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THE FORMALDEHYDE DERIVATIVES OF AMINO ACIDS AND THE MODE OF ACTION OF A FORMALDEHYDE-TYROSINE DERIVATIVE AGAINST INFLUENZA A VIRUS *IN OVO*

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An effective chemotherapeutic agent against the smaller viruses has not yet been found. The limited activity which has been claimed for a variety of substances (reviewed by Hurst, 1953; Dickinson, 1954) is usually only shown at or near the toxic dose, or else activity is confined to tissue culture or bacteriophage tests. Lack of knowledge about protein and nucleic acid synthesis in general suggests that it will be some time before a rational approach to virus chemotherapy can be made. Some problems of protein synthesis are discussed by Chantrenne (1953) and Synge (1953).

Gale and Folkes (1953) found that chloramphenicol, chlortetracycline and oxytetracycline inhibited protein synthesis by washed suspensions of *Staphylococcus aureus*. These antibiotics are effective inhibitors of the growth of the larger viruses, and it may be, though there is no direct evidence, that they inhibit protein synthesis by the larger viruses. Chloramphenicol also inhibited the multiplication of T1 phage of *Escherichia coli* (Edlinger, 1951; Bozeman, Wisseman, Hopps, and Danauskas, 1954), but only at concentrations affecting bacterial growth. Further evidence that it may be possible to attack viral protein synthesis is suggested by the work of Ackerman (1952) and Ackerman and Maassab (1953), who found that certain α-aminosulphonic acids affected the proliferation of influenza virus. Synthetic lysine polypeptides had slight activity against influenza A and mumps viruses (Rubini, Rasmussen and Stahmann, 1951; Green and Stahmann, 1953). Eaton, Magasanik, Perry, and Karibian (1951) found that the basic amino acids, arginine, lysine, and ornithinine had a slight action against influenza and mumps in tissue culture.

As part of a programme of investigations into the chemotherapy of virus diseases, amino acids and various derivatives (particularly formaldehyde derivatives) have been investigated, using influenza A and a bacteriophage of *Pseudomonas pyocyanea* as test viruses. One of the disadvantages of studying formaldehyde (F) derivatives is that many are very unstable; however, derivatives prepared at 70°, although more complex since more bonds react, are usually more stable. If dissociation occurred the free F formed would be markedly toxic and mask any other activity. However, traces of free F are readily detected on bacteriophage assay plates as zones of host (*Ps. pyocyanea*) inhibition; in such experiments anti-phage activity cannot be assayed.

A formaldehyde-tyrosine preparation (TF) has been found to be active against influenza virus in eggs but not in mice. The mode of action of this substance in eggs is described.

METHODS

Formaldehyde Derivatives.—The methods of Fraenkel-Conrat *et al.* (1945, 1946, and 1948) were used. One g. amino acid in 10 ml. buffered F solution (containing 10 ml. 40% formaldehyde soln. + 60 ml. 0.1 M phosphate-citrate buffer *p*H 8.0) was put in a tightly capped 1-oz. bottle for four days at 70°, with occasional shaking. After cooling, 5–6 vol. ice-cold acetone were added and the precipitate removed, redissolved, and reprecipitated by acetone once or twice (depending on the yield). Supernatant fluids were not investigated. Yields varied, but there was usually sufficient material for testing (Table I); glycine, leucine, and tyrosine gave good yields. The derivatives were usually colourless or yellow oils, but crystalline solids (e.g., valine and β-phenylalanine derivatives) were sometimes obtained. The method for the tyrosine derivative is given in detail later: this was the only compound showing activity. A cysteine derivative, thiazolidine-4-carboxylic acid, was prepared by the method of Ratner and Clarke (1937).

Copper Derivatives.—Amino acids and hydrolysates were boiled with excess basic copper carbonate until there was no further effervescence. After filtration the soluble complexes were evaporated and where possible crystallized from hot water. Insoluble derivatives were not investigated.

