Deoxyribonucleic Acid Photoreactivating Enzyme. A Quantitative Chemical Assay and Estimation of Molecular Weight of the Yeast Enzyme*

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ABSTRACT: We describe a simple chemical assay for photoreactivating enzyme, which uses [^{3}H]thymine-labeled, acetophenone-photosensitized *Escherichia coli* DNA, containing more than 30% of its radioactivity as cyclobutane thymine–thymine dimers, as the substrate. Characteristics of this assay are compared with those of the more common *Haemophilus*-transformation bioassay. The molecular weight of yeast photoreactivating enzyme in crude preparations is estimated by gel filtration to be 72,000 and by sucrose gradient analysis to be about 63,000.

Deoxyribonucleic acid photoreactivating enzyme catalyzes in the light the direct remonomerization of pyrimidine dimers induced in DNA by ultraviolet (uv) radiation (Setlow and Setlow, 1963; Wulff and Rupert, 1962; Cook, 1967). Since such dimers disrupt the various functions of DNA (for review, see J. K. Setlow, 1966), their monomerization *in situ* constitutes a direct repair event. The activity of photoreactivating enzyme *in vivo* underlies the biological repair phenomenon of *direct photoreactivation* (Jagger and Stafford, 1965), which has been observed in nearly all plant and animal phyla (for review, see Cook, 1970). The light requirement for the overall reaction is absolute.

Photoreactivating enzyme activity was first demonstrated *in vitro* by Goodgal *et al.* (1957), who showed that the transforming activity of uv-irradiated DNA from *Haemophilus influenzae* could be partially restored by incubation in the light in the presence of an extract of *Escherichia coli*. Since that time transformation systems have been the basis for the most commonly used assay for the activity from any source. The principal advantage of the transformation assay is its high sensitivity, since only a few nanograms of irradiated DNA are required. The principal disadvantages are the possible inactivation of the transforming function by nucleases in the material being assayed, and the necessity for maintaining competent bacteria.

It is obviously desirable to have an assay of equal sensitivity that measures directly the conversion of the substrate to its product. In the past this has been feasible with radioactive techniques (Wulff and Rupert, 1962; Setlow and Carrier, 1966) but not at all simple, since even heavily uv-irradiated DNA contains only a few per cent of its pyrimidines as dimers (Wulff, 1963). Accurate quantitation of the dimers must be made against a high background of monomeric pyrimidines, as well as a residuum of other nonphotoreactivable photoproducts. In careful work this usually requires at least two-dimensional chromatography.

The demonstration by Lamola and Yamane (1967) and Lamola (1969) that, by use of the photosensitizer acetophenone, nearly 40% of the thymine in *E. coli* DNA can be converted to cyclobutane thymine–thymine dimers with the quantitatively trivial production of other photoproducts has made available a new substrate for photoreactivating enzyme *in vitro*. [^{3}H]Thymine-labeled, acetophenone-treated DNA can be used in an assay that has the same order of sensitivity as the *Haemophilus* assay but is very much less susceptible to the action of nucleases and does not require the maintenance of competent bacteria. Moreover, this assay measures directly the chemical conversion of the substrate. This paper describes the assay in detail and our use of it in estimating the molecular weight of yeast photoreactivating enzyme. Although the experiments described here deal only with yeast, we have found the assay convenient and useful in studies of the activity in many diverse tissues (Cook, 1972).

Materials and Methods

Yeast Enzyme Preparation. The preparation of crude yeast enzyme followed basically the first part of the procedure of Muhammed (1966). Commerical Baker's yeast was dried 2–3 days at 37°. The dry material was added to 4 times its weight of 0.067 M potassium phosphate buffer (pH 7.1) and stirred 6 hr at 37°. The lysed cells were centrifuged (3000*g*, 20 min) and the pellet discarded. The supernatant was brought to 33% saturation with ammonium sulfate at 4° and stirred overnight. The suspension was centrifuged at 15,000*g* for 20 min, and again the pellet was discarded. The supernatant was brought to 60% saturation with ammonium sulfate, again stirred overnight at 4°, and centrifuged; the supernatant was discarded. The final pellet was dissolved in 0.04 M potassium phosphate buffer and dialyzed against three changes of this buffer in the cold. Most of the experiments described in this paper were done with this crude material, which had a protein concentration (Lowry *et al.*, 1951) of 17 mg/ml. The activity in this material has been stable at −20° for more than 1 year. In preparation for some of the molecular weight determinations, the crude material was adsorbed to phosphocellulose in a column, washed with 10 bed volumes of 0.04 M potassium phosphate buffer, and eluted with 0.33 M K_2HPO_4 (Muhammed, 1966). The pH of the eluate was restored to 7.1. There was no spectrally apparent contamination with nucleic acid.

