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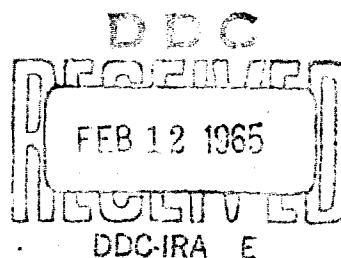
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# TECHNICAL MANUSCRIPT 189

## PROTECTIVE EFFECT OF THIOUREA ON ULTRAVIOLET-IRRADIATED EEE VIRUS

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TECHNICAL MANUSCRIPT 189

PROTECTIVE EFFECT OF THIOUREA ON  
ULTRAVIOLET-IRRADIATED EEE VIRUS

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#### ABSTRACT

Inactivation of suspensions of partially purified eastern equine encephalitis (EEE) virus by ultraviolet light was markedly retarded by the addition of thiourea. In the presence of 0.5 M thiourea, infectivity was reduced only 0.5 log in 10 minutes by the same UV radiation that produced a 7-log decrease in titer in 2 minutes in control samples. Although thiourea absorbs strongly in the ultraviolet region, the protection is not related entirely to the light-absorbing character of the molecule. The effect appears to be specific for compounds containing the double-bonded carbon-sulfur group. Phenylthiourea and sodium diethyldithiocarbamate were more effective than thiourea when compared at concentrations of  $5 \times 10^{-3}$  M. Sulfhydryl and disulfide compounds, such as cysteine, cysteamine, and cystamine, were completely ineffective. The mechanism of stabilization may involve an interaction of thiourea and viral nucleic acid since 0.1 M thiourea also prevented the UV inactivation of the purified infectious ribonucleic acid of EEE virus. In contrast to thiourea, urea and guanidine increased the rate of inactivation of EEE virus by UV.

## I. INTRODUCTION

In an earlier report<sup>1</sup> thiourea was shown to be more effective than cysteine in stabilizing partially purified suspensions of EEE virus during storage at 4 C, and to be effective in completely preventing the inactivation of EEE virus by sodium ascorbate, or by the sulfhydryl group reagent, p-chloromercuribenzoate. It was further shown that the nonsulfur analogs of thiourea, urea and guanidine, had an effect opposite to that of thiourea; i.e., the nonsulfur compounds caused inactivation of EEE virus.

In continuing studies on the stabilizing effects of thiourea, we have observed that thiourea also protects EEE virus against inactivation by UV irradiation. Details of these studies are presented in this report.

## II. MATERIALS AND METHODS

The virus used in all tests was the Louisiana strain of EEE virus.<sup>2</sup> A seed prepared from embryos of eggs infected with EEE virus was used for the inoculation of Maitland-type chick embryo cultures. The culture medium consisted of 0.5% lactalbumin hydrolyzate, 10% calf serum, 0.075% sodium bicarbonate, and 0.002% phenol red in Hanks' balanced salt solution. The medium was sterilized by filtration through an asbestos pad in a Seitz apparatus. After inoculation with virus, Roux bottles containing 100 ml of medium were shaken at 37 C; fluids were removed after 24 to 30 hours and stored at -65 C. Virus was removed from thawed tissue culture fluid by one cycle of differential centrifugation (10,000 rpm for 15 min, 25,000 rpm for 60 min) in the type 30 rotor of the Spinco model L centrifuge. Sediments of the high-speed centrifugation were rinsed with phosphate buffer to remove traces of tissue culture fluid, and then resuspended in 0.02 M phosphate buffer, pH 7.8. Virus in this partially purified condition was used in all experiments.

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1. Wachter, R.F., and J.F. Comer. 1964. Relationship between the structure of compounds and their effect on the infectivity of eastern equine encephalitis virus. Bacteriol. Proc., p. 143. (Abstr.)
  2. Howitt, B.I., L.K. Bishop, R.H. Gorrie, R.E. Kissling, G.H. Hauser, and W.L. Trueting. 1948. An outbreak of equine encephalomyelitis, Eastern type, in Southwestern Louisiana. Proc. Soc. Exptl. Biol. Med. 68:70-72.

