complexes of actinomycin C_3 with mononucleotides, deoxydinucleotides, and an oligonucleotide using optical spectral technique. These authors found that AMP formed a 2:1 complex with actinomycin C_3, consistent with the present results for actinomycin D. However, they report finding a 1:1 stoichiometry for the actinomycin C_3–dAMP complex. The present results indicate that both dAMP and AMP form 2:1 complexes with actinomycin D.

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Ultraviolet Irradiation of the Components of the Wheat Embryo *in Vitro* Protein Synthesizing System†

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ABSTRACT: In order to evaluate the sensitivities of the biological activities of plant RNA molecules to ultraviolet (uv) light, various components of protein synthesis were isolated from wheat embryos, irradiated, and then tested for their ability to support amino acid incorporation in an *in vitro* system. Messenger RNA was the component most sensitive to uv irradiation. The inactivation cross sections for two messengers, tobacco mosaic virus (TMV)-RNA and polyuridylic acid, were 50×10^{-5} and 68×10^{-5} mm²/erg, respectively, compared to cross sections for wheat ribosomes, transfer RNA, and S100-DEAE (mixed enzymes without transfer RNA activity) of 7.0×10^{-5}, 1.0×10^{-5}, and 2.2×10^{-5} mm²/erg, respectively. Wheat ribosomes were significantly more uv sensitive than *Escherichia coli* ribosomes. The ability of TMV-RNA to cause infection in tobacco was eight times more uv sensitive than was its messenger activity.

Short-wave ultraviolet radiation (uv[1]) strongly interacts with RNA and chemically alters its constituent pyrimidine nucleotides, forming pyrimidine hydrates, cyclobutane-type pyrimidine dimers, and other, uncharacterized photoproducts. Uv also disrupts the biological activities of RNA molecules. Previous studies of tRNA in amino acid activating assays and of tRNA, mRNA, and ribosomes in amino acid incorporating assays have indicated the sensitivity of these RNA species to uv-induced changes in their structures (Grossman, 1962; Wacker *et al.*, 1962; Swenson and Nishimura, 1964; Harriman and Zachau, 1966; Kagawa *et al.*, 1967; Ottensmeyer and Whitmore, 1968; Tokimatsu *et al.*, 1968; Wada *et al.*, 1968; Aoki *et al.*, 1969; Eker and Berends, 1970; Remsen and Cerutti, 1972). The studies have shown that uv has both inactivating and, in some cases, mutagenic effects on RNA.

Uv radiation may be a particularly significant stress to the protein synthetic system in higher plants, since plants, as obligatory photosynthetic organisms, are often exposed to relatively high doses of uv light from the sun. The idea that plant RNA-containing components may suffer from uv-induced inactivation is supported by the fact that certain plants possess mechanisms for photoreactivating uv-inactivated RNA viruses and RNA-viral genomes (Kleczkowski, 1971; Murphy and Gordon, 1971). However, this idea has not been firmly established. For one thing, there are no reports of photoreactivation of uv-damaged cellular (nonviral) RNA. For another, there is little information available about the uv sensitivity of plant RNA species. The uv-irradiation studies mentioned above have emphasized procaryotic systems. The effects of uv on plant ribosomes or on mes-

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[1] Abbreviations used are: uv, short-wave ultraviolet radiation (190–300 nm); TMV, tobacco mosaic virus.

sengers translated by plant ribosomes have not yet been described.

In order to help evaluate the uv sensitivity of RNA molecules in plants, and in order to form the basis of a search for photoreactivation of uv-damaged nonviral RNA molecules, we have studied the effects of uv on the components of an *in vitro* amino acid incorporating system prepared from wheat embryos. This system, devised and characterized by Marcus and his coworkers (Marcus *et al.*, 1968; Weeks and Marcus, 1971), is able to use either polyuridylic acid or tobacco mosaic virus (TMV)-RNA as a messenger to direct amino acid incorporation. We chose this system in part because the RNA-containing components, tRNA, mRNA, and ribosomes could be resolved and irradiated separately, and in part because we felt that the TMV-RNA would serve as a good model for natural messenger RNA in a plant cell. The following paper reports the sensitivities to uv of wheat ribosomes, wheat tRNA, and wheat S100-DEAE when assayed with polyuridylic acid as messenger and the sensitivities of polyuridylic acid and TMV-RNA when assayed as messengers with the other components.

Experimental Section

Materials. Seeds of spring wheat, variety "Fortuna," were supplied by Mr. Arthur Dubbs of the Central Agricultural Research Center, Moccasin, Mont. Embryos were prepared from the seeds according to the method of Johnston and Stern (1957), with the use of the cyclohexane–carbon tetrachloride mixture (10:25) for the density separation of embryos from endosperm. Purified embryos were dried and stored at 4°. Wheat germ, used for preparation of soluble RNA and for some polyuridylic acid directed phenylalanine incorporation experiments, was purchased locally. Polyuridylic acid, ATP, GTP, creatine phosphate, creatine phosphokinase, labeled amino acids, and yeast-soluble RNA were obtained commercially. Wheat-soluble RNA was prepared from wheat germ by a modification of the method of Holley (1967). TMV (strain U1) was propagated in *Nicotiana tabacum* var. *Turkish*. The virus was isolated by a modification of the methods of Steere (1963) and Gooding and Hebert (1967). RNA was purified from the virus by the procedure of Diener and Schneider (1968).