Substrate for the Photoreactivation Assay. [^{3}H]Thymine-labeled DNA, isolated by the method of Marmur (1961) from *E. coli* 15 T$^-$, was the gift of W. L. Carrier. The DNA was

* From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830. *Received September 8, 1971.* Research sponsored by the U. S. Atomic Energy Commission under contract with the Union Carbide Corp.

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irradiated in the presence of 0.01 M acetophenone with uv light of wavelength greater than 310 nm (Lamola and Yamane, 1967; Lamola, 1969) until more than 30% of its thymine was converted to pyrimidine dimers. After irradiation, the DNA in 0.01 M sodium phosphate buffer at pH 6.8 was absorbed to hydroxylapatite and washed with 10 column volumes of 0.12 M sodium phosphate buffer to remove any single-stranded nucleic acids (Bernardi, 1965; Miyazawa and Thomas, 1965). The double-stranded DNA was eluted in 0.4 M sodium phosphate buffer and dialyzed against 0.04 M buffer to reduce the salt concentration. Finally, the molecular weight was determined in neutral sucrose gradient (5–20%) with bacteriophage λ-DNA (mol wt 33 × 10^6) (Caro, 1965) as marker (Studier, 1965). R. B. Setlow assisted with the molecular weight determination.

Photoreactivation Assay. A bank of six fluorescent blacklights (General Electric) with peak wavelength at about 380 nm are mounted on the floor of an incubator set at 36°. For reaction vessels, flat-bottomed glass vials about 2 cm in diameter stand in temperature-equilibrated water in large Petri dishes, which in turn rest on a glass shelf 15 cm above the lights. With all of the lights on, the intensity (> 100 ergs mm^{-2} sec^{-1}) is well above the saturating level for the reaction, so that the position of the reaction vials with respect to the lights is not critical.

To each reaction vial is added a mixture of substrate DNA in 0.04 M potassium phosphate buffer and KCl or NaCl in such amounts and concentrations that the vial contains less than 1 μg of DNA in 0.9 ml of a salt solution of the desired ionic strength, buffered to pH 7.1 with phosphate buffer at a final concentration of less than 0.005 M. The reaction is very sensitive to ionic strength (Harm and Rupert, 1970; Cook, 1972), being fastest and the assay therefore most sensitive when the ionic strength is 0.17–0.19. On both sides of this optimum the rate dependency is so steep that it is important for the the ionic strength to be maintained constant for comparable assays.

The substrate mixture is allowed to come to temperature with the blacklights on, and the reaction is started by the addition, with thorough mixing, of 100 μl of the enzyme preparation, which also is in a solution of appropriate ionic strength, pH, and temperature. Owing to the temperature lability of this enzyme in many organisms, the prewarming step should be as brief as possible.

Samples of 100–200 μl are taken at timed intervals from the reaction mixture and added to an equal volume of cold 10% tricholoracetic acid. The acid-insoluble material is hydrolyzed in 98% formic acid at 175° (Carrier and Setlow, 1971), and the formic acid is evaporated. The residue is taken up for chromatography in about 50 μl of a mixture of 1 mg/ml each of thymine and cytosine in water and streaked across the origin of a 1-in.-wide strip of Whatman No. 1 paper. The chromatograms are developed in saturated ammonium sulfate–1.0 M sodium acetate–2-propanol (40:9:1, v/v) (Wacker *et al.*, 1960) for about 3 hr, when they have run about 16 cm and the pyrimidine markers are well separated. The peak radioactivity of the pyrimidine dimer is found near the leading edge of the cytosine marker (Figure 1). The paper strip is sliced across the trailing edge of the cytosine marker. A cut forward toward the solvent front and twice the length of the cytosine marker includes all of the dimer radioactivity. A cut of the same length backward toward the origin includes all of the monomeric thymine radioactivity. Each cut is put into its own counting vial, to each of which is added 1 ml of water and 10 ml of dioxane scintillation fluid containing 5 g/l. of Permablend (Packard Instrument Co.) as scintillator. Since the amount of paper (about 2.5 × 5 cm) stuffed into each vial is enough to cause a certain amount of quench in the counting, it is convenient to have the same amount of paper in each vial of a pair. The fraction of dimers in each sample is computed, after background correction, as ((counts per minute in dimer cut)/(counts per minute in dimer cut + counts per minute in thymine cut)). Further computations are described below in the Results section.