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Infectivity was determined by titration in 14-day embryonated eggs inoculated via the amniotic cavity.

A volume of 1.0 ml of the partially purified virus suspension was mixed in a 60-mm glass petri plate with an equal volume of a 0.02 M phosphate buffer solution of the compound being tested. Control samples were prepared in the same manner by mixing equal volumes of the virus suspension and 0.02 M phosphate buffer, pH 7.8. Unless otherwise indicated, samples were allowed to stand at room temperature for 60 or 90 minutes before being exposed to UV. The samples were irradiated with constant agitation under a 15 watt General Electric germicidal lamp at a distance of 8 inches. The lamp intensity at 2537 Å was 271 microwatts per square centimeter at this distance. At specified time intervals portions of the samples were withdrawn and diluted in Bacto heart infusion broth for assay of infectivity.

### III. RESULTS

#### A. PROTECTIVE EFFECT OF DIFFERENT CONCENTRATIONS OF THIOUREA

Samples of the virus suspension were mixed with thiourea to give final thiourea concentrations of 0.5 M, 0.1 M, and 0.01 M. At intervals of 1, 3, 5, 10, and 20 minutes 0.1 ml samples were removed and diluted for assay of infectivity. As shown in Fig. 1, inactivation of partially purified EEE virus by ultraviolet light was markedly retarded by thiourea, even with the relatively prolonged exposure times of 10 and 20 minutes. Much greater protection was given by the higher concentrations of thiourea. With 0.5 M thiourea an infectivity decrease of only 0.5 log was observed in 10 minutes whereas virus without thiourea lost 7 logs of infectivity in 2 minutes. The shape of the inactivation curve obtained with 0.1 M thiourea suggests an initial lag phase (3 minutes), followed by a sharp decrease in titer between 3 and 5 minutes, and then a plateau of very gradual decrease in infectivity between 10 and 20 minutes. Limited data indicate that about 3 logs of infectivity remained at the end of 60 minutes in the presence of 0.1 M thiourea.