Methods. AMINO ACID INCORPORATION. Extracts of wheat embryos were prepared following the procedures of Weeks and Marcus (1971) and of Marcus *et al.* (1968). Either wheat germ or wheat embryo (0.5 g) was ground with sand plus 5 ml of a solution containing 0.05 M KCl, 0.001 M magnesium acetate, 0.002 M $CaCl_2$, and 0.12 M $KHCO_3$. The $KHCO_3$ concentration of this extraction buffer was chosen to give optimum TMV-RNA directed amino acid incorporating activity with our sample of Fortuna embryos. After grinding, the suspension was centrifuged at 23,000*g* for 10 min. To each milliliter of supernatant were added 0.01 ml of 0.1 M magnesium acetate and 0.025 ml of 1 M Tris buffer, pH 7.6. For use with TMV-RNA messenger, the augmented supernatant (S23) was dialyzed for 2 hr against buffer containing 0.001 M Tris-acetate, pH 7.3, 0.002 M magnesium acetate, 0.05 M KCl, and 0.005 M mercaptoethanol. For experiments with the polyuridylic acid messenger, ribosomes and soluble factors were separated by centrifugation at 100,000*g* for 60 min. Ribosomes were resuspended in buffer containing 0.001 M Tris-acetate, pH 7.6, 0.02 M KCl, 0.001 M magnesium acetate, 0.3 M sucrose, and 0.005 M mercaptoethanol and were centrifuged at 23,000*g* for 10 min to remove denatured protein. The upper part of the supernatant either was used without further treatment (S100) or was supplemented with KCl to 0.3 M and then passed through a column (0.5 cm × 4 cm) of DEAE-cellulose equilibrated with 0.001 Tris-acetate, pH 7.1, containing 0.002 M magnesium acetate, 0.3 M KCl, and 0.005 M mercaptoethanol (S100-DEAE).

Standard wheat incubation mixtures for amino acid incorporation with the polyuridylic acid messenger contained in 0.5 ml: 25 mM Tris-acetate, pH 8.1, 44 mM KCl, 15 mM mercaptoethanol, 1 mM ATP, 0.025 mM GTP, 5.0 mM magnesium acetate, 8 mM creatine phosphate, 25 μg of creatine phosphokinase, 5 μg of chloramphenicol, 25 μg of yeast tRNA (unless otherwise noted), 50 μg of creatine phosphokinase, 50 μg of polyuridylic acid, 150 μg of ribosomal RNA (as ribosomes), 100 μg of S100 protein, and 0.1 μCi of L-[^{14}C]phenylalanine (92 μCi/μmol).

Standard wheat incubation mixtures for amino acid incorporation using the TMV-RNA messenger were as described above, but with the following alterations: 3.6 mM instead of 5.0 mM magnesium acetate; 30 μg of TMV-RNA in place of polyuridylic acid; 3500 μg of (protein) S23 (dialyzed) in place of ribosomes and S100; and 0.1 μCi of L-[^{14}C]leucine (310 μCi/μmol) instead of L-[^{14}C]phenylalanine. In addition a mixture providing 10^{-5} M final concentrations of all necessary amino acids except leucine was added.

Ribosomes and S100 from *Escherichia coli*, strain MRA 600 (RNase I$^-$), were kindly provided by T. Bickel and J. Hershey of the Biochemistry Department, University of California, Davis. To remove proteases, S100 was dialyzed against 0.01 M Tris buffer, pH 7.5, containing 0.01 M magnesium acetate and 0.02 M NH_4Cl, and then applied to a column of DEAE-cellulose equilibrated with the dialysis buffer. A protein fraction eluting with 0.01 M Tris buffer, pH 7.5, containing 0.01 M magnesium acetate and 0.26 M NH_4Cl was used for amino acid incorporation.

Standard *E. coli* incubation mixtures for amino acid incorporation contained in a volume of 0.5 ml: 50 mM Tris-acetate pH 8.1, 100 mM NH_4Cl, 5 mM mercaptoethanol, 1 mM ATP, 0.025 mM GTP, 12 mM magnesium acetate, 8 mM creatine phosphate, 25 μg of creatine phosphokinase, 150 μg of *E. coli* tRNA, 50 μg of polyuridylic acid, ribosomes, 20 μl of S100-DEAE (OD_{280} 17.3), and 0.43 μCi of L-[^{14}C]phenylalanine (10 μCi/mol).

Amino acid incorporation mixtures were incubated at 30°. At various times, 0.10-ml samples were withdrawn, precipitated with trichloroacetic acid, and washed in batches according to the procedure of Mans and Novelli (1961). The radioactivity of washed disks was determined in toluene scintillation fluid (4.0 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis[2-(5-phenoxazolyl)]benzene/l. of toluene) with a Nuclear-Chicago liquid scintillation spectrometer system.

