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Efficiency of Photolytic Binding of Ethidium Monoazide to Nucleic Acids and Synthetic Polynucleotides

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SUMMARY

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Radiolabeled 3-amino-8-azido-5-ethyl-[^{14}C]-6-phenyl phenanthridine (ethidium monoazide) was photolyzed *in situ* in the presence of calf thymus DNA, RNA, poly A, calf thymus histones and nucleoid structures prepared from lymphocytes. Non-linear least squares analysis of the binding data enabled us to predict the amount of drug adduct formed at infinite polymer concentration. Photolytic efficiency could then be calculated since the input drug concentration was constant and therefore represented the non-covalent complex expected at infinite polymer concentration. The efficiency of photolysis for native, heat-denatured and formaldehyde-denatured DNA was 0.23, 0.26 and 0.45, respectively, and for RNA it was found to be 0.29. The value for poly A was 0.42. The intensity of light was not a limiting factor under the conditions of these experiments.

INTRODUCTION

Photoaffinity drug binding is a promising new approach for studying the molecular details of drug-nucleic acid interactions and for defining the mechanisms for nucleic acid dictated biological processes. Studies in this laboratory have used a photosensitive azide analogue of ethidium to study mutagenesis in yeast and *Salmonella* (1-5) and to provide a covalent probe for following DNA repair (6). The covalent attachment of the drug moiety on photolysis provides a means of correlating a variety of biological processes with the kinetic and molecular details of drug binding. Ideally, the results from photoaffinity labeling would accurately represent *in vivo* drug binding if the following criteria were met: (1) the initial non-covalent binding of the photosensitive analogue is identical to that of the parent drug; (2) the activated state is sufficiently reactive and non-specific so that covalent attachment is dictated by the non-covalent complex rather than by a dependence on the reactivity of moieties within the attachment site; (3) the covalent adducts formed produce appropriate biological responses; and (4) the photolytic conversion to the covalent adduct is an efficient process. Previous studies from this laboratory indicate that the azide analog selected for the present investigation meets the first three criteria. The azide was found to produce cytoplasmic mutations in yeast in the same fashion as the parent ethidium, suggesting identity of initial drug binding of the two compounds in these cells (1). Furthermore, ethidium azide was found to compete with the unmodified drug for intracellular binding and for the enhanced mitochondrial mutagenesis resulting from such binding (5). Also, the nitrene (produced by photol-

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ysis of ethidium azide) is highly reactive and relatively nonselective (7).

The present experiments are concerned with the question of the efficiency of the covalent reaction on photolysis of ethidium monoazide with native and denatured DNA, RNA, nucleoids, histones and poly A. The results show that photolytic attachment to nucleic acids produced by relatively low intensity irradiation with an ordinary fluorescent lamp is an efficient process. The efficacy of such a low intensity visible light source means that the destructive effects of radiation *per se* can be minimized in studies of intact cells.

MATERIALS AND METHODS

Radiolabeled [^{14}C]ethidium monoazide (3-amino-8-azido-5-ethyl-6-[^{14}C]-6-phenylphenanthridine) was synthesized using a modification of methods reported previously with [^{14}C]ethidium bromide (specific activity 17.7 mCi/mM, Modichem Developments, Ltd., Manchester, England) as the starting material (8). Ethidium bromide, 11 mg, was dissolved in 0.1 N HCl (1.0 ml) at 5° and a solution of $NaNO_2$ in water (2 mg in 1.0 ml) was added slowly with stirring. The reaction was allowed to proceed at room temperature for 15 min. Sodium hydroxide, 10 N, was added (0.1 ml) to the reaction mixture, and a precipitate occurred immediately. The precipitate was filtered off and dissolved in 2.0 ml of 0.001 N HCl. This volume was applied to a column (15 × 2 cm) containing Cellex-CM cation exchange cellulose (Bio-Rad Laboratories) and eluted with 0.001 N HCl. Ethidium diazide separated as a light yellow band from ethidium monoazide which was observed as an orange band. The diazide eluted first, and 60 ml were collected followed by monoazide, of which 225 ml were collected. Yields for mono- and diazide were determined spectrophotometrically and were 59% and 10%, respectively.