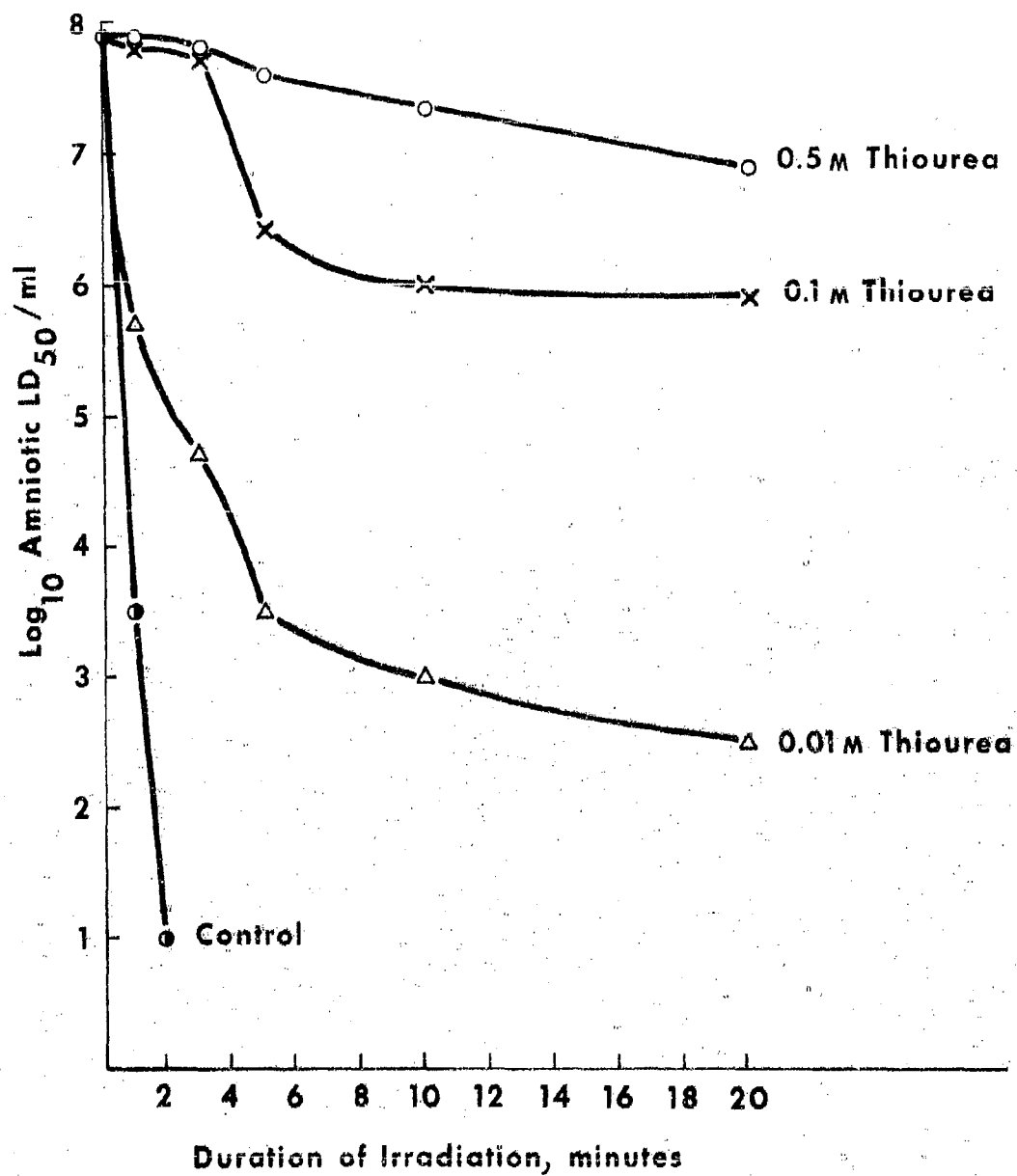


Figure 1. Protective Effect of Thiourea Against Inactivation of EEE Virus by Ultraviolet Irradiation.

## B. COMPARISON OF THIOUREA AND RIBONUCLEIC ACID (RNA)

Since thiourea shows strong absorbancy in the ultraviolet region, tests were conducted to determine whether protection of EEE virus by thiourea was related entirely to the light-absorbing character of the compound. Samples of the virus were mixed with solutions of thiourea or of yeast RNA that had approximately the same absorbancy at a wave length of 260 millimicrons. The results of infectivity assays following exposure of the virus samples to UV irradiation for 60 seconds are shown in Table 1. In the presence of 0.1 M thiourea the virus showed essentially no infectivity change, whereas, in the presence of  $10^{-3}$  M RNA, a loss of titer of almost 4 logs occurred.

TABLE 1. COMPARISON OF THIOUREA AND RNA FOR PROTECTION OF EEE VIRUS AGAINST UV INACTIVATION

| Sample                              | Infectivity for Embryonated Egg <sup>a, b</sup> /<br>After 60 seconds exposure |
|-------------------------------------|--------------------------------------------------------------------------------|
| Virus control                       | 2.0                                                                            |
| Virus + thiourea (0.1 M)            | 7.2                                                                            |
| Virus + RNA ( $2 \times 10^{-3}$ M) | 3.5                                                                            |

a. Log<sub>10</sub> amniotic LD<sub>50</sub> per ml.

b. Titer before exposure:  $10^{7.3}$  LD<sub>50</sub> per ml.