TABLE I

ANTI-VIRAL ACTION OF AMINO ACIDS AND THEIR FORMALDEHYDE DERIVATIVES

Amino Acid, etc.	Activity		Formaldehyde Derivatives		
			Yield	Activity	
	Influenza	Phage	(+ to +++)	Influenza	Phage
Glycine ..	0	0	+++	0	0
DL-Alanine ..	0	0	+	0	0
DL-Leucine ..	0	0	+++	0	0
DL-Norleucine	0	0	–	—	—
DL-Isoleucine ..	0	0	+	0	0
DL-Lysine ..	0	0	+	0	0
L-Arginine ..	0	0	++	0	0
DL-Valine ..	0	0	+	0	0
DL-Serine ..	0	0	++	0	0
DL-Proline ..	0	0	++	0	0
DL-Glutamic acid	0	0	+	0	0
DL-Aspartic ,,	0	0	+	0	0
DL-Asparagine	0	0	+	0	0
DL-Tryptophane	0	0	+	0	0
L-Tyrosine ..	0	0	+++	A(4–16)	0
L-Histidine ..	0	0	+	0	0
DL-Methionine	0	0	+	0	0
L-Cystine ..	0	0	+	0	0
L-Cysteine ..	0	0	+	0	0
Glycylglycine	0	0	++	0	0
Polylysine (mol. wt. 1600) ..	0	A(5)	+	0	0
Polylysine (mol. wt. >16,500)	0	A(5)	+	0	0
DL-β-Phenylalanine ..	0	0	++	0	0
Nitroarginine	0	0	+	0	0
Glycocyamine	0	0	++	0	0
Betaine ..	0	0	–		
Taurine ..	0	0	+	0	0
Sarcosine ..	0	0	+	0	0
DL-Benzoylalanine ..	0	0	+	0	0
Acetamide ..	0	0	+++	0	0

A=active. 0=inactive at ½ the dose toxic to the host, or at 10 mg./egg. ()=activity factor expressing the chemotherapeutic ratio. — =no derivative obtained.

Other Derivatives.—The following were prepared: nitroarginine (Kossel and Kennaway, 1911); acetamidomethylene compound of alanine (Fraenkel-Conrat, 1948) and that of leucine by a similar method at *p*H 3.0; hydantoins and *N*-carbamyl derivatives of tyrosine and leucine (Dakin, 1910) and of arginine (Boon and Robson, 1935).

Tests against Influenza A Virus.—0.2 ml. of a series of 4-fold dilutions of a 10% soln., or suspension, of the drug in water were injected into the allantoic sac, unless otherwise stated, of 10–12 day embryonated eggs, followed 1 hr. later by 10–100 egg-infecting doses (EID) of influenza A virus (PR8 strain). After a further 48 hr. incubation the allantoic fluids were tested for their ability to haemagglutinate fowl red blood cells, by the method of Salk (1944). Control eggs had titres (expressed as reciprocals) of 1024 or 2048. Treated eggs with titres less than 10 were titrated for infectivity. All infectivity titres refer to 0.25 ml. volumes of inocula. Tests for possible inhibition of haemagglutination by the drug were made by adding 4 u. haemagglutinin to 0.25 ml. allantoic fluid from treated eggs, plus 0.25 ml. of 0.5% fowl cells. Tests for false haemagglutination by the compounds were made by adding 0.25 ml. fluid from treated eggs to 0.25 ml. blood.

In the tests, groups of three or four eggs were used and activity was only considered significant if it occurred in all eggs of a group at a dose which did not kill the embryo. Many compounds, including F itself, showed sporadic activity at or just below the toxic dose in these, and other, investigations, but the study of such border-line activity needs large numbers of eggs.

