R., and Vogt, M. (1954). *J. exp. Med.*, **99**, 183.
Eagle, H. (1955a). *Proc. Soc. exp. Biol., N.Y.*, **89**, 96.
—— (1955b). *J. exp. Med.*, **102**, 37.
Earle, W. R. (1943). *J. nat. Cancer Inst.*, **4**, 165.
Edmonds, E. J., Volkman, C. M., and Beerstecher, E. (1956). *Proc. Soc. exp. Biol., N.Y.*, **92**, 80.
Fox, S. W., and Warner, C. (1954). *J. biol. Chem.*, **210**, 119.
Francis, T., and Moore, A. E. (1940). *J. exp. Med.*, **72**, 717.
Gard, S., and Heller, L. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 68.
Hahn, F. E., Wisseman, C. L., and Hopps, H. E. (1954). *J. Bact.*, **67**, 674.
Hurst, E. W., Melvin, P., and Peters, J. M. (1952). *Brit. J. Pharmacol.*, **7**, 455, 473.
—— and Hull, R. (1956). *Pharmacol. Rev.*, **8**, 199.
Matthews, R. E. F., and Smith, J. D. (1955). *Advances in Virus Research*, vol. 3, p. 51. New York: Academic Press Inc.
Reed, L. J., and Muench, H. (1938). *Amer. J. Hyg.*, **27**, 493.
Salk, J. E. (1944). *J. Immunol.*, **49**, 87.
Shaw, K. N. F., and Armstrong, M. D. (1955). *Proc. Soc. exp. Biol., N.Y.*, **88**, 673.
—— and Fox, S. W. (1953). *J. Amer. chem. Soc.*, **75**, 3417.
Simmonds, S., Dowling, M. T., and Stone, D. (1954). *J. biol., Chem.*, **208**, 701.
Tyrrell, D. A. J. (1955). *J. Immunol.*, **74**, 293.
Waymouth, C. (1955). *Texas Rep. Biol. Med.*, **13**, 522.