## C. REQUIREMENT OF DOUBLE-BONDED CARBON-SULFUR GROUP IN MOLECULE OF PROTECTIVE COMPOUND

Stabilization of EEE virus against inactivation by UV irradiation appears to be specific for compounds that contain the double-bonded carbon-sulfur group. This is illustrated by the data in Tables 2 and 3. Portions of the partially purified virus suspension were mixed with equal volumes of 0.2 M thiourea, urea, thiosemicarbazide, or semicarbazide and allowed to stand for 10 minutes at room temperature before being exposed to UV light for 15 seconds. As shown in Table 2, thiourea and thiosemicarbazide prevented the sharp loss of infectivity observed for the control sample, whereas urea and semicarbazide afforded no protection to the virus. Further indication of the specificity of structure of the stabilizing molecule was found when the sulphydryl and disulfide compounds, cysteine, cysteamine, and cystamine, were tested for possible protective effect. Cysteine was tested at a final concentration of 0.1 M; cysteamine and cystamine at a concentration of 0.01 M; and thiourea at both concentration levels. Mixtures

of virus and compounds were allowed to stand for 60 minutes at room temperature before being exposed to UV. As indicated in Table 3, the sulfhydryl compounds, cysteine and cysteamine; and the disulfide compound, cystamine, showed no protective effect in contrast to the protection given by thiourea.

TABLE 2. EFFECT OF COMPOUNDS WITH C=S AND C=O BONDS ON THE INACTIVATION OF EEE VIRUS BY UV IRRADIATION

| Compound Added <sup>a</sup> / | Infectivity for Embryonated Egg <sup>b,c</sup> / After 15 seconds exposure |
|-------------------------------|----------------------------------------------------------------------------|
| None                          | 4.0                                                                        |
| Thiourea                      | 8.3                                                                        |
| Urea                          | <3.0                                                                       |
| Thiosemicarbazide             | 8.0                                                                        |
| Semicarbazide                 | <4.0                                                                       |

a. Concentration: 0.1 M.

b. Log<sub>10</sub> amniotic LD<sub>50</sub> per ml.

c. Titer before exposure: 10<sup>8.2</sup> LD<sub>50</sub> per ml.

TABLE 3. COMPARISON OF COMPOUNDS WITH DIFFERENT SULFUR GROUPINGS FOR PROTECTIVE EFFECT ON EEE VIRUS AGAINST UV INACTIVATION

| Compound Added     | Infectivity for Embryonated Egg <sup>a,b</sup> / After 30 seconds exposure |
|--------------------|----------------------------------------------------------------------------|
| None               | 1.0                                                                        |
| Cysteine, 0.1 M    | <1.0                                                                       |
| Cysteamine, 0.01 M | <3.0                                                                       |
| Cystamine, 0.01 M  | <3.0                                                                       |
| Thiourea, 0.01 M   | 6.1                                                                        |
| Thiourea, 0.1 M    | 7.3                                                                        |

a. Log<sub>10</sub> amniotic LD<sub>50</sub> per ml.

b. Titer before exposure: 10<sup>7.5</sup> LD<sub>50</sub> per ml.

#### D. COMPARISON OF PROTECTIVE EFFECTS OF THIOUREA, PHENYLTHIOUREA, AND SODIUM DIETHYLDITHIOCARBAMATE

To investigate further the specificity of the carbon-sulfur group, and to determine whether changes in the thiourea molecule altered its effect, thiourea, phenylthiourea, and sodium diethyldithiocarbamate were compared for their relative stabilizing effect on EEE virus during UV irradiation. Because of the limited solubility of phenylthiourea the three compounds were tested at a concentration of 0.005 M. Samples were removed for assay of infectivity at intervals of 30, 60, 120, and 180 seconds. Phenylthiourea and sodium diethyldithiocarbamate stabilized the virus to about the same extent, and both compounds were significantly more effective than thiourea at the 0.005 M level (Fig. 2). When compared at the 0.1 M level, sodium diethyldithiocarbamate and thiourea were equally effective for 5 minutes, but sodium diethyldithiocarbamate gave greater protection at more extended intervals.