ULTRAVIOLET IRRADIATIONS. Solutions were irradiated in open flat-bottomed beakers with a low-pressure mercury vapor lamp filtered with Corning glass 9863 (Ultraviolet Products, Inc., San Gabriel, Calif., Model C-81). The primary emission from this lamp is at 254 nm. Incident irradiance at the surface of the samples, measured with a short-wave uv meter (Ultraviolet Products, Model J-225), was usually 1000 ergs/(mm^2 min). All uv doses quoted in the Results include a correction for self-absorption by the sample. They were calculated according to the formula (Kagawa *et al.*, 1967)

$$D = \frac{I_0 t}{2.3Al}(1 - 10^{-Al})$$

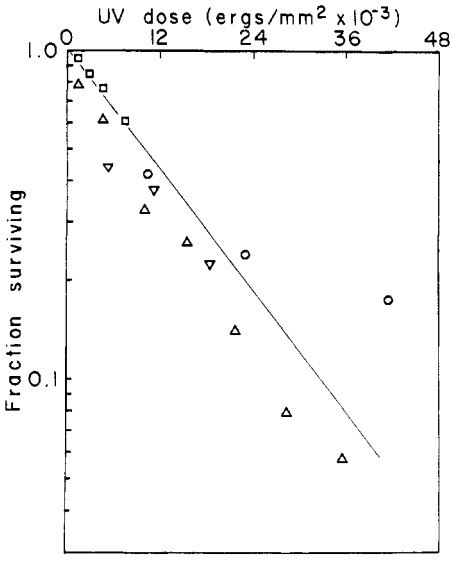

FIGURE 1: Inactivation of wheat ribosomes by uv. [^{14}C]Phenylalanine incorporating activity was measured in a polyuridylic acid directed system. Different symbols represent different experiments.

in which A is the optical density of the sample at 254 nm, l is the average thickness of the sample layer in centimeters, t is the period of irradiation, I_0 is the incident irradiance at the surface of the sample, and D is the effective average dose received by the sample. The solutions were stirred continuously, and the beakers were kept on ice, during irradiation.

INFECTIVITY OF VIRAL RNA. The infectivity of unirradiated and irradiated TMV-RNA was assayed by the procedure described by Murphy and Gordon (1971). Infectivity is defined as the ability of the RNA to form local lesions on the host, *N. tabacum* var. *Xanthi*.

Results

Protocol for Measuring Uv Inactivation. The measurement of the extent of inactivation of any component (or group of components) of an amino acid incorporating system requires conditions in which that component is a limiting factor in the overall rate of amino acid incorporation by the system. In order to satisfy this condition in uv irradiation experiments with the wheat embryo system, we adopted the following protocol. A solution containing one component of the system was irradiated with short-wave uv light at a constant dose rate, usually 1000 ergs/(mm² min). Samples were taken from this solution before irradiation and at different times during irradiation. For each sample, a series of reaction tubes was prepared containing different amounts of the sample. Each reaction tube received the standard amount (saturating or optimal) of each of the other (unirradiated) components needed for amino acid incorporation. The reaction tubes then were incubated in a 30° water bath. Aliquot volumes (0.1 ml) were withdrawn from each tube at three times during incubation and their amounts of trichloroacetic acid insoluble ^{14}C-labeled amino acid were measured.

From the resulting data, the calculation of the rate of uv inactivation of the irradiated component required three computational steps. First, the rate of amino acid incorporation in each reaction tube was calculated. For the wheat embryo system, the initial portion of the curve relating amino acid incorporation (counts per minute) with incubation period was linear; the slope of this line was used as a measure of the rate of the reaction. Second, titration curves were prepared for each sample. A titration curve indicated the rate of amino acid incorporation as a function of the amount of the sample, the variable component, added to the reaction tube. The titration curves immediately showed whether the sample was present in limiting or in saturating amounts. "Titers" of the samples were determined from the slopes of those portions of the appropriate titration curves in which samples were limiting. Third, the titers of the irradiated samples, expressed as fractions of the titer of the unirradiated control sample, were graphed as a function of the uv dose they received. The rate of inactivation or "inactivation cross section," σ, was expressed as the negative of the slope of the line of formula $\log_e y = \sigma D$, selected for the best least-squares fit of the inactivation data (y = fraction of control titer; D = uv dose).

Ribosomes. Wheat embryo ribosomes were irradiated in standard diluent (0.01 M Tris-acetate, pH 7.6, 0.02 M KCl, 0.001 M magnesium acetate, 0.3 M sucrose, and 0.005 M mercaptoethanol) containing in addition 50 μg/ml of chloramphenicol. The ribosomes were diluted to a concentration of 1 mg/ml (RNA) and placed under the short-wave uv lamp for periods ranging up to 3 hr. This corresponds to doses of up to 40,000 ergs/mm² after correction for self-absorption by the solution. The ribosomes were then assayed according to the above protocol in the standard polyuridylic acid directed [^{14}C]phenylalanine incorporating system.

For all samples of unirradiated and uv-irradiated ribosomes, the incorporation of phenylalanine into acid-insoluble material was linear with time over the period from 5 to 25 min of incubation. Titration curves for unirradiated and irradiated ribosomes showed that they were limiting incorporation when present in amounts below 50–100 μg. Figure 1 shows a comparison of titers of unirradiated and irradiated ribosomes plotted as a function of uv dose. These data were obtained from four independent experiments. Inactivation of the ribosomes followed logarithmic kinetics: the inactivation cross section was 7.0×10^{-5} mm²/erg, corresponding to one biological hit (37% survival) after about 12,000 ergs/mm² of uv irradiation.

Wheat ribosomes appeared to be more sensitive to uv than ribosomes from *E. coli*. Wacker *et al.* (1962) obtained 45% of control activity from *E. coli* ribosomes irradiated with 35,000 ergs/mm² (uncorrected for self-absorption of the solution) and tested in a polyuridylic acid directed incorporating system. Kagawa *et al.* (1967) showed that inactivation cross sections depended on the strain of *E. coli* used, on the conditions of irradiation (low or high Mg concentration), and on other experimental factors. They measured cross sections of 5×10^{-5} (strain B) and 1.7×10^{-5} (strain H) mm²/erg for polyuridylic acid directed polyphenalanine incorporation after irradiation in their "high Mg" (0.01 M) buffer and 4.9×10^{-5} (strain B) and 3.5×10^{-5} (strain H) mm²/erg after irradiation in "low Mg" (0.25×10^{-3} M) buffer. All these values are below that which we found for wheat ribosomes.

