ratio of 1:2 made up at *p*H 6, stored for several hours, and then adjusted to 5.5 just before it was used had a small but measurable suppressing effect (about equal to the 1:½ mixture at *p*H 7).

Obviously both the molarity of the ascorbic acid and the *p*H of the mixture are important factors. The latter is borne out by the fact that treatment of root tips with mixtures of colchicine and ascorbic acid at *p*H 5.5 produces an enhancement of the colchicine effect, the degree of which is dependent on the molarity of the ascorbic acid. Interestingly enough, pretreatment of root tips with ascorbic acid at two times the molarity of colchicine had virtually the same effect as combining the two compounds at *p*H 5.5 before use. While the latter might be considered to be an in vitro effect, the former must be an in vivo one.

Obviously, before a compound is designated as an enhancer or antagonist, the conditions of treatment must be stated. L-Ascorbic acid falls into both categories, depending, in part at least, on *p*H. However, alkaline *p*H's which produce rapid hydrolysis of the lactone ring (*8*) render the ascorbic acid ineffective. The effect of L-ascorbic acid on the colchicine reaction is determined by three factors (i) the *p*H, (ii) the relative molarity, and (iii) the integrity of the ascorbic acid molecule. Exactly how these factors are related mathematically remains to be determined.

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18 December 1957

Reaction of Epinephrine with Ethylenediamine

The condensation of epinephrine with ethylenediamine (EDA) to form a fluorescent product was first reported by Natelson, Lugovoy, and Pincus (*1*). Weil-Malherbe and Bone (*2*) adapted this reaction to the fluorimetric quantitation of epinephrine and norepinephrine in blood.

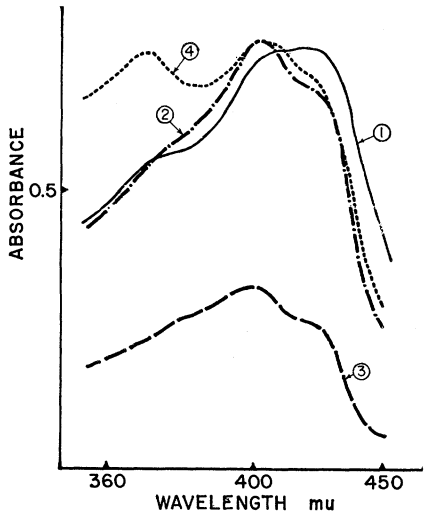

Fig. 1. Absorption spectra of fluorescent derivatives in isobutanol. Curve 1 is the product from 0.8 μM epinephrine, 1×10^{-2} mole EDA, 1×10^{-3} mole EDA dihydrochloride; curve 2 is the product from 0.8 μM epinephrine, 1×10^{-3} mole EDA, 1×10^{-4} mole EDA dihydrochloride; curve 3 is the product from 0.8 μM epinephrine, 1×10^{-4} mole EDA, 1×10^{-5} mole EDA dihydrochloride; curve 4 is the product from 0.8 μM epinephrine, 1×10^{-2} mole EDA, 1×10^{-3} mole EDA dihydrochloride, heated at 70°. Spectra were obtained using a Beckman DK-2 recording spectrophotometer.

While considerable attention has been given to improving the method of Weil-Malherbe and Bone, little work has been reported on the mechanism of this reaction and the nature of the products formed. Burn and Field (*3*) reported the formation of two fluorescent derivatives from norepinephrine by heating at 50° to 70°C with 5 percent EDA for 1.5 to 2 hours at *p*H 11. They obtained two fluorescent derivatives from epinephrine under similar conditions.

A study of the reaction of various catechols with EDA is underway in our laboratory. As part of this study, the reaction of epinephrine with EDA was carried out under various conditions of temperature, *p*H, and EDA concentration, and the products were examined by spectrophotometric and chromatographic techniques. The absorption spectrum of the products obtained under the conditions established by Weil-Malherbe and Bone (*2*) is represented by curve 1 of Fig. 1. The peaks at 400 and 415 mμ and the shoulder at 370 mμ each represent a different fluorescent derivative as shown by chromatographic separation on filter paper (*4*) and by spectrophotometric measurement of the eluted products. The compound with $\lambda_{max.}$ 370 mμ had an R_f value of 0.22 and fluoresced blue-white; that with $\lambda_{max.}$ 400 mμ, R_f 0.35, fluoresced blue-green; and the derivative with $\lambda_{max.}$ 415 mμ, R_f 0.49, fluoresced yellow.