#### E. EFFECT OF UREA AND GUANIDINE ON THE INACTIVATION OF EEE VIRUS BY UV IRRADIATION

In tests to compare urea and guanidine with thiourea for their effect on the UV inactivation of EEE virus, it appeared that these nonsulfur analogs of thiourea did not protect the virus against inactivation but instead increased the rate of inactivation. A number of experiments were carried out in which EEE virus was exposed to UV light in the absence and in the presence of urea. The results presented in Table 4 represent the average values from 7 tests using 0.1 M urea with no standing time before exposure. It can be seen that viral inactivation proceeded much more rapidly in the presence of urea than in the control samples. A similar difference was found for 0.01 M urea and also for guanidine at 0.01 M and 0.1 M concentrations.

TABLE 4. EFFECT OF UREA ON THE INACTIVATION OF EEE VIRUS BY ULTRAVIOLET LIGHT

| Sample               | Infectivity for Embryonated Egg <sup>a</sup> /<br>Time of exposure to UV, seconds |     |     |
|----------------------|-----------------------------------------------------------------------------------|-----|-----|
|                      | 0                                                                                 | 30  | 60  |
| Virus Control        | 7.4                                                                               | 5.7 | 3.3 |
| Virus + Urea (0.1 M) | 7.4                                                                               | 3.7 | 1.6 |

a. Log<sub>10</sub> amniotic LD<sub>50</sub> per ml.