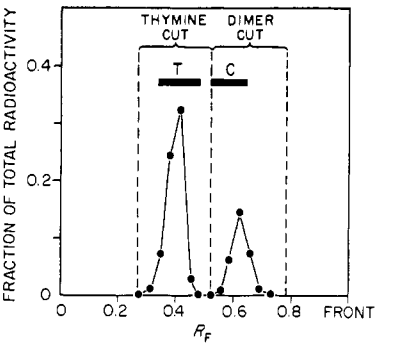

FIGURE 1: Chromatography of [^{3}H]thymine-labeled, acetophenone-photosensitized, hydrolyzed *E. coli* DNA in ammonium sulfate–sodium acetate–2-propanol. Uv-opaque markers were thymine (T) and cytosine (C). For this figure, the radioactivity was measured in sequential cuts of 0.5 cm from a 15-cm chromatogram and plotted as indicated by the points. In the two-cut assay, as indicated in the upper part of the figure, the dimer cut is twice the length of the cytosine marker running forward from the trailing edge of that marker toward the solvent front. The thymine cut is of the same length, running backward from the trailing edge of cytosine toward the origin.

It was occasionally useful to use a simpler "microassay" involving, instead of a complete curve with a series of time points, a single point taken at a fixed time. The components of the reaction mixture were essentially as above, except that the volumes were scaled down by a factor of ten and all the reactions were carried out in a clear Plexiglass microtiter plate that could hold up to 80 samples. All other procedures were as described above. The limitations of the microassay are given below.

Molecular Weight Estimation by Gel Filtration. A column 1.5 × 90 cm, containing Sephadex G-150 (Pharmacia), was equilibrated with the elution buffer (1.0 M KCl–0.04 M potassium phosphate buffer–0.001 M EDTA, pH 7.1). The column was loaded with 3 ml of a mixture of the desired protein solution (less than 10 mg of protein/column), blue dextran (mol wt 2 × 10^6), and [^{14}C]thymine (1–2 × 10^5 cpm) and eluted with the elution buffer at 4°. The void volume (V_0) was determined by the blue dextran, and the internal volume (V_i) by the elution of the radioactive thymine. The column was calibrated by measuring elution volumes (V_e) of purified proteins of known molecular weight, which were monitored by their absorbancy at 280 nm, except hemoglobin, which was followed at 417 nm. The proteins were: egg white lysozyme (Sigma); bovine plasma albumin (Mann); chymotrypsinogen (Mann); ovalbumin (Mann); hemoglobin from freshly lysed human erythrocytes; and rabbit muscle triose phosphate isomerase, a gift from F. C. Hartman. The molecular weight of the latter was taken as 54,000 (Norton *et al.*, 1970). The distribution coefficient, K_D, of each protein in the gel was calculated from the relationship: $K_D = (V_e - V_0)/(V_i - V_0)$.