In order to confirm the lower uv sensitivity of *E. coli* ribosomes relative to wheat ribosomes, we first repeated our ribosome-irradiation experiments using the same protocol with an *E. coli* polyuridylic acid–amino acid incorporating system. Ribosomes from *E. coli* MRA 600 (RNase I$^-$), a strain derived from strain B, were irradiated at 0.6 or 1.2 mg/ml in 0.01 M Tris-acetate buffer, pH 7.5, containing 0.01 M magnesium acetate, 0.02 M KCl, 0.3 M sucrose, and 0.005 M mercaptoethanol. Samples were then assayed for polyphenylalanine incorporating activity.

TABLE I: Uv Inactivation of Wheat Ribosomes in Buffers Containing Different Mg Concentrations.[a]

	Mg Concn (M)		
Expt	0.001	0.036	0.010
1	0.32	0.26	0.30
2	0.36	0.38	0.39

[a] [^{14}C]Phenylalanine incorporating activity of the ribosomes was measured in a polyuridylic acid directed system. Values listed represent the fraction of control (unirradiated) titer remaining in a sample of ribosomes after an irradiation of approximately 25,000 erg/mm^2.

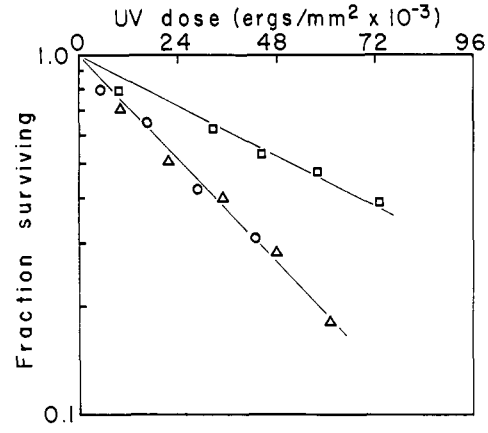

FIGURE 2: Inactivation of *E. coli* ribosomes by uv. Different symbols represent different experiments.

In contrast to the wheat embryo system, incorporation in the *E. coli* system was not linear with the period of incubation: the rate of incorporation decreased steadily throughout the period of reaction. The decrease occurred with both unirradiated and irradiated ribosomes; it occurred with various salt, S100, and ribosome concentrations and in the presence of increased energy-generating components; it occurred even though the S100 was treated with DEAE-cellulose chromatography to remove proteases. Because of the nonlinear reaction kinetics, we could not use a linear initial rate of incorporation as a measure of the rate of reaction. Instead, we used total incorporation at a single time point (usually 25 min). This matches the procedure of Kagawa *et al.* (1967). Titration curves constructed with this convention for measuring the rate of reaction gave clear titers for both unirradiated and irradiated samples of ribosomes. We believe the inactivation rate calculated with these titers should be comparable to that found for the wheat ribosomes.

Figure 2 shows the inactivation of the *E. coli* ribosomes, measured in three independent experiments. Kinetics of inactivation were logarithmic: the cross section measured in one experiment was 1.3×10^{-5} mm^2/erg; the cross section in the other two experiments was 2.8×10^{-5} mm^2/erg. We have no explanation for the difference between the experiments. These values for inactivation cross section are lower than the comparable "high Mg" value obtained by Kagawa *et al.* (1967) (5×10^{-5} mm^2/erg) and considerably lower than the value for wheat ribosomes.

We also irradiated wheat ribosomes in different Mg concentrations. In our original experiments wheat ribosomes were irradiated in 0.001 M Mg, while *E. coli* ribosomes were irradiated in 0.01 M Mg. It seemed possible that the higher uv sensitivity of wheat ribosomes resulted from the lower Mg concentration in the irradiated solution, just as strain H *E. coli* ribosomes were more sensitive at lower than at higher Mg concentrations (Kagawa *et al.*, 1967). In order to test this possibility, wheat ribosomes were irradiated in buffers similar to the one used before, but containing concentrations of 0.001, 0.0036, and 0.01 M magnesium acetate. The ribosomes were then assayed as described above. The data, presented in Table I, indicated that the ribosomes in the three Mg concentrations were equally susceptible to uv damage. The wheat ribosomes irradiated at 0.01 M Mg were thus more sensitive to uv than were *E. coli* ribosomes at the same Mg concentration.

Transfer RNA. Samples of wheat germ soluble RNA were diluted to 0.1 mg/ml in 0.001 M magnesium acetate and irradiated for periods as long as 3 hr, corresponding to uv doses of up to 151,000 ergs/mm^2. The unirradiated and irradiated samples were tested for their ability to support polyuridylic acid directed [^{14}C]phenylalanine incorporation in the standard system with yeast tRNA omitted and with S100-DEAE in place of S100. With S100-DEAE, amino acid incorporation observed without added tRNA was less than 5% of that observed with saturating amounts of tRNA. This experiment tested the effect of uv on phenylalanyl-specific tRNA species only. It did not indicate which of the various functions of the tRNA (amino acid acceptance, ribosome binding, amino acid transfer) were inhibited by irradiation.