Calf thymus DNA and calf thymus histones were obtained from Sigma Chemical Co. Poly A was purchased from Schwarz Mann, and RNA, soluble A grade, from Calbiochem. Lymphocytes were obtained from heparinized blood samples of normal volunteers by separating in 5% dextran as described by Evans and Norman (9). Nucleoids were prepared from lymphocytes by the method of Cook and Brazell (10). The cells were lysed in a mixture containing Triton X-100 and 1.95 M NaCl. The lysis mixture contained NaCl, EDTA, Tris, and Triton X-100 in amounts which, on addition of one volume PBS (pH 7.2) containing cells, 1×10^7/ml, to three volumes of the mixture, gave final concentrations of the components of 1.95 M, 0.1 M, 2 mM and 0.5%, respectively. The cells were allowed to lyse for 15 min at room temperature and then an equal volume of PBS was added. The solution was centrifuged at 5000 rpm for 30 min at 4° in the S34 rotor in a Sorval RC 2-B centrifuge.

For drug exposure, reagents were dissolved in saline-citrate (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) except for the lymphocytes which were suspended in MEM spinner modified tissue culture medium at 2.4×10^6 cell/ml. The [^{14}C]-ethidium monoazide was added to a final concentration of 10 μM, and the solutions were allowed to stand 1 hr in the dark prior to photolysis. Solutions were then irradiated in plastic tubes for 15 min at a distance of 2 cm from a 30 watt G.E. daylight fluorescent lamp (Buchler Instruments). Following photolysis, cells and nucleoids were lysed and collected on nucleopore filters, 0.8 μM pore diameter, and washed successively with PBS, 5% TCA containing 1% sodium pyrophosphate, and 95% ethanol. The calf thymus DNA, calf thymus histones, RNA and poly A ware precipitated in 20% trichloroacetic acid following photolysis and also collected on nucleopore filters. The air-dried filters were then transferred to scintillation vials containing 10.0 ml Aquasol (New England Nuclear) for counting.

RESULTS AND DISCUSSION

The determination of photolytic efficiency requires that the extent of covalent adduct formation be determined using conditions under which the concentration of non-covalent complex can be determined precisely. Moreover, the confident assignment of a single value requires that the binding sites be identical, a condition sel-

dom met in drug-polymer interactions, or that all classes of non-covalent binding result in the same photolytic efficiency.

The difficulties in determining the precise concentrations and distributions of the non-covalent adducts obtained at various finite drug concentrations were avoided in the present study by the following strategy. Based on simple equilibrium considerations, we reasoned that experiments using increasing amounts of polymer added to a constant amount of drug could be extrapolated to infinite polymer concentration where the drug bound non-covalently should be equal to the total drug added. Moreover, at infinite polymer concentrations, the drug binding sites occupied would be homogeneous and would represent only those sites with the highest affinity for the drug. The efficiency of photolytic binding for this set of sites could then be estimated by taking the ratio of the amount of drug bound covalently at infinite polymer concentration to the total amount of drug available. Experimentally, the amount of covalent adduct formed photolytically from a constant amount of added drug ([^{14}C] labeled) was determined over a wide range of polymer concentration by counting the radioactivity remaining after precipitating and washing the complex.

The results of experiments with native, heat-denatured, formaldehyde-denatured DNA and RNA are shown in Fig. 1. Apparently the counts bound approach a limiting value at high polymer concentrations in every case. We found that all of the data for DNA and RNA could be represented by the simple hyperbolic expression:

$$C = \frac{P}{m + (I)(P)} \qquad (1)$$

where C = counts per minute of bound drug, P = polymer concentration in units of moles/liter, and m and I are constants which are characteristic properties of the individual curves. This equation has the same form as the classical binding curve for a set of identical, independent binding sites. However, we do not mean to imply that such a simple model applies to these curves in the strict theoretical sense. In other words, we do not attach any mechanistic significance to the parameters m and I, apart from using them to estimate the initial slopes and asymptotic limits for the data represented in Fig. 1. That the data in Fig. 1 conform to equation (1) is shown by the close agreement between the observations and the predicted curves. Equation (1) has the same form as the Langmuir adsorption isotherm (11). In the present studies, we justify applying equation (1) to the data by considering that at equilibrium, the drug is distributed between the polymer molecule surface phase and the solution phase. When equation (1) is written in the usual double reciprocal form:

$$\frac{1}{C} = \frac{m}{P} + I, \qquad (2)$$

it is apparent that I is the reciprocal of C at infinite P. The parameter m is easily shown to be the reciprocal of the initial slope for the binding curves by differentiating equation (1) and finding the limiting value at $P = 0$.