Sucrose Gradient Analysis of Molecular Weight. Sucrose gradients (5–20%, 3.6 ml) were made up in the same buffer

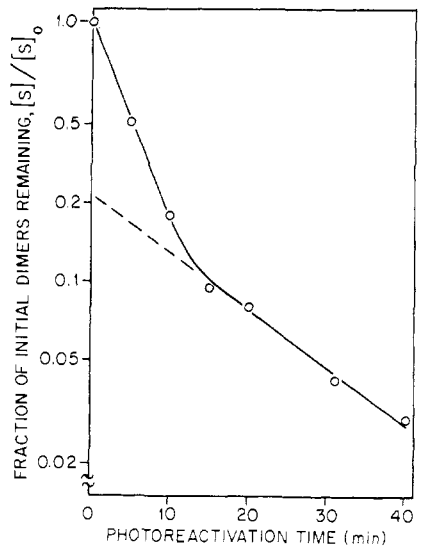

FIGURE 2: Time course of photoreactivation by yeast photoreactivating enzyme of thymine–thymine dimers in acetophenone-photosensitized *E. coli* DNA. Reaction mixture contained, in 1 ml, 0.17 mg of yeast protein and 30 ng of acetophenone-treated DNA at 36°. Ionic strength was 0.092.

salts as used for the gel filtration. The gradients were overlaid with 100 μl of buffer, containing 800 μg of yeast protein and 75 μg of rabbit muscle triose phosphate isomerase as marker. The samples were spun in an SB-405 rotor in an International B-60 centrifuge at 4° and at 56,000 rpm for 17 hr. Because of the high uv absorbancy of the crude yeast preparations, it was necessary to assay the isomerase by its activity, and for this it was first necessary to inactivate the endogenous yeast isomerase. This was done by treating the yeast preparations with chloroacetol phosphate, a specific ligand for isomerase which reacts covalently with the active site (Hartman, 1971). When no further isomerase activity was detectable, the ligand itself was decomposed by adding β-mercaptoethanol to 0.01 M. The sulfhydryl reagent does not inhibit the photoreactivating activity nor the subsequently added rabbit muscle isomerase. The isomerase activity was assayed by the procedure of Beisenherz (1955; see Norton *et al.*, 1970).

The samples from both the Sephadex columns and the sucrose gradients were diluted with water to reduce the ionic strength for assay of photoreactivating enzyme. Microassays were performed, and total recoveries of activity were between 80 and 110%.

Results and Discussion

Properties of the Substrate. When the acid-insoluble radioactivity was assayed by the filter disk technique (Bollum, 1959), the substrate DNA used here had a specific activity of 3.25 $\times 10^5$ cpm/μg of DNA. The acetophenone-treated DNA was double stranded, according to the criterion of its behavior on hydroxylapatite (Bernardi, 1965; Miyazawa and Thomas, 1965). From multiple analyses using either the two-cut technique described above or higher resolution chromatography as in Figure 1, the fraction of [^{3}H]thymine radioactivity in cyclobutane pyrimidine dimers (or cochromatographing with them) was 0.355 ± 0.015 of the total. In the two-cut technique, there is approximately a 5% error in any single determination. When the chromatographs were developed with butanol–acetic acid–water as solvent (Smith, 1963), we observed, as did Lamola (1969), that less than 2% of these dimers were of the mixed thymine–cytosine type. The total dimer production depends of course on the extent of the acetophenone photosensitization treatment. For the substrate used here, then, a single nanogram of DNA had about 100 cpm in dimer activity and about 200 cpm in thymine activity. These amounts may be easily measured without unreasonably long counting, and as little DNA is required as is commonly used in the *Haemophilus* assay.

By analysis in neutral sucrose gradients, the DNA had a molecular weight of 6×10^5, from which it may be estimated that each substrate-containing DNA molecule contained 500 thymines, of which 175 were in the form of pyrimidine dimers.

The substrate was exposed under optimum conditions to the photoreactivating activity of the yeast enzyme preparation. After 15 min the light (which yielded more than 90% photoreactivation), a small sample was withdrawn for dimer analysis, and fresh enzyme was added to the reaction mixture. This process was repeated 6 times. Even with such exhaustive treatment, we were not able to reduce the radioactivity in the "dimer cut" below 0.017 of the total. The photoreactivable sector of the substrate therefore is $(0.355 - 0.017)/(0.355) = 0.95$. We do not know the nature of the small amount of nonphotoreactivable radioactivity. It could perhaps be pyrimidine dimers in a position where they are not susceptible to photoreactivating enzyme. In any event, we made appropriate corrections for this nonphotoreactivable material (see below).

