Some Effects of Certain Resorcinol Derivatives on Eastern Equine Encephalomyelitis Virus. Factors Influencing *in vitro* Screening Technic.* (20119)

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The present report is concerned with compounds which have a marked *in vitro* inactivating effect on the Eastern Equine Encephalomyelitis (EEE) virus and which are for the most part non-toxic, ranging in acute toxicity from 15 mg to 50 mg/100 g body weight.

The study has been divided into two phases. In the first, the conditions of interaction were investigated by noting the effects of temperature, concentration, pH and protein in the chemical-virus system. On the basis of this information, the second phase, *in vitro* screening of various compounds, was possible under simple standard conditions.

Materials and methods. The EEE virus used in this study was obtained through the courtesy of Dr. Karl Habel, U. S. Public Health Service. It was maintained at −30°C as pools of 20% mouse brain suspensions (by weight) in distilled water. Throughout the investigation, CFW mice (18-20 g) were used both as source animals and as indicators for the presence of virus. The specific nature of the virus pools was frequently checked by neutralization test with known antiserums. In preparing virus for each inactivation experiment a vial of a given pool was thawed, the emulsion diluted 4 times with sterile distilled water and centrifuged at 13,000 r.p.m. for 5 minutes in a Servall angle centrifuge. Model SS1. The supernate was diluted 5 times to a final virus dilution of 1:100 and was maintained in cracked ice until mixed with each compound to be tested. To make certain that deterioration of virus would not be confused with inactivation, the control solution was always the last one to be mixed and therefore the last to be injected into mice. Under the usual experimental conditions, the titer of the virus pool was approximately $10^{-8.0}$. Of the *14 resorcinol derivatives*, all but the 4-chloro- and 4-bromo-resorcinol† were synthesized in this laboratory. Agreement of melting points (where possible) with previous reports was taken as a criterion of purity. In the case of the substituted fluorescein dyes, purity of the compounds was established by quantitative analysis performed by an independent laboratory and by paper chromatography. The 2,7 diiodotetraiodofluorescein is a new compound and the method of synthesis of this compound as well as new methods of synthesis for the 5-nitro-beta-resorcylic acid, 3,5 diiodo-beta resorcylic acid and octoiodofluorescein were developed in this laboratory and are being reported by one of us(1).

Inactivation technic. One-half gram of the compound to be tested was partially dissolved at room temperature (22-24°C) in 2.0 ml of sodium hydroxide (1.0 M) plus 23 ml disodium hydrogen phosphate solution (M/15). Twenty-four to 30 hours later, when the inactivation experiment was carried out, this 2% solution was diluted to 1% with distilled water and the desired pH adjustment was made by the addition of hydrochloric acid or sodium hydroxide solution. To test inactivation, 1:100 dilutions of both virus and compound were mixed and the 1:200 compound-virus solution was allowed to incubate under specific experimental conditions. The incubation took place in the absence of light so that photodynamic action would not be a factor. When virus activity was to be tested at predetermined time intervals, 1.0 ml of the compound-virus mixture was diluted with 4.0 ml of 10% inactivated rabbit serum and 0.03 ml of 10-fold serial dilution was injected intracerebrally into 5 CFW mice per dilution. The LD_{50} of each chemical-virus system was thus deter-

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† These compounds were obtained from the Eastman Kodak Co.

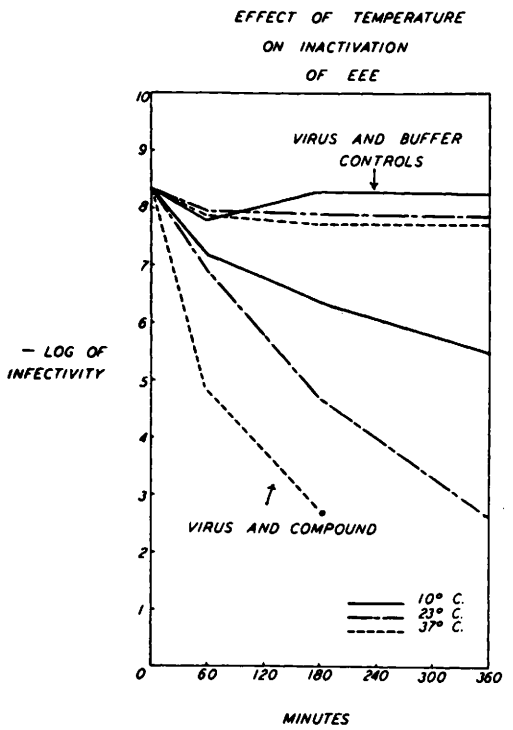

FIG. 1. Lower 3 lines represent virus and 2,7-diiodotetraiodofluorescein mixtures. Corresponding controls are represented by the upper 3 lines. Test periods are 60, 180 and 360 min. (At 37°C the testing limit was reached at 180 min.)