Tests against Bacteriophage.—A virulent phage, Pc, of *Pseudomonas pyocyanea* was used as the test virus. The lactate medium and method of Chantrill, Coulthard, Dickinson, Inkley, Morris, and Pyle (1952) were used for quantitative tests, the results of which were expressed as $\frac{\text{mg./ml. inhibiting phage}}{\text{mg./ml. inhibiting host}}$, abbreviated to a factor. For preliminary testing, to rule out inactive compounds, and to note host inactivators (usually due to F liberation), cup plate tests, using lactate medium with 0.5% leucine and 1.5% agar, were employed; the phage and host content were arranged to give almost confluent lysis. After diffusion overnight at 5° plates were incubated for 18 hr. at 37°. Zones of phage inhibition appeared as zones of host growth.

RESULTS

Results appear in Table I and indicate that significant anti-viral activity was shown only by the formaldehyde-tyrosine preparation. Several other formaldehyde derivatives showed anti-influenza activity at the dose toxic to some eggs, but these were not studied.

The copper derivatives of glycine, DL-glutamic acid, DL-alanine, DL-aspartic acid, L-arginine, DL-valine and glycylglycine were all inactive. The *N*-carbamyl derivatives and hydantoins of DL-leucine, L-tyrosine, and L-arginine, the *N*-(*N*-acetamidomethylene) derivatives of alanine and leucine, and thiazolidine carboxylic acid were also inactive.

Polylysine was included in these tests because it had been reported to inhibit influenza A and mumps viruses *in ovo* (Rubini, Rasmussen and Stahmann, 1951; Green and Stahmann, 1953). Both samples tested (mol. wt. 1600 and >16,500) were inactive. When the allantoic fluids of treated eggs were titrated by the haemagglutination technique, no difference in the titres was found. Both samples agglutinated fowl cells, when diluted in saline (down to concentrations of 1 μg./ml.), but

the allantoic fluid of eggs receiving even 3.2 mg. did not cause agglutination. Since the maximum non-toxic dose to eggs was 0.8 mg., the tests were not invalidated. The slight anti-phage action was not impressive in view of the marked antibacterial action.

The Action of Formaldehyde-Tyrosine on Influenza A Virus

TF was the only substance found significantly active against influenza A virus *in ovo* ; the chemotherapeutic ratio was 4–16. The action of TF was almost certainly not due to formaldehyde (F) liberation, for the following reasons:

1. A solution of F was only active at a dose toxic to some of the eggs or at half this dose.

2. The activity of TF solutions was unaffected by boiling, whereas one would expect dissociation to increase.

3. TF was inactive against bacteria in cup-plate tests, whereas traces of F could be detected.

Preparation and Properties.—The preparation required certain experimental conditions. Tyrosine had to be in excess ; reducing its proportion to 1/10th resulted in a yield of yellow crystals, which were inactive. When the reaction temperature was reduced from 70° to 37°, inactive pink crystals were produced. Probably the initial reaction was followed by further coupling with excess tyrosine. Eventually the following process was standardized.

Batches of L-tyrosine (2 g.) were suspended in buffered F (20 ml.) as in the standard process (see Methods). During incubation at 70° a considerable amount of free tyrosine disappeared and the liquor turned first pink and then bright yellow. After cooling and filtering off the unchanged tyrosine the filtrate was cooled to +5° and then added, with vigorous agitation, to six times its volume of cold acetone. The yellow precipitate was centrifuged down, redissolved in the minimum amount of water and again precipitated by cold acetone ; some batches were precipitated three times with acetone. The final precipitate was suspended in dry acetone, filtered off and washed with acetone and ether. For preparing 25-g. batches of TF, 25 separate 2-g. amounts of tyrosine were used, as the yields were poor if larger quantities were handled. Yields of the reprecipitated material were usually about 60% of the weight of the original tyrosine.

The active substance was a yellow amorphous powder which melted gradually, just above room temperature, to a brown liquid. It became resinous on standing for some weeks at room temperature, but this gummy material was still active. It was very soluble in water, a 25% solution being readily prepared, but insoluble in common organic solvents.