Changes in EDA concentration affected the relative proportion of the fluorescent derivatives formed in this reaction. A stepwise reduction of the EDA concentration resulted in a gradual increase in the ratio of the absorbance at 400 mμ to that at 415 mμ. Curve 2 of Fig. 1 represents the effect of a tenfold decrease in EDA concentration. A 100-fold decrease in EDA concentration resulted in lesser amounts of all three derivatives (curve 3, Fig. 1). The highest EDA concentration (curve 1, Fig. 1) gave a *p*H of 10.6. All other concentrations were adjusted to this *p*H with saturated trisodium phosphate solution.

The effect of increased temperature is illustrated by curve 4 in Fig. 1. At 70°C a significantly greater amount of derivative with $\lambda_{max.}$ 370 mμ was formed. Heating for longer than 20 minutes at 50°, 70°, or 100°C decreased the yield of all three derivatives.

Maximum amounts of the blue-green and yellow fluorescent compounds were formed at *p*H 10.6, lower yields being obtained at *p*H 9.5 or 11.0.

Weil-Malherbe and Bone furnished evidence that adrenochrome is an intermediate in this reaction (*2*). This fact has been verified in the present study. Adrenochrome and epinephrine yielded identical products with EDA as demonstrated by absorption spectra and paper chromatography.

Bubbling oxygen through the reaction mixtures increased only slightly the yields of fluorescent material. In fact, substitution of adrenochrome for epinephrine, in which case oxygen is not required (*2*), does not increase the yields of fluorescent compounds. This is contrary to the results of Burn and Field (*3*) who reported that the oxygen dissolved in the solution was not sufficient for maximum yields of fluorescent material.

Norepinephrine, in contrast to epinephrine, formed only two fluorescent derivatives under the same conditions; one having R_f 0.33 fluoresced blue-green, the other, R_f 0.49, fluoresced yellow (*4*). The spectrum of this fluorescent material was similar to that reported by Burn and Field (*3*), which showed a single peak at 420 mμ in isobutanol. The difference in the reaction of epinephrine and norepinephrine with EDA cannot be explained at this time (*5*).

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4. Fluorescent material from 900 mg of catechol

amine was streaked on Whatman No. 3 MM paper and developed in 0.2*N* ammonia saturated with isobutanol according to the method of I. Gray and J. G. Young [*Clin. Chem.* 3, No. 4, 239 (1957)].

5. A portion of these data was presented at the 67th meeting of the Tennessee Academy of Science, 22 Nov. 1957. This work was supported in part by a grant from the National Heart Institute.

23 December 1957

Plaque Morphology and Pathogenicity of Vesicular Exanthema Virus

Two recent reports have described a correlation of plaque-type mutants of polioviruses with reduced virulence for monkeys and mice, respectively (*1*). However, variation of plaque morphology with antigenic type has not been described. This report describes intertype variations in plaque morphology among seven types of vesicular exanthema of swine virus, as well as intratype variations which have been correlated with differences in virulence for the natural host.

Plaque formation is obtained by infecting monolayers of a transmissible swine kidney cell, PK-2a (*2*), by means of the prescription bottle method of assay (*3*). The nutrient agar is similar to that described by Youngner (*4*). After incubation of infected cultures at 36° or 37°C, plaques can be seen as early as 24 hours. These continue to increase in size and number for 7 to 10 days, although 90 to 95 percent of the plaque count is obtained within 96 hours. Marked variation in plaque morphology is observed not only between virus types but also within virus types. Characteristic plaques of two of the seven virus types are shown in Fig. 1. With the exception of the G_{55} virus, two types of plaques are seen: a large, clear, round plaque which appears after 24 to 48 hours' incubation and increases in diameter to 5 to 8 mm within 96 hours, and a minute, opaque plaque, frequently irregular in shape, usually not visible until 72 to 96 hours have passed, which rarely exceeds 1.5 mm in diameter, even with 7 to 10 days' incubation. Pronounced differences in the ratio of the number of minute and large plaques, as well as in the size of plaques, are found between virus types. Some of the plaque characteristics observed are listed in Table 1.