mined. The controls consisted of virus in buffer solution (Na_2HPO_4, M/15) adjusted to the same pH as the compound to be tested. All virus mixtures were handled in the same manner, each injection of animals per dilution taking place within 10 minutes of withdrawal of the aliquot to be tested. The inactivation effect of a given chemical was then calculated by comparing LD_{50} values of the chemical and virus with control and virus. With this technic, *in vitro* inactivation of approximately 6 logs or 1 million mouse intracerebral units could be measured.

Experimental. I. Conditions affecting inactivation of EEE virus with 2,7-diiodotetraiodofluorescein. Effect of temperature on inactivation. Using the routine technic, the course of inactivation of EEE by 2,7-diiodotetraiodofluorescein was followed at 3 different temperatures: 10°, 23°, and 37°C. The results seen in Fig. 1 show the greatest inactivation occurred at 37°C. It is noteworthy that the order of inactivation after one hour at 37°C has, for all practical purposes, proceeded to completion, since the initial LD_{50} of the chemical-virus mixture has been reduced within that time from 500 million infectious mouse units per 0.03 ml to 95 thousand infectious mouse units. This represents a reduction of infectivity of over 99%.

Effect of dye concentration. Where concentration of dye was reduced from 1.0% to 0.4% (Table I), other factors remaining constant, it was apparent that the greatest degree of inactivation occurred with the 1% solution, the highest concentration tested. The different inactivating effects of the 3 dye concentrations were more clearly seen at the 360-min. test interval.

Effect of pH. 2,7-diiodotetraiodofluorescein solution was routinely prepared so that a final pH range of approximately 6.0, 7.0, 8.0, and 9.0 was obtained in 4 aliquots. Comparable virus controls were prepared for each pH. The compound-virus and control-virus mixtures were incubated at 22°-24°C for one, 3, and 6 hours, and virus activity was measured at each of these time intervals. As can be seen from Fig. 2, the most rapid and significant inactivation occurred at pH 7.0 and 8.0. At pH 9.0 and pH 6.0, the action of dye on virus was masked because of the rapid deterioration of the control in one hour consistent with the results of other investigators(2,3).

Effect of added protein. To study the effect of protein on inactivation, 1% 2,7-diiodotetraiodofluorescein solution was adjusted to pH 8.0, as above, and diluted at room temperature (22°-24°C) with equal parts of virus in water, virus in 25% rabbit serum, or virus in 50% rabbit serum. Table II illustrates

TABLE I. Effect of 2,7-diiodotetraiodofluorescein Concentration on Inactivation of EEE Virus.

Inactivation mixture (% dye and virus)	Change of LD_{50}* with time (min.) 60	180	360
1.0	6.1	4.1	3.3
0.7	6.3	5.5†	4.7
0.4	6.5†	5.5†	5.3
Control (Na_2HPO_4, M/15) and virus	7.7	7.5	7.3

* Negative log.
† Estimated LD_{50} (no end point).

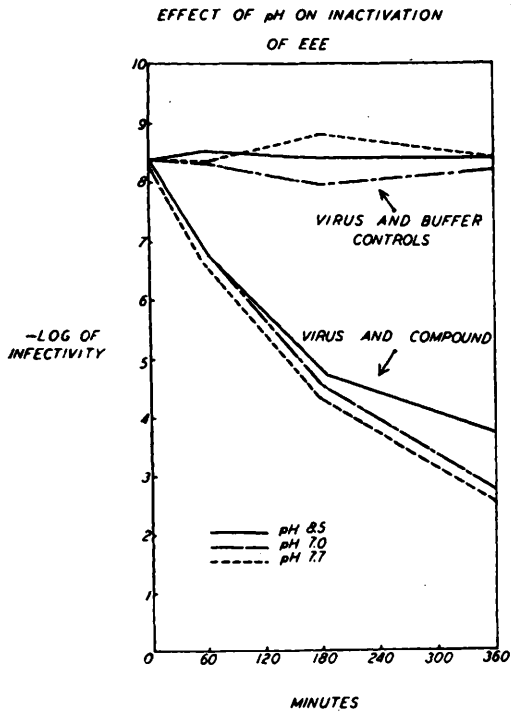

FIG. 2. Lower 3 lines represent virus and 2,7-diiodotetraiodofluorescein mixtures. Corresponding controls are represented by the upper 3 lines. Test periods are 60, 180 and 360 min.