Action on Influenza A Virus in ovo

Nine batches of TF, prepared on different occasions, all showed activity at 10 mg./egg ; up to 80 mg. of some batches could be given via the allantoic sac or yolk sac without causing death of the embryo. One batch was active at all doses from 5–80 mg./egg. Its action was not affected by serum broth and it was active when given 1, 2, and 4 hr. after, or 24–48 hr. before, infection. Eighty mg./egg via the yolk sac was inactive against virus inoculated via the allantoic sac or chorioallantoic membrane.

Twenty mg. TF/egg was active against 100 or 10,000 EID virus both in the 48 hr. allantoic sac test and also when the test was prolonged to 96 hr. Against either dose of virus, the haemagglutinin titres of allantoic fluid from treated eggs were always <2, whereas controls were 1024–2048. For the 100 EID inoculum, the infectivity titres were: treated 10^0–10^2, controls 10^6–10^8. A second dose of virus, given to eggs that had been treated (and infected) for 48 hr., did not grow. The washed cells of the chorioallantoic membrane of treated eggs were still capable of supporting the growth of virus in tissue culture (Mrs. M. J. Thompson, personal communication). The treated allantoic fluid did not inhibit haemagglutination.

Action on Extracellular Virus in ovo (*Contact Test*)

1. Five eggs, infected 48 hr. previously and incubated, were treated with 20 mg. TF/egg and incubated for 24 hr. to find the effect on free virus. The titre of the allantoic fluid fell from 10^6–10^7 to 10^4 ; haemagglutination titres fell from 1024–2048 to <2, <2, 2, 2, 4. Control eggs showed no fall in haemagglutination titre and were still infective at 10^{-6}.

2. Eggs which had been given 20 mg. TF/egg 1 hr. previously were infected with 10,000 EID virus and re-incubated for 1 hr.; the allantoic fluids were then removed and 0.25 ml. samples injected into groups of 3 eggs. No virus was detected in 2 treated eggs, but it was present in the 3rd egg. The 3 control eggs were infective at 10° but not at 10^{-2} (except for 1/3 eggs). This drop was expected because of adsorption on to the cells, the tests being designed to indicate effects on adsorption. Using 10–100 EID, no virus was detected in either control or treated eggs at 1 hr.

Action on Extracellular Virus in vitro (*Contact Test*)

Neither 50 mg. TF/ml. nor 10 mg./ml. inactivated 1,000 EID virus within 15 min. at 37°

in M/15 phosphate-citrate buffer, *p*H 7.2. However, when the contact period was 1 hr. the titre fell to 10 EID. These experiments were typical of many *in vitro* contact tests, giving different results according to the conditions.

Chemotherapeutic Tests in Mice

TF was not toxic to mice when given subcutaneously in a single dose of 5 mg./g. Small-scale *in vivo* tests against influenza A were done, giving the drug subcutaneously, intraperitoneally, intranasally, and as an aerosol, in varying doses.

Mice, in groups of 10 or 20, were infected with mouse-adapted influenza A virus (PR8) intranasally under light anaesthesia. Treatment was given twice daily, starting 3 hr. before infection, and continued for 7–10 days, when survivors were killed and the lungs assessed for consolidation (values 0–4, death with lungs totally consolidated, 5). A daily dose of 4 mg./g. was toxic parenterally, but 2 mg./g. was well tolerated. The drug was very toxic when given as a 2% solution intranasally. Given as an aerosol (1% solution for 1 hr. twice daily), it was better tolerated but had no protective value. A preliminary test, giving the drug subcutaneously in 2 daily doses of 1 mg./g. each, had suggested a reduction of lung lesions (controls, 6/10 survivors, average lung assessment 3.6; treated, 8/10 survivors, average lung assessment 1.7; $P < 0.01$ for difference in lung-lesion assessment). These results were not confirmed when the test was repeated (controls, 8/20 survivors, average lung assessment 4.2; treated, 4/21 survivors, average lung assessment 4.7).

Discussion

The commonly used allantoic sac test of anti-viral activity is useful if regarded as a primary screening test. The few compounds showing activity can then be investigated further; the results usually eliminate the need for large-scale animal tests, especially if the mouse toxicity figures indicate that the concentrations active in eggs are not likely to be attained in animals.