Assay. It has been shown in the extensive studies by Harm *et al.* (summarized, 1971) that the reaction may be described by

$$[E] + [S] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [ES] \xrightarrow{k_pI} [E] + [P]$$

where the reversible formation of the enzyme–substrate complex occurs in the dark (with $k_2/k_1 \approx 10^{-10}$–10^{-11} M, depending on reaction conditions), and the photolytic step occurs with a rate constant which depends on the product of a photolytic constant k_p and the light intensity I. Under the conditions of our assay, k_pI is maintained high enough so that [ES] is always negligibly small; *i.e.*, increasing I does not further increase the overall reaction rate. Since k_2 is very small relative to k_1, the dark dissociation of ES is also negligible, and the overall reaction is essentially a function only of reactant concentrations and k_1. With [ES] always small, [E] remains virtually constant at $[E]_0$, and the disappearance of the substrate may be described by the pseudo-first-order expression

$$d[S]/dt = -k_1[E]_0[S] \quad (1)$$

$$\ln [S]/[S]_0 = -k_1[E]_0t \quad (2)$$

In practice, $[S]/[S]_0$ is calculated by first determining the fraction f of total radioactivity in the dimer cut and allowing for nonphotoreactivable radioactivity; thus, for the particular substrate used here,

$$[S]/[S]_0 = \frac{f - 0.017}{0.355 - 0.017} = \frac{f - 0.017}{0.338}$$

Three sets of observations relative to the application of eq 2 to this reaction must be considered; linearity, independence of slope from substrate concentration, and dependence on enzyme concentration.

LINEARITY. According to eq 2, $\ln [S]/[S]_0$ is expected to be linear with time at a given enzyme concentration. Figure 2

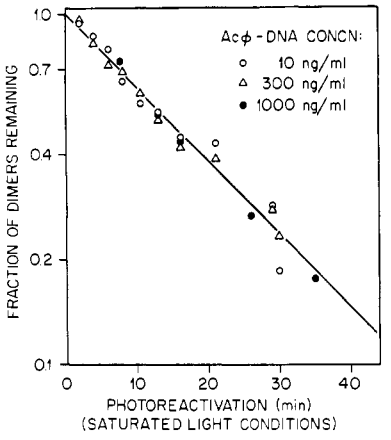

FIGURE 3: Independence of photoreactivation rate constant (slope of repair curve) from substrate concentration under saturating light conditions. Acetophenone-treated (Ac-Φ) DNA in concentrations from 10 to 1000 ng per ml, as indicated. Yeast protein concentration, 0.17 mg/ml; ionic strength, 0.06. Note differences in ionic strength between this experiment and those depicted in Figures 2 and 4.

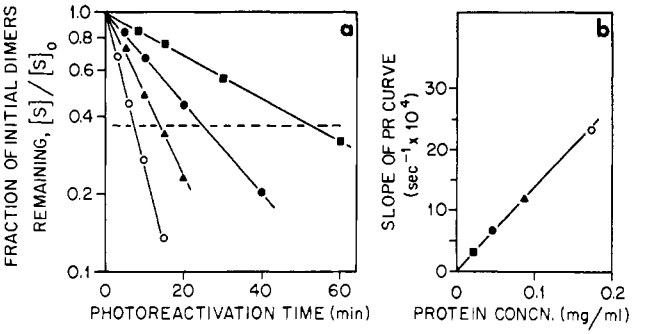

FIGURE 4: Rates of photoreactivation of thymine–thymine dimers in acetophenone-treated DNA as a function of yeast protein concentration. DNA concentration, 120 ng/ml; ionic strength, 0.092. Other conditions as described in the Materials and Methods section. (a) Repair curves with time at four protein concentrations. (b) Slopes of curves in part a as a function of protein concentration. Slopes calculated as the reciprocal of the time in seconds to reach 37% photoreactivable radioactivity remaining.

shows an experiment in which the shape of the curve was checked with care, particularly at times after the reaction was more than 90% complete. It is evident that the curve shows a marked break, and that in the later stages the reaction slows by a factor of 2 or more. An artifactual effect of this kind could arise in calculating the data if too small a value were used for the nonphotoreactivable fraction. In order to avoid the possibility of such an artifact, we have recalculated several experiments with that term deliberately overestimated, but the break in the curve is still evident. By extrapolating the second slope to the ordinate, we observe that 20% or more of the dimers are being repaired at the slower rate. This effect, which has also been observed in the *Haemophilus* assay by Harm and Rupert (1970), cannot be ascribed to the differential rates of repair of different kinds of pyrimidine dimers, since in this case only 2% or less of the dimers are mixed cytosine–thymine, and cytosine–cytosine dimers are not labeled at all. We can only conclude that some of the dimers are less accessible to the enzyme than others. The conformational basis for this difference is not known. As may be seen in Figure 2 and and subsequent figures, at least the first 80% of the repair takes place at a rate which may be characterized by a single slope. In subsequent discussion, we shall be concerned only with this initial slope.