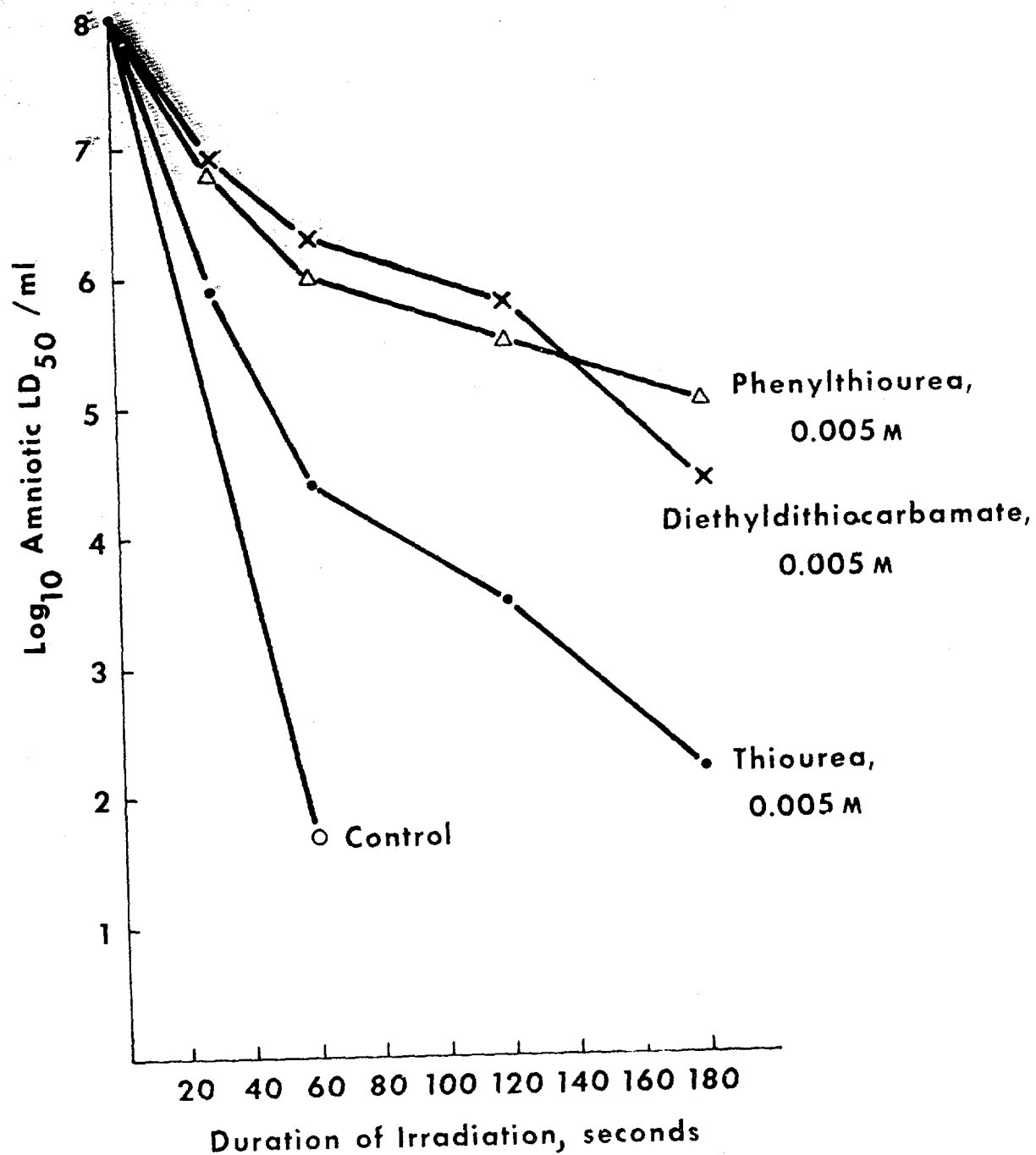


Figure 2. Protective Effect of Thiourea, Phenylthiourea, and Sodium Diethyldithiocarbamate Against Inactivation of EEE Virus by Ultraviolet Irradiation.

#### F. EFFECT OF THIOUREA ON THE UV INACTIVATION OF INFECTIOUS RNA OF EEE VIRUS

To investigate the mechanism by which thiourea protects EEE virus against UV inactivation, the infectious RNA of EEE virus was exposed to UV irradiation in the presence of thiourea. The sample of RNA was prepared by phenol extraction of chick embryos infected with EEE virus and was purified by ethanol and sodium chloride precipitation steps as described by Colón and Idoine.<sup>3</sup>

The rather striking stabilizing effect of 0.1 M thiourea is shown in Fig. 3. Following an initial decrease in infectivity of 0.5 log, there is a stabilization effect such that less than 0.5 log of titer is lost by 120 seconds. After 5 minutes of irradiation 3.5 log of infectivity still remained.

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3. Colón, J.I. and J.B. Idoine. 1964. Factors affecting plaque formation by the infectious ribonucleic acid of the equine encephalitis viruses. J. Infect. Diseases 114:61-68.