Ignoring the host defence system, which is not well developed in the egg, the possible modes of action of an anti-viral compound are either by the direct inactivation of extracellular virus (contact action), or by the prevention of adsorption, multiplication or release of virus. If the drug is given by the same route as the virus, even several hours after infection, a contact action cannot be excluded. The usual arbitrary *in vitro* contact tests, varying in time and medium, may be very misleading, particularly as these tests have usually to be made on a high concentration of virus (to allow dilution of the drug before injection into the egg). In chemotherapeutic tests only 10–100 EID are given, and even a slight contact action may reduce the virus concentration to a sub-infective level. A compound exerting its effect by contact action on free virus can be detected by the fact that no free virus is present when the tests are read at 48 hr. The only alternative explanation for complete absence of free virus assumes that the unadsorbed fraction of the inoculum contains too little virus to be detected, and also that viral multiplication (or release) is completely suppressed.

An effect on adsorption can be detected by finding the level of virus 1–4 hr. after the injection of an inoculum sufficiently heavy to leave a measurable residual level after adsorption. If no adsorption occurs the initial inoculum remains free and is higher than in control eggs. Henle (1953) stated that adsorption was practically complete in 1 hr., but Horsfall (1954), whose results had not been published when the TF work was done, found the minimum free virus level at 4 hr. If the virus titre is lower than in control eggs a contact action is again indicated.

If the virus titre at 48 hr. is equal to or above the residual virus at 1 hr. but significantly less than in control eggs, then an inhibition of viral multiplication (or release) is indicated—assuming that the cells of the allantoic sac are still capable of supporting viral growth (tested in tissue culture). The titre may rise on further incubation, indicating an effect on the *rate* of multiplication, or possibly the gradual inactivation of the drug; this effect, however, may be associated with a drug concentration which is toxic to the host and is then of little interest. On all types of action there may be a superimposed contact action and this is illustrated by the results for TF, which was one of the very few compounds active in the primary test. It had a chemotherapeutic ratio in eggs of 4–16 and was active in the presence of serum broth. It was stable and easy to prepare and therefore suitable for further investigation.

TF-treated eggs contained small amounts of free virus at 48 hr., the amount being greater than at 1 hr. after infection; for 10–100 EID inocula no virus was found at 1 hr., but the titre was 10^0–10^2 at 48 hr., compared to 10^6–10^7 for control eggs, and it did not rise on further incubation. Contact action was indicated by: (1) the results at 1 hr., after 10,000 EID virus (titre less than in control eggs); (2) the *in ovo* contact test. It is not possible to discriminate between an effect on adsorption and

one on multiplication, in view of the contact action. However, the ratio of titres of control fluids to TF treated fluids in the *in ovo* contact test was 10^6–$10^7/10^4$ by infectivity (1024–2048/2 by haem-agglutination), while the corresponding ratio in the chemotherapeutic tests was 10^6–$10^7/10^0$–10^2. The difference in the ratios, together with the slight increase in virus between 1 and 48 hr., suggests that only part of the action of TF was due to inactivation of free virus.

TF was inactive when given by the yolk sac. It may be a large molecule, since it appears to be formed by repeated reactions of excess tyrosine with intermediate products; permeability effects may account for its inactivity when given by this route. The effective concentration, calculated from egg studies, could only just be (theoretically) attained in mice at the maximum non-toxic dose; for this reason only small-scale animal tests were done.

Summary

1. Amino acids and derivatives (mainly formaldehyde derivatives) were tested against influenza A virus in eggs and against a bacteriophage of *Ps. pyocyanea*.

2. A formaldehyde-tyrosine derivative was active against influenza *in ovo*; the chemotherapeutic ratio was 4–16. It was not active in mice.

3. Studies on the mode of action of this substance suggested that only part of the effect was due to inactivation of extracellular virus.

4. By considering the results of various *in ovo* experiments it is often possible to decide whether a substance found active *in ovo* is worth testing in animals.

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