The theoretical possibility that the large or minute plaques might be produced by a contaminating virus was excluded by the following series of experiments with E_{54} type virus. Stocks of "pure" large plaque formers (Lpf) and minute plaque formers (Mpf) of E type virus were prepared after three successive plaque purifications. The stocks could be considered pure only in a relative sense. As yet there is no suitable selective technique by which a small number of large plaque formers can be detected in the presence of a large number of minute plaque formers, and vice versa. Thus, viral suspensions which contained 1 large plaque former among 1000 minute plaque formers or 1 minute plaque former among 1000 large plaque formers were considered 99.9 percent pure. To determine whether both plaque variants were capable of producing vesicular exanthema, groups of swine were inoculated intradermally on the lip with 10^7 plaque-forming units of wild type (mixed) E_{54} virus, plaque purified E (Lpf) and purified E (Mpf). The ratios of minute plaque formers to large plaque formers in these three inocula were approximately 50:1, 0.001:1; and 1000:1, respectively. Temperatures were taken daily, and the presence or absence of primary and secondary vesicles was noted over an 18-day period. When possible, fresh vesicle coverings were obtained to determine the plaque type of the recovered virus. Two weeks after inoculation all animals were bled to obtain convalescent serum for neutralization tests.

Inoculation of the wild type virus and of the large plaque formers produced typical vesicular exanthema in four out of four animals, especially severe in the case of the latter. Four animals which received 10^7 plaque-forming units of minute plaque formers (which may have contained as many as 10^4 large plaque formers) exhibited no elevation of temperature over a 7-day period after infection, but each developed a single small lesion at the site of inoculation on the 6th or 7th day. No secondary lesions were observed. The absence of a febrile reaction prior to vesicle formation, the mildness of the primary lesions, and the absence of secondary vesicles constitute a picture of extreme atypical vesicular exanthema. These results indicate that the large plaque former is highly virulent for swine and that the minute plaque former either is essentially avirulent or is greatly reduced in pathogenicity.

Suspensions were made of vesicle coverings from infected swine and tested for plaque type of the recovered virus. Only the large plaque former was recovered, even from animals infected with the minute plaque former. This result substantiates the hypothesis that the large plaque former is the virulent virus particle and suggests that the mild disease produced in the swine inoculated with the minute plaque former was actually caused by the 0.1 percent of the large plaque former in the inoculum.

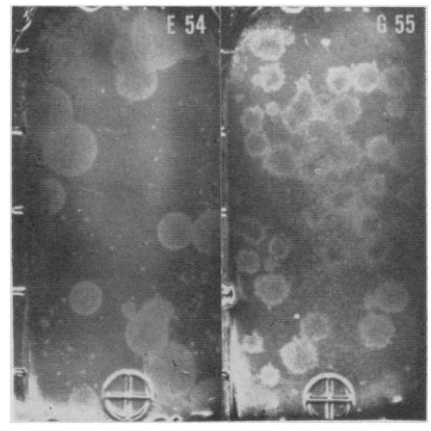

Fig. 1. Plaque morphology of vesicular exanthema of swine virus, types E_{54} and G_{55}, on transmissible swine kidney monolayers.

Cross-neutralization tests carried out by plaque assay method demonstrated the antigenic identity of the large plaque former and the minute plaque former. Convalescent sera from the three groups of infected swine were diluted 1 to 50, mixed with an equal volume of diluent containing about 10^8 plaque-forming units of either wild type E_{54} virus, E (Lpf) or E (Mpf), held 1 hour at room temperature, and assayed for residual infective virus. Serum from each group of infected swine neutralized 90 to 95 percent of the plaque-forming activity of all of the three virus suspensions, indicating close immunological relationship.

There appears to be no published report of the occurrence of such extreme differences in plaque morphology among different antigenic types of a single virus

Table 1. Characteristics of plaque variants of vesicular exanthema of swine virus on transmissible swine kidney cells.

Virus	Plaque size at 96 hr (mm)		Hour when 90% plaque count was obtained	No. of minute plaques per large plaque
	Large	Minute		
		Group I		
A_{48}	5–10	1	72	2
D_{53}	5–8	1	72	10
E_{54}	5–10	0.5	96	100
G_{55}	5–7		72	< 0.001
		Group II		
B_{51}	2–3	1	96	100
C_{52}	2–4	1	96	100
F_{55}	2–3	0.5	72	1–3