INDEPENDENCE OF THE SLOPE FROM INITIAL SUBSTRATE CONCENTRATION. According to eq 2, so long as the data are expressed as a fraction of the initial substrate concentration the slope of the repair curve is expected to be independent of the substrate concentration. That this expectation is realized may be seen in Figure 3, depicting an experiment in which the substrate concentration was varied by a factor of more than 100. This independence very much eases restrictions on conditions for determining enzyme concentrations. A corollary is that competitive inhibition of the reaction by unlabeled substrate will not be observed under these conditions. Of course, at high total substrate concentration the rate of dark formation of ES may be fast enough so that [ES] is no longer negligible, and the apparent rate constant for the overall reaction is reduced accordingly. The observation of competitive inhibition, however, is more easily made and more readily quantitated at low light intensity, where [ES] approaches $[E]_0$ (data to be published).

DEPENDENCE OF THE SLOPE ON ENZYME CONCENTRATION. As expressed in eq 2, under saturating light conditions the slope of the repair curve depends directly on enzyme concentration; hence the aptness of the system as an assay (Figure 4). It has been our experience that at low concentrations the reaction does not remain linear beyond about 60 min, probably due to temperature lability of the enzyme. A further restriction, alluded to above, is the ±5% uncertainty in the estimation of dimers.

The assay is most satisfactory when 20–80% of the dimers is monomerized in 2–40 min. These numbers represent a more than 100-fold concentration range in which the enzyme can be directly assayed, and of course this range can be extended by appropriate dilution.

Using a modification of the flash photolysis technique first described by Harm and Rupert (1968), we have determined the absolute number of enzyme molecules in some of our preparations (data to be published), and from these numbers we have also estimated k_1. At the peak of the ionic strength curve and at 37°, we find a value of 1.08×10^8 l. mole^{-1} sec^{-1}, which is very close to the value calculated by Harm and Rupert from the less direct *Haemophilus* assay (see Harm *et al.*, 1971). They were using a DNA which contained only about 1% dimers. Apparently, the very large number of dimers in our material does not so alter the DNA as to markedly affect its interaction with the enzyme.

When it is desired to take many samples, as in assaying through a gradient, the microassay (Materials and Methods) with all samples taken at a fixed time may be convenient. Since both time and k_1 are then invariant, eq 2 becomes simply: $[E]_0 = \ln([S]_0/[S]) \times$ constant. This method is useful in finding peaks of activity, provided $[S]_0/[S]$ does not exceed 9 (*i.e.*, 90% repair), but the ±5% uncertainty in a single determination leads to a base line in regions of no activity which may fluctuate around zero by as much as 6% of the peak value. Negative values in such measurements are plotted as zero activity in the experiments described below.

Molecular Weight Estimation. GEL FILTRATION. In early experiments with crude yeast preparations on Sephadex columns, all of the photoreactivating enzyme activity came through with the void volume, which had a cloudy appearance. We thought that this might be due either to aggregation of proteins or to nonspecific binding of the enzyme to nucleic acids in the preparation. The effect was overcome by eluting the