the inverse relationship between the degree of inactivation and the amount of protein present in the dye-virus mixture. It is to be noted that the routine dye-virus mixture in water inactivated the virus at the same rate as in the previous experiment carried out under similar conditions, *i.e.*, a reduction of one and 4 logs of virus activity occurred at one and 3 hours respectively, while complete measurable inactivation was seen at 6 hours. In view of the known combining power of serum for halogenated fluoresceins(4-6), these results were not surprising.

TABLE II. Influence of Protein on Inactivation of EEE Virus by a Fluorescein Dye.

Inactivation mixture	Change of LD_{50}* with time (min.) 60	180	360
Dye and virus in:			
water	6.9	4.3	<2.5†
12.5% rabbit serum	7.9	6.9	6.3
25% " "	7.5	7.1	>6.5†
Control (Na_2HPO_4, M/15) and virus	7.9	8.3	7.9

* Negative log.
† Estimated LD_{50} (no end point).

II. Screening of resorcinol derivatives by inactivation of EEE virus. As a result of the first phase, it was apparent that a major degree of *in vitro* inactivation occurred when the EEE virus and 2,7-diiodotetraiodofluorescein were mixed at pH 8.0 and remained in contact for 3 hours at room temperature (22°-24°C). Therefore, to obtain comparative data, screening of other compounds was carried out under the same experimental conditions.

Inactivation of EEE by resorcinol derivatives. Compounds with heterogeneous groupings were selected so that information could be obtained concerning the specific effect substitution might have on the process of virus inactivation. As can be seen from Table III, of the 6 compounds tested in this group, the greatest degree of inactivation was obtained in the 3,5-diiodo-beta-resorcylic acid-virus system with an LD_{50} change from $10^{-7.9}$ to $10^{-4.7}$ within the 3-hour test period. The only other inactivation of possible significance occurred with the use of 4,6-diiodoresorcinol. As in phenolic chemistry(7), where the order of reactivity of the halogens increases as one moves down the periodic table, the bromo and chloro compounds were not active against the EEE virus under the given experimental conditions. Using the same screening technic, a group of fluorescein dyes was also tested. In Table IV, it can be seen that the iodine molecule again is a significant factor in contributing to the inactivating capacity of the fluorescein nucleus. No attempt is made to differentiate quantitatively between chlorine and bromine in this respect, because the study has been carried out on a per cent concentration rather than on the basis of mole weight. However, both bromine and chlorine were not conspicuous in their activity. It is also of interest that the largest molecule, *i.e.*, the octoiodofluorescein with a molecular weight of more than 1300, gave the most dramatic inactivation, even though its concentration was only 0.007 M.

Discussion. The present study has been limited to a simplified *in vitro* method and has attempted to demonstrate the usefulness of this technic. The need for studying various

TABLE III. Inactivation of EEE Virus by Resorcinol Compounds.*

Compound	Mole wt	Moles/ 1% sol.	LD_{50} (neg. log value)	Acute toxicity (min. lethal dose), mg/100 g body wt
5-Nitro-beta-resorcylic acid	198.12	.051	7.5	35
4-Chlororesorcinol	144.57	.069	7.3	15
4-Bromoresorcinol	189.03	.053	7.1	45
2,4-Dihydroxyacetophenone	152.14	.066	7.5	22
3,5-Diiodo-beta-resorcylic acid	405.96	.025	4.7	>50
4,6-Diiodoresorcinol	361.95	.028	6.5	40
Control (Na_2HPO_4, M/15)			7.9	

* pH of compounds = 8.0.
Contact period between virus and compound = 3 hr. Exp. at 22°-24°C.