Initial estimates of m and I were obtained from double reciprocal plots. Using these estimates and equation (1), error mean squares were calculated from the binding data. The values of m and I which minimized the error mean square for each curve were then found by an iterative scheme of successive approximations based on the method of Marquardt (12). The fraction of total drug bound non-covalently to the polymer prior to photolysis depends on the dissociation constant of the polymer-drug complex as well as the concentration of polymer. Assuming a finite dissociation constant and a constant, finite level of drug input, it seems reasonable to expect that at infinite polymer concentration, the concentration of unbound drug would be negligible compared to the amount bound. The input drug level was constant at 5.82×10^5 cpm. Fractional values can be obtained for photolytic efficiency by dividing 5.82×10^5 into the reciprocal of the I values. This method of estimating photolytic efficiency assumes that as the polymer concentration approaches infinity, the equilibrium distribution of the drug between polymer surface and solution phase is such that the amount of drug remaining in solution is negligible

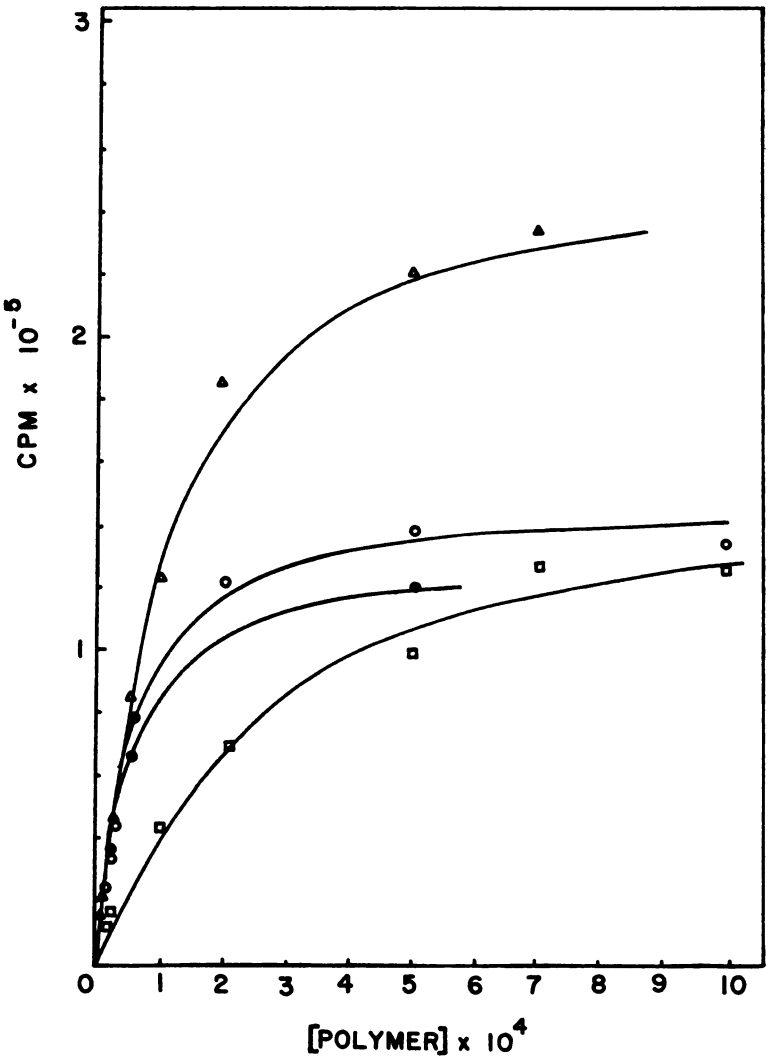

FIG. 1. *Effects of polymer concentration on photolytic binding of [^{14}C]ethidium monoazide to calf thymus DNA* (●), *heat-denatured DNA* (○), *formaldehyde-denatured DNA* (△), *and RNA* (□)

For denaturation, DNA solutions that were 1% with respect to formaldehyde or that contained no formaldehyde were heated at 90° for 10 min and then cooled rapidly to 5°. Binding experiments were then performed as described in the text. The lines are predicted curves calculated with equation (1) and the values of the parameters I and m for the respective polymers listed in Table 1. Polymer concentration is in units of moles/liter, and the ethidium monoazide concentration is 10 μM. Samples were 2 cm from the light source, and the time of exposure was 15 min.

compared to the amount bound. These ratios for this experiment and for the experiments described below are listed in Table 1. For comparison, the final values obtained by computer analysis are presented with those obtained by an initial manual double reciprocal plot. The native DNA and heat-denatured DNA gave values for the photolytic efficiency of 0.23 and 0.26, respectively, while that for formaldehyde-denatured DNA was 0.45. The m values, whose reciprocal is the initial slope of the hyperbolic curve, are also listed in Table 1. They (m^{-1}) represent changes in the CPM attached covalently per polymer unit as the polymer concentration approaches zero.