For all samples of unirradiated and irradiated soluble RNA, initial rates of amino acid incorporation were linear; titration curves gave clear titers for limiting amounts of each sample. Figure 3 shows the inactivation of the tRNA biological activity, measured in four independent experiments. The kinetics of uv inactivation were approximately logarithmic, though the inactivation curve may have had some downward curvature. The inactivation cross section, assuming logarithmic kinetics, was 1.0×10^{-5} mm^2/erg, corresponding to one biological hit with about 100,000 ergs/mm^2 of irradiation.

The uv inactivation of tRNA from *E. coli* and from other organisms has been studied in many laboratories (Swenson and Nishimura, 1964; Harriman and Zachau, 1966; Wada *et al.*, 1968; Aoki *et al.*, 1969). The inactivation cross section

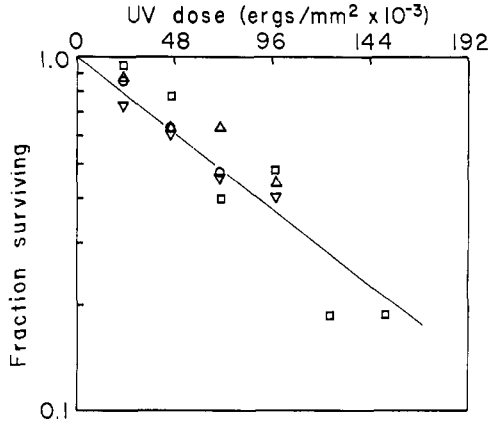

FIGURE 3: Inactivation by uv of the tRNA activity of a wheat germ soluble RNA preparation. Activity was measured as the stimulation of [^{14}C]phenylalanine incorporation by a polyuridylic acid directed wheat system. Different symbols represent different experiments.

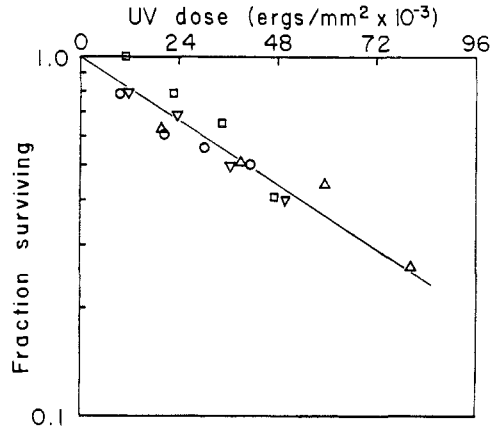

FIGURE 4: Inactivation by uv of the ability of wheat S100-DEAE to stimulate [^{14}C]phenylalanine incorporation in a polyuridylic acid directed wheat system. Different symbols represent different experiments.

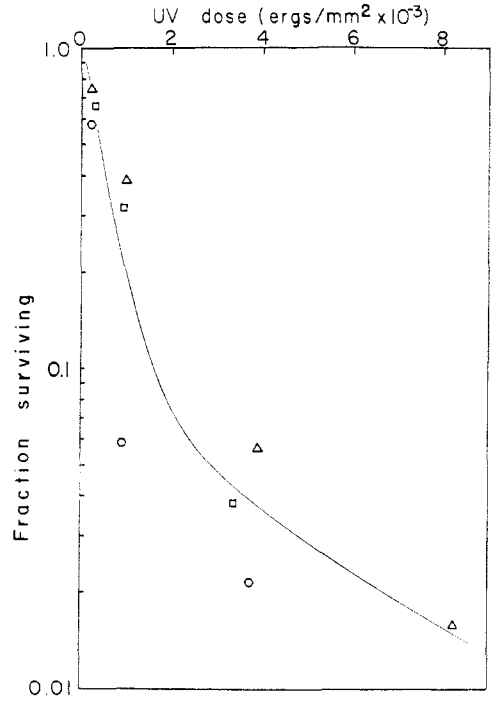

FIGURE 5: Inactivation of polyuridylic acid messenger activity by uv. Messenger activity was measured as [^{14}C]phenylalanine incorporation in a wheat ribosome system. Different symbols represent different experiments.

depends on the amino acid specificity of the tRNA, on the organism from which the tRNA was isolated, on the composition of the solvent during irradiation, and on the biological activity assayed. Using conditions of irradiation and assay similar to ours, Aoki *et al.* (1969) obtained an inactivation cross section for *E. coli* phenylalanyl-tRNA of approximately 2×10^{-5} mm^2 erg; Harriman and Zachau (1966) found a value of 1.2×10^{-5} mm^2 erg. It seems that the uv sensitivity of wheat phenylalanyl specific tRNA is less than or equal to the sensitivity of the corresponding *E. coli* tRNA species.

Protein Factors. Supernatant fractions, either S100 or S100-DEAE, were diluted to concentrations giving $OD_{280,1cm}$ values of 0.075–0.175 and were irradiated for periods of up to 2 hr. Control and irradiated samples were then used to stimulate amino acid incorporation in the polyuridylic acid directed system (containing an excess of yeast tRNA). In this situation, the essential components that were irradiated probably included phenylalanine activating enzyme and elongation factors T1(E) and T2(E) (Seal *et al.*, 1972).

Initial rates of amino acid incorporation were linear with time for all samples, both control and irradiated; titration curves gave clear titers, showing limiting amounts were present for all samples. Figure 4 shows the uv inactivation of the soluble factors observed in four independent experiments. The kinetics of inactivation were logarithmic; the inactivation cross section was 2.2×10^{-5} mm^2/erg, corresponding to one biological hit after about 46,000 erg/mm^2 of uv irradiation.