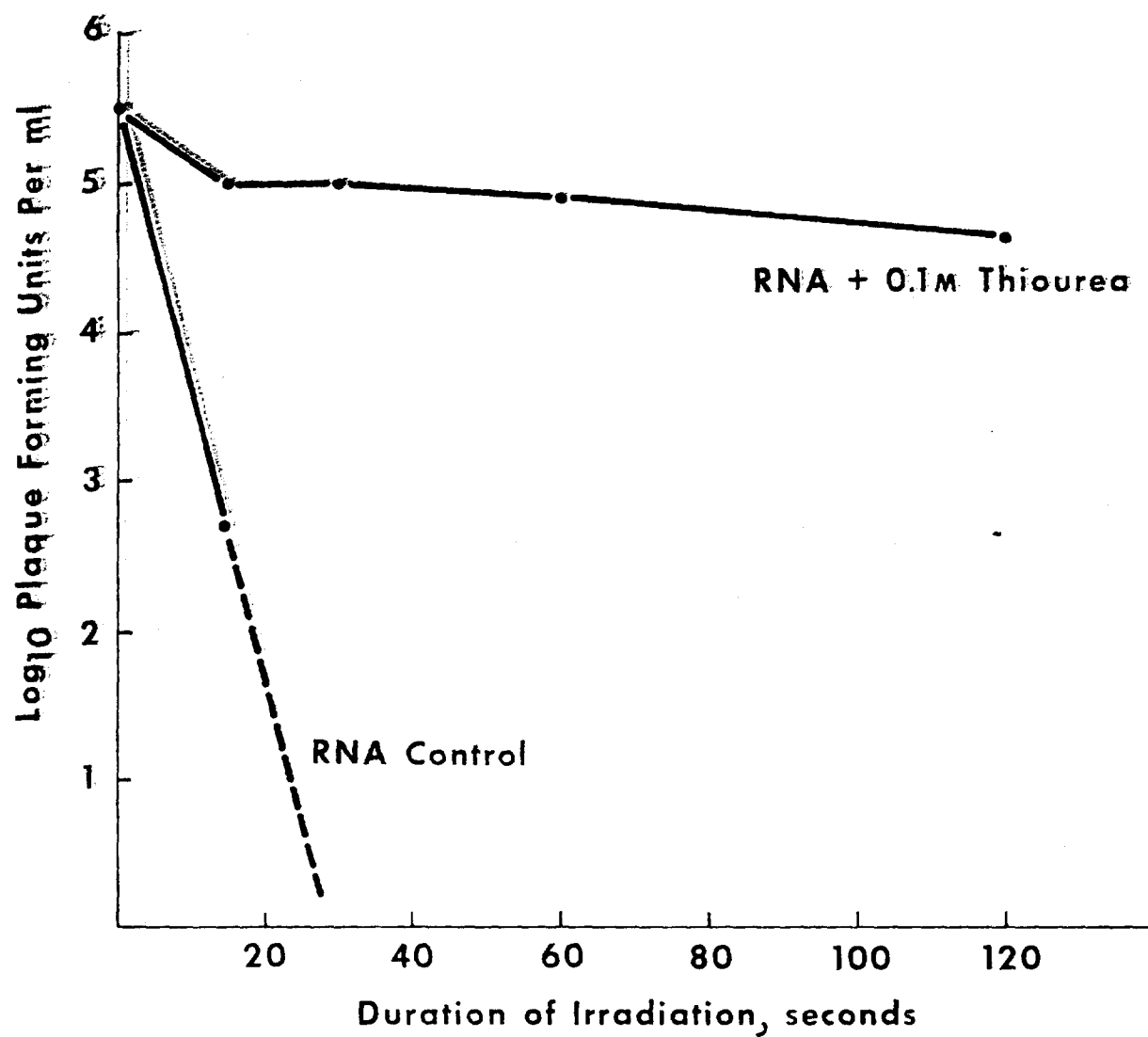


Figure 3. Effect of Thiourea on the UV Inactivation of Infectious RNA of EEE Virus.

#### IV. DISCUSSION

The stabilizing effect of thiourea on partially purified EEE virus that we reported earlier<sup>1</sup> included stabilization during storage at 4 C and protection against inactivation by ascorbate and p-chloromercuribenzoate. Stabilization to extended storage was given by cysteine and a number of other sulfur-containing compounds and also by some compounds that contain no sulfur. Inactivation of virus by ascorbate or by p-chloromercuribenzoate could be prevented by cysteine as well as by thiourea. The nonsulfur analogs of thiourea (urea and guanidine) increased the rate of inactivation of EEE virus, in contradistinction to the thiourea effect.

In contrast to the relatively broad spectrum of compounds that were found to stabilize EEE virus during storage, stabilization of virus against UV inactivation has been restricted, at the current status of these studies, to compounds having structures related to that of thiourea. It may be significant to note that the thiourea analogs, urea and guanidine, which enhanced the inactivation of EEE virus during storage, also increased the rate of inactivation by ultraviolet light.

Since thiourea has been shown to protect isolated infectious RNA, and since protection of the virus does not depend entirely on the light-absorbing property of thiourea, it is concluded that the mechanism of thiourea protection of EEE virus involves some interaction of thiourea with the viral nucleic acid. Although thiourea shows strong absorbancy at a wave length of 260 millimicrons, the compound has an absorption maximum at 236 millimicrons. More tests need to be performed with other compounds that absorb light in this region to establish possible relationships of the carbon-sulfur group, light absorbancy, and stabilizing potential. Since thiourea had previously been shown to stabilize EEE virus more effectively than compounds with other molecular structures, stabilization of EEE virus by thiourea probably is related both to the specific configurational compatibility of the molecule with the structure of the virus particle and to the light-absorbing capacity of thiourea.