TABLE 1

Binding of [^{14}C]ethidium monoazide

Photolytic binding of [^{14}C]ethidium monoazide at infinite polymer concentration. The saturation values (I^{-1}) for infinite polymer concentration, and the initial slope (m^{-1}) of the hyperbolic curve were determined by non-linear least squares analysis. Photolytic efficiency was calculated by dividing each saturation value by the input drug concentration, 10 μM (5.82 × 10^5 cpm). Details for reciprocal plots and experimental methods are given in the text.

Polymer	Saturation value ($I^{-1} \times 10^{-5}$; cpm)	Initial slope (m^{-1})	Efficiency
Native DNA	1.31	2.62[a]	0.23 (0.23)[e]
Heat-denatured DNA	1.49	2.96[a]	0.26 (0.29)
Formaldehyde-denatured DNA	2.64	2.53[a]	0.45 (0.43)
RNA	1.67	0.60[a]	0.29 (0.22)
Nucleoids	1.09	0.040[b]	0.19 (0.18)
Histones	0.356	5.49[c]	0.06 (0.06)
Poly A	2.46	[d]	0.42

[a] Expressed as CPM/mole nucleotide/ml × 10^{-9}.

[b] Expressed as CPM/cell/ml.

[c] Expressed as CPM/mg/ml × 10^{-5}.

[d] Since the poly A data were analyzed with the logistic curve, no value was obtained for the initial slope.

[e] The respective values in parentheses were obtained by a manual graphical analysis of the double reciprocal plot.

The results show that for native and denatured DNA similar saturation values and initial slopes are obtained. The saturation value for RNA is similar to that observed for native DNA, but the initial slope of the RNA binding curve is significantly less.

Experiments were then performed to determine whether light intensity was a significant factor in the efficiency of the photolytic binding process under our experimental conditions. These experiments should determine whether dissociation of the drug-DNA complex prior to complete photolysis introduces a significant error in interpretation of the photoaffinity labeling studies. Experiments were carried out in which both the time of irradiation and the distance of the sample from the light source were varied. When the data from these experiments were plotted with time as the variable, curves were obtained of a simple saturation form with binding approaching a limit at long times of photolysis. We surmised, therefore, that their results could also be analyzed by the nonlinear least squares method with time as the independent variable to determine the maximum extent of adduct formation at $T = \infty$, and the initial rate of photolysis at $T \rightarrow 0$. The results presented in Fig. 2 for different distances and times of irradiation include the raw data (symbols) and the derived predicted curves (solid lines). There were significant differences in the rates of photolysis as determined from the initial slopes of the binding curves. At a target source distance of 2 cm, the initial rate of photolysis was double and triple the rates at distances of 10 and 20 cm, respectively. Despite these differences in the rates of photolysis resulting from variation in light intensity at the different target distances, the final extents of adduct formation on prolonged irradiation were identical. Thus, the intensity of illumination was not a limiting factor in determining the maximum photolytic efficiency under the experimental conditions used. These findings suggest that this probe may be useful for *in vivo* studies since illumination sources of low intensity may be used without compromising the efficiency of photolytic drug attachment.

Experiments on the photolytic binding of the azide analogue to poly A and calf thymus histones are presented in Fig. 3. The binding curve for poly A showed very little interaction with low polymer concentrations and was characteristic of a cooperative type binding process with increasing complex formation at the higher polymer concentrations. This observation was consistent with that of Waring, who found that the non-covalent interactions of ethidium bromide with poly A did not show a simple saturation curve (13). Binding data for poly A suggested a sigmoidal relationship, the simplest form of which is the well known logistic curve (14). We analyzed the poly A data using the form of the logistic curve given in the legend of Fig. 3. The optimum value of the derivative $d(\text{cpm})/d(\text{mg/ml})$ is found at the point where $CPM = (CPM)_{max}/2$. This occurs at the polymer