The only data on other systems available for comparison are those of Wacker *et al.* (1962). These authors found no inactivation (actually some stimulation) when they irradiated *E. coli* S100 with 35,000 ergs/mm^2 of short-wave uv.

Messenger RNA. The uv sensitivities of two different RNA messengers, polyuridylic acid and TMV-RNA, were measured in somewhat different assay systems. Polyuridylic acid was diluted to about 0.5 mg/ml in water and irradiated for periods extending up to 30 min. Samples removed after various uv doses were used to direct [^{14}C]phenylalanine incorporation in the standard wheat system, with ribosomes, S100, and the other essential factors.

For all samples, initial rates of incorporation were linear with time. Titration curves indicated both control and irradiated polyuridylic acid were limiting in the reaction when added in amounts below 1–20 μg/reaction tube. Figure 5 shows inactivation, calculated from limiting portions of titration curves, as a function of uv dose. There was considerable variation among the three independent experiments; also, there was a strong suggestion of upward curvature. The inactivation cross section calculated from the data by assuming logarithmic kinetics was 68×10^{-5} mm^2/erg, corresponding to one biological hit with 1500 ergs/mm^2. This is a much higher cross section than any mentioned above. The deviations from logarithmic kinetics would make the initial inactivation cross section for polyuridylic acid even greater.

The uv inactivation of the messenger activity of polyuridylic acid in *E. coli in vitro* amino acid incorporating systems has been studied by Grossman (1962), Wacker *et al.* (1962), Singer and Fraenkel-Conrat (1970), Ottensmeyer and Whitmore (1968), and Eker and Berends (1970). The inactivation cross sections reported by these groups vary considerably. Inactivation cross-section values of approximately 0.15×10^{-5} and 6×10^{-5} mm^2/erg can be calculated from the data of Grossman (1962) and Wacker *et al.* (1962), respectively. Singer and Fraenkel-Conrat (1970) reported that polyuridylic acid irradiated with a uv dose sufficient to decrease its OD_{260} by 9% retained 78% of its messenger activity: this implies a cross section below 5×10^{-5} mm^2 erg. These values are quite low. On the other hand, the other two groups have presented higher values. Values of at least 16×10^{-5} and 77×10^{-5} mm^2/erg can be inferred from the data of Ottenmeyer and Whitmore (1968) and Eker and Berends (1970), respectively; initial rates would be even higher, since both groups found nonlogarithmic kinetics of inactivation similar to ours. We do not know why the different investigators have obtained different results; however, experimental conditions have varied among the laboratories, so because of some as yet unrecognized factor the experiments may not be strictly comparable.

TMV-RNA was irradiated in water at a concentration of 0.072 mg/ml. The dose rate was 250 ergs/(mm² min); applied doses were up to 7500 ergs/mm². Samples of unirradiated and irradiated RNA were used to direct [^{14}C]leucine incorporation in a system containing wheat S23 and other components needed for protein synthesis. In this system, amino acid incorporation depends on a specific initiation step and is thought to reflect natural protein synthesis more accurately than do *in vitro* systems directed by synthetic polynucleotide messengers (Marcus, 1970; Marcus *et al.*, 1970a,b).

For both irradiated and unirradiated samples, rates of incorporation were linear with time after the initial 5-min lag that is normal for this system (Marcus, 1970). Irradiation apparently affected the rate of incorporation without changing the preliminary lag time, although the possibility of minor changes in lag time could not be excluded, particularly for RNA samples that had received relatively large doses of uv. Titration curves for the RNA samples were based on rates of incorporation as usual. Figure 6 shows inactivation kinetics, based on titration curves from three independent experiments. The inactivation kinetics were similar to those for polyuridylic acid: inactivation was rapid and there was significant upward-curving deviation from logarithmic inactivation. The inactivation cross section calculated assuming logarithmic inactivation was 50×10^{-5} mm²/erg. For comparison, Figure 6 also shows the inactivation of the infectivity of TMV-RNA. The kinetics for this process are approximately logarithmic and indicate an inactivation cross section of about 4×10^{-3} mm²/erg.

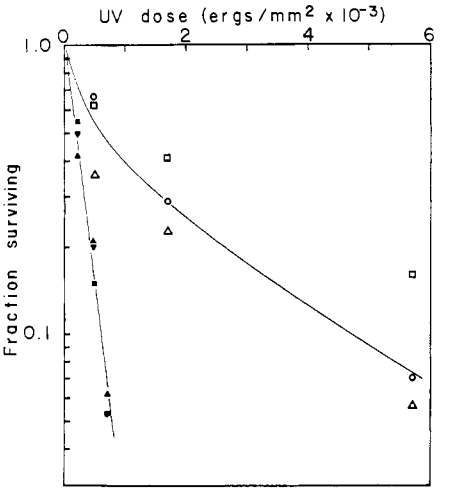

FIGURE 6: Inactivation of TMV-RNA by uv. Messenger activity was measured as [^{14}C]leucine incorporation in a wheat system (open symbols). Infectivity was measured by the formation of local lesions on Xanthi tobacco leaves (closed symbols).