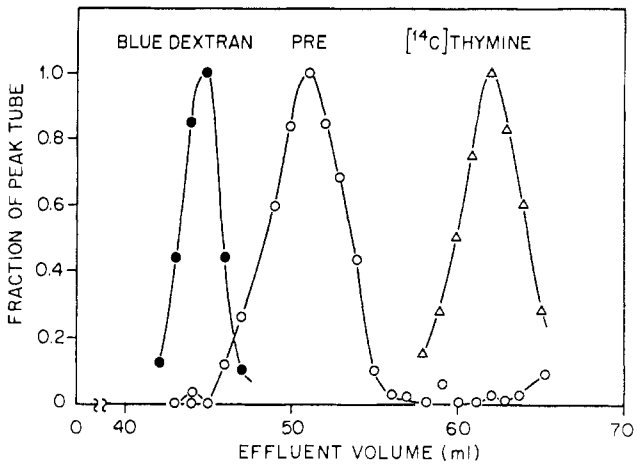

FIGURE 5: Elution of yeast photoreactivating enzyme activity and markers from Sephadex G-150. Flow rate, 8 ml/hr; K_D for yeast activity in this experiment, 0.36.

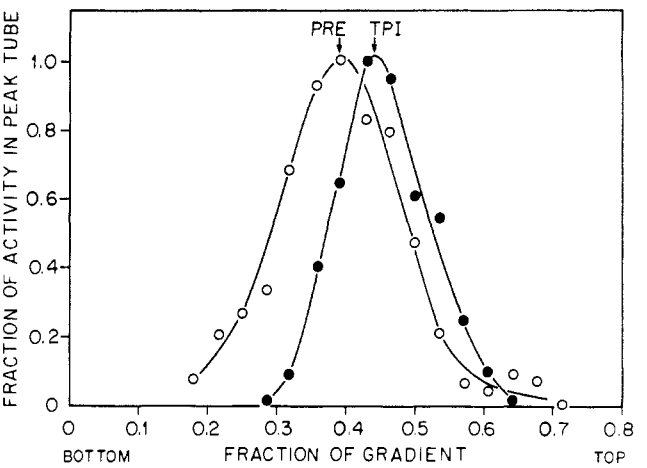

FIGURE 6: Cosedimentation of yeast photoreactivating enzyme (PRE) activity and rabbit muscle triose phosphate isomerase (TPI) activity in 5–20% sucrose gradient. Conditions given in text.

macromolecules from the columns in solutions of high ionic strength (1.0 M KCl plus buffers, see Materials and Methods). Under these conditions, yeast enzyme was eluted from G-150 as a single peak (Figure 5). In two determinations of yeast enzyme elution from G-150, the activity peak was found at positions corresponding to molecular weights of 71,000 and 73,000 when the column was calibrated with lysozyme, chymotrypsinogen, triose phosphate isomerase, and hemoglobin (but see Conclusions).

SEDIMENTATION IN SUCROSE GRADIENTS. The high ionic strength buffer was again used to avoid aggregation in 5–20% sucrose gradients. The material sedimented was either the crude yeast preparation, the crude preparation treated with pancreatic DNase (50 μg/ml, 24 hr at 4°), or the nucleic acid free preparation which had been more highly purified by phosphocellulose chromatography. The marker we used was triose phosphate isomerase activity with a molecular weight of 54,000 (Norton *et al.*, 1970). The molecular weight of the yeast photoreactivating enzyme activity (Figure 6) was calculated from the relationship given by Martin and Ames (1961): (distance from meniscus of protein[1]/distance from meniscus of protein[2]) = $(Mw_1/Mw_2)^{2/3}$. The molecular weights so calculated for the activity from the cruder preparations were 60,000 and 62,000; from the DNase preparation, 62,000; and from phosphocellulose-purified material, 69,500.

Conclusions

It has been shown by Ackers (1964) that the behavior of proteins in gel-filtration columns correlates well with their Stokes' radius and less well with their molecular weight. For example, ovalbumin and bovine plasma albumin in G-150 do not fall on the line which describes the relationship between K_D and the logarithm of the molecular weight of the more spherical proteins. If only the albumins were used for the calibration of the G-150 column, the estimated molecular weight for the yeast enzyme would be near 60,000. For these reasons, and in the absence of better physical data for this impure protein, the better estimate of the molecular weight comes from the sucrose-gradient determinations, which give a mean of 63,000.

Earlier, the molecular weight had been estimated by Muhammed (1966) to be nearer 30,000, but his determination was made optically with the analytical ultracentrifuge, using a preparation which is now known to have been no more than 1% photoreactivating enzyme.