TABLE IV. Inactivation of EEE Virus by Fluorescein Dyes.*

Compound	Mole wt	Moles/ 1% sol.	LD_{50} (neg. log value)	Acute toxicity (min. lethal dose), mg/100 g body wt
Fluorescein	332.30	.030	8.3	50
4,5-Diiodofluorescein	584.14	.017	6.3	40
Tetrachlorofluorescein	470.14	.021	5.9	35
Tetrabromofluorescein	647.98	.015	7.5	40
Tetraiodofluorescein	836.28	.012	5.7	45
4,5-Diiodotetraiodofluorescein	1088.12	.009	5.1	35
2,7-Diiodotetraiodofluorescein	1088.12	.009	6.3	30
2,4,5,7-Octoiodofluorescein	1339.96	.007	3.5	40
Control (Na_2HPO_4, M/15)			8.3	

* pH of compounds = 8.0.
Contact period between virus and compound = 3 hr. Exp. at 22°-24°C.

conditions of virus-chemical interaction is apparent since failure to consider this factor has resulted in negative reports for such acid dyes as eosin, eosin B, eosin Y, erythrosin and rose bengal(8-11).

From the structure of the molecules observed in this study, it seems that the most active molecules contain iodine atoms as shown by the high inactivating capacity of 4,6-diiodoresorcinol, 3,5-diiodo-beta-resorcylic acid and octoiodofluorescein. That the inactivation by the iodinated resorcinols is not due to free iodine is evident from the following. Greater inactivation occurred at pH 7.0 and 8.0 than at 9.0, the pH at which iodine is more easily freed. Furthermore, resorcinol derivatives without iodine have been found to possess great inactivating capacities(12). However, iodination enhanced the inactivating capacity of resorcinol derivatives to a greater extent than other forms of halogenation.

Conclusions. 1. Various resorcinol derivatives of a non-toxic nature have been shown to be active against EEE virus under *in vitro* conditions. 2. Some factors affecting inactivation of EEE virus by 2,7-diiodotetraiodofluorescein have been presented and their importance in an *in vitro* study on compound-virus interaction stressed. 3. An *in vitro* screening technic has been demonstrated permitting the study of the effect of compound configuration on virus activity.

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Chelators and Resinous Exchangers. (20120)

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Cation exchange resins of the carboxylic and sulfonic types have found wide application in medical practice for the purpose of sodium removal in cardiac decompensation cases. A major deterrent to a still broader use is the bulk of such agents required for therapeutic efficacy. Clearly, any improvement in capacity would tend to reduce required intake and hence increase patient cooperation.

It was postulated that capacity would be augmented through the removal by chelating agents of polyvalent cations leaving only the monovalent cations for exchange with the resin. Even if this ideal state could be approximated, improvement should ensue. Ethylenediamine tetra-acetic acid or any similar agent will chelate only with polyvalent cations and it is these ions which possess the greater affinity for cation exchange resins, and therefore reduce *in vivo* capacity.

Experimental. The experimental design paralleled in large measure that employed by McChesney and his associates(1-3), Ch'en and Freeman(4). and others. Natrinil, a commercial product, which is a purified cation exchanger carboxylic in a very finely divided state (90% through 200 mesh), 20% in the potassium cycle. and 80% in the hydrogen cycle was used. The *in vitro* capacity was *ca.* 10.0 meq./g. The ethylenediamine tetra-acetic acid was a very pure sample which was used without further purification. The diet had the following composition:

	g
Casein	1500
Salt mix (Hubbel, Mendel, Wakeman)	300
Crisco	1050
Sucrose	3000
Cod liver oil	225
Sodium chloride	75
Yeast	600
Roughage or resin as desired	750
Total	7500

As indicated above, any quantity of roughage may be replaced by resin as the experiment would require. In this way the total quantity of bulk was kept roughly constant. The resin was incorporated into the diet at a 5% level and the EDTA when used at a 1% level.

A pair of rats was placed in each metabolism cage and given the experimental diet and water *ad libitum* for 3 days, were then placed in clean cages and the diet was continued with careful check on the food consumption. The feces were collected every day and pooled until the end of the experiment. The urine was allowed to accumulate in containers containing 3 ml of concentrated nitric acid. The rats were kept on this diet for 4 days and were then placed in clean cages on the same or new diet for the conditioning period (3 days). The collection was made in the usual way. The feces were thoroughly dried at 80-105°C, then ground and thoroughly mixed.

After a review of the literature on flame photometry it was decided to check the results reported by Mosher *et al.*(7) and the agreement was good. Methods were then developed using the idea of radiation buffers as proposed by West *et al.*(8). It was found that the results obtained by this procedure were completely reproducible. Methods for the various elements are summarized briefly below. The urine was checked to make certain that it was