Discussion

None of the fractions irradiated in this study can be considered a homogeneous target for uv inactivation. The S100 fraction contained several protein components essential for incorporation; the tRNA-containing fraction may have included more than one isoaccepting species of phenylalanyl-specific tRNA (Guderian, 1970). The two ribosomal subunits, whether or not they were associated, probably interacted with uv independently of each other; in addition, different partial functions of any one subunit may have been independently sensitive to radiation damage (Kagawa *et al.*, 1967; Tokimatsu *et al.*, 1968; Yasuda and Fukutome, 1970). Even the messengers, polyuridylic acid and TMV-RNA, probably had some heterogeneous character; for instance, their ability to bind to ribosomes and their ability to direct extended elongation of polypeptide chains may have been affected by uv differently. Because of the complexity of the irradiated fractions, the inactivation kinetics that we obtained cannot be given simple interpretations. Each inactivation curve may represent the inactivation of a single most-sensitive component of the fraction, or the curve may represent the inactivation of a group of components or functions of approximately equal sensitivity. Certain situations of sufficiently high complexity may show inactivation kinetics that approximate simple single-hit kinetics such as were observed with wheat and *E. coli* ribosomes and with the S100 fraction (Kleczkowski, 1970). It is not possible to distinguish between simple or complex alternative inactivation mechanisms from the results with ribosomes or S100.

The nonlogarithmic inactivation kinetics observed with polyuridylic acid and with TMV-RNA argue definitely against a simple interpretation of the process of inactivation of these molecules. The upward curvature suggests that a fraction of the molecules, or a fraction of the messenger activity of each molecule, was resistant to uv inactivation. However, this is still conjectural.

The choice of solvent and solutes can affect both the rate and the mechanism of inactivation of a macromolecule. Singer (1971) has shown that 10^{-3} M Mg^{2+} greatly changes the relative amounts of pyrimidine hydrates and pyrimidine dimers formed during uv irradiation of TMV-RNA. Smith and Meun (1968) have reported that sulfhydryl reagents (cysteine) may add to irradiated polynucleotides. It is probable that our *in vitro* irradiations do not exactly reflect the situation that occurs in irradiated intact cells.

There are several components of the protein synthetic system the uv sensitivities of which we did not test, and yet which are extremely important to the process. These include the tRNA species and the activating enzymes for amino acids other than phenylalanine; they also include the initiation factors needed to start protein synthesis with natural messengers (Marcus, 1970). Work by Weeks and Marcus (1971) suggests, in addition, that endogenous wheat mRNA may have a structure (and thus a uv sensitivity) very different from that of TMV-RNA. Further studies will be needed to identify how these components will affect the net uv sensitivity of protein synthesis in plant cells.

With the above uncertainties in mind, we can make the following conclusions.

Wheat embryo ribosomes, assayed for overall polyuridylic acid directed polyphenylalanine incorporation, were approximately two–three times more sensitive to irradiation with short-wave uv than were *E. coli* ribosomes. The exact relative sensitivities depended on the strain of *E. coli* used for comparison and on the conditions of irradiation of the *E. coli* ribosomes. The higher sensitivity of the wheat ribosomes might be a significant reflection of the structural differences between procaryotic and eucaryotic ribosomes. Perhaps a larger fraction of the RNA of eucaryotic ribosomes is essential for the ribosome's structural or functional integrity.

The uv-inactivation cross section for TMV-RNA, measured on the basis of infectivity, was several times greater than the inactivation cross section measured on the basis of messenger activity (Figure 6). The higher uv sensitivity of TMV-RNA's infectivity, relative to that of its messenger activity, might be explained by any of the following hypotheses. (a)

TABLE II: Uv Inactivation Cross Sections for the Various Components of Protein Synthetic Systems.[a]

Component	σ
Ribosomes (wheat)	7.0×10^{-5}
Ribosomes (*E. coli*)	$1.3\text{–}2.8 \times 10^{-5}$
$tRNA_{Phe}$	1.0×10^{-5}
S100-DEAE	2.2×10^{-5}
Polyuridylic acid (mRNA)	68×10^{-5}
TMV-RNA (mRNA)	50×10^{-5}
TMV-RNA (infectivity)	400×10^{-5}

[a] Values are given in mm^2/erg. The reciprocal of the value represents the uv dose needed to reduce the activity of the component to $1/e$ (37%) its original value.

TMV-RNA has several cistrons. Each works independently of the others as a messenger; each is equally sensitive to uv; the functions of all cistrons are necessary for a successful infection. (b) Infection involves some property of the RNA in addition to messenger activity, for instance, template activity for RNA synthesis; this property is more sensitive to uv than is messenger activity. (c) Infection requires the *accurate* translation of TMV-RNA. Low doses of uv disturb the fidelity of translation, while higher doses are necessary to reduce messenger activity in terms of total amino acid incorporation. These hypotheses are simplifications; the actual explanation is probably more complex. As yet there is no evidence to support or refute any of these, or other, hypotheses. It is noteworthy that Remsen and Cerutti (1972) have obtained similar data with an R17 bacteriophage–*E. coli* system. These workers irradiated intact R17 phage and then extracted its RNA: in this situation uridine hydrates are the only uv photoproducts found in the RNA. They reported that five times as many uridine hydrates were required to inactivate a given fraction of the RNA's messenger activity as were required to inactivate the same fraction of its infectivity. The authors suggested that hydrates might inactivate infectivity by interfering with the formation of the double-stranded replicative form of the RNA. This suggestion corresponds to hypothesis b above.