The yeast enzyme appears to be somewhat smaller than that from the blue-green alga *Anacystis nidulans*, for which Saito and Werbin (1970) have estimated a molecular weight of 93,000.

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Inhibition of Transfer Ribonucleic Acid Methylase Activity from Several Human Tumors by Nicotinamide and Nicotinamide Analogs*

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ABSTRACT: Previous studies (Halpern, R. M., Chaney, S. Q., Halpern, B. C., and Smith, R. A. (1971), *Biochem. Biophys. Res. Commun. 42*, 602) have demonstrated that a low molecular weight inhibitor of tRNA methylase exists in normal rat liver. The isolation of this substance and subsequent structure elucidation has revealed it to be nicotinamide. In these present studies, we have demonstrated that nicotinamide is capable of inhibiting tRNA methylase activity obtained from a human tumor cell line grown in tissue culture (KB cells). Furthermore, several structural analogs of nicotinamide, for example, thionicotinamide, 6-aminonicotinamide, and pyridine-3-carboxaldehyde, were also capable of inhibiting the KB cell enzyme. Finally, we have demonstrated that nicotinamide is able to inhibit tRNA methylase activity in extracts prepared from several human malignancies: three adenocarcinomas of the bowel, a reticulum cell sarcoma, and a seminoma. Nicotinamide is without effect on the tRNA methylase activity prepared from normal tissues removed from the areas surrounding the tumors.

The greater tRNA methylase activity in extracts prepared from malignant cells (Berquist and Mathews, 1962; Tsutsui *et al.*, 1966; Chaney *et al.*, 1970) as well as from cells isolated from fetal tissue (Simon *et al.*, 1967; Hancock, 1967; Kerr, 1970) has been attributed to the absence of inhibitors of that enzyme in those cells. In 1970, Kerr identified a nondialyzable heat-labile, trypsin-sensitive protein from adult normal tissue and Novikoff tumor, which was capable of inhibiting tRNA methylation. Recently we have shown that a dialyzable inhibitor (Chaney *et al.*, 1970) is present in normal adult tissue and absent in the malignant tissues which we have examined. We have purified the dialyzable inhibitor and subsequent analysis has revealed it to be nicotinamide (Halpern *et al.*, 1971). Kerr has recently reported the isolation of two substances from normal adult tissue which "in concert" are capable of causing inhibition of tRNA methylase. One of these is a low molecular weight fraction (mol wt <700) which by itself is inactive, but when added to the second high molecular weight substance has increased inhibitory activity. The high molecular weight component is apparently absent from embryonic and tumor tissues (Kerr, 1971).

These present studies were undertaken to determine whether or not analogs of nicotinamide possessed inhibitory activity against tRNA methylase isolated from adult rat liver, a W-256 tumor, and W-256 tumor cells grown in tissue culture.[1] In addition, we wished to examine whether the tRNA methylase prepared from human malignant cells was capable of being inhibited by nicotinamide; therefore, we prepared tRNA methylase from KB cells grown in culture and from several human malignancies: three adenocarcinomas of the bowel, a reticulum cell sarcoma, and a seminoma. All were strikingly inhibited by nicotinamide. Kinetic evaluation of the effects of nicotinamide as well as some of the more active analogs against KB cell and W-256 cell tRNA methylase are also presented.

Materials and Methods

Preparation of tRNA Methylase. KB CELLS. KB cells were grown using a roller cell apparatus to a density of 5×10^6/ml. Cells were harvested and washed by suspension in isotonic sterile saline. They were then resuspended at a density of 0.01 g/ml in Tris–magnesium chloride buffer (Tris, 0.01 M, pH 8–magnesium chloride 0.01 M). The cells were broken by homogenization at 4° in a Sorvall OmniMixer run at top speed for approximately 1 min. After centrifugation at 4° for 10 min at 10,000*g*, the supernatant was removed and centrifuged at 105,000*g* for 60 min at 4°. The 105,000*g* supernatant was removed and used as a cell-free tRNA methylase preparation.

* Contribution from the Departments of Chemistry and Medicine and the Molecular Biology Institute, University of California at Los Angeles, Los Angeles, California, Chemistry Department Publication No. 2861, and from the ICN Nucleic Acid Research Institute, Irvine, California 93664. *Received July 8, 1971.*

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