The messenger RNA molecules, polyuridylic acid and TMV-RNA, were by far the most uv-sensitive components tested in our experiments (Table II). The two were about equally sensitive. Their uv inactivation was rapid enough to suggest that the uv radiation in summer sunlight might damage a significant proportion of mRNA molecules in exposed plant cells. This suggestion is based on the following considerations. Sunlight at Davis, Calif., provides a maximum uv dose rate equivalent (in terms of inactivation of TMV-RNA infectivity) to 4 ergs/(mm^2 min) of 254-nm radiation (Murphy, 1973). Therefore, 1 hr of sunlight could inactivate 30–40% of the messenger activity in cells, assuming the cells were not shielded from uv, *e.g.*, epidermal cells (Figures 5 and 6). This might not be rapid inactivation if the natural turnover rate of the messengers were high, but the half-lives of several plant messengers or presumptive messengers have been found to be greater than 1 hr (Waters and Dure, 1966; Key and Ingle, 1964; Chroboczek and Cherry, 1966; Click and Hackett, 1967; Gayler and Glasziou, 1968). Sunlight uv inactivation would significantly increase the rate of catabolism of these messengers. Cells that depended on such messengers might be seriously damaged by sunlight, and they might be materially protected by RNA-photoreactivating mechanisms like those known to act on uv-damaged viral RNA genomes that infect plants.

Whether uv-damaged mRNA in plant cells actually can be repaired is yet unknown. We hope that the results reported here will aid in finding a final solution to this question.

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Effects of High pH and Sodium Dodecyl Sulfate on the Hidden Tyrosines of Human Serum Albumin†

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ABSTRACT: A stop-flow technique has been developed for distinguishing between the tyrosines of human serum albumin which are freely accessible to solvent (11 or 12 out of 18) and those which are hidden in the interior of the native molecule. When the latter are unmasked by raising the pH or by adding various quantities of sodium dodecyl sulfate, reversible changes in degree of ionization and in degree of unfolding both occur and may be distinguished from one another. Exposure to high pH enhances both. Exposure to detergent leads to more complex results; small amounts suppress both ionization and unfolding, and large amounts (up to 300 equiv) enhance unfolding. Unfolding produced at acid pH (N → F transition) has also been examined by this method. The pK of the accessible tyrosines, which may occur in two sets, is anomalously high. Preliminary explorations of the effects of low and high ionic strengths, temperature, and defatting procedures on the ionization and unfolding have also been made. The results with bovine serum albumin differ in detail from those with the human protein. Rate measurements of all the experiments show that a fast first-order process dominates the kinetics; the dependence of rate on pH increases rapidly at pH >11.4. The effects of detergent concentration are complex: *e.g.*, 20 equiv of dodecyl sulfate result in a slowed rate, only slightly dependent on pH, even when the reaction goes to completion at high pH, but the highest detergent concentrations enhance the rate at any pH.

It is now well known that some of the acidic and basic groups of many native proteins in solution are inaccessible to solvent or other solutes. Thus these groups do not contribute to protein titration curves if the latter are determined at very short time intervals after mixing. Some of the phenolic groups of tyrosine are almost always found in this "masked" category (Steinhardt and Reynolds, 1969, Table VI). Since dissociating a proton requires that the protein undergo a prior conformation change (which exposes the groups to solvent), their apparent dissociation constants contain factors contributed by the equilibria controlling these conformation changes. The discrepancy between the measured titration constants and those of normally accessible groups has often led to their being described as "abnormal" or "anomalous" tyrosines. However, since the conformation change is sometimes observably time dependent (for an example, see data on ribonuclease (Tanford *et al.*, 1955)), and since the ionization of phenolic groups is accompanied by large easily measured time-dependent changes in their ultraviolet (uv) spectra, the origin of the "abnormal" titration behavior of these tyrosines is fairly obvious. The use of stop-flow devices is well suited to reveal the time-dependent nature of the abnormal tyrosine dissociations when the reactions are more rapid than with ribonuclease. A suitable wavelength is 243 nm at which the extinction coefficient of tyrosine phenolic groups is close to zero, but at which the extinction coefficient of the corresponding phenoxy ion may be greater than 11,000 (Wetlaufer, 1962).

The time-dependent change in extinction coefficient (absorption) may be used to study the conformation change itself, the equilibria determining the initial and final states, and the kinetics of the reaction that transforms the native conformation to an unfolded state. The technique is not limited to studying denaturation by bases at pH values high enough to ionize the tyrosine. As long as the pH is high enough to cause partial ionization of the tyrosines which are already exposed in the native state (the "outside tyrosines"), the method may be applied to denaturation by sodium dodecyl sulfate or other detergents, and even to denaturation by acid. Application to denaturation by other means would probably also be feasible.

The technique depends on the ability to rapidly mix protein solutions with base, and on the fact that ionization changes may be regarded as instantaneous as compared with the time required for conformation changes. With sufficiently rapid mixing, the *initial* optical density can be used, after correction for instantaneous ionization changes brought about by the changes in pH, to characterize the initial conformation state at the *initial* pH, *i.e.*, the fraction of the total tyrosines which were free to ionize at the initial pH, *i.e.*, before the addition of base. The difference between the initial and infinite time optical densities at the same pH is then proportional to the increase in the number of ionizing groups brought about by the conformation change. The entire increase in optical density at a given high pH is due to the *increment* in the fraction of tyrosine groups which are exposed, and is given numerically by $\Delta OD/\alpha$, where Δ represents the increment from zero to infinite time and α is the degree of ionization of individual groups at the final pH of the experiment. When denaturation is brought about by, *e.g.*, the addition of detergent, there need be no change in pH. The actual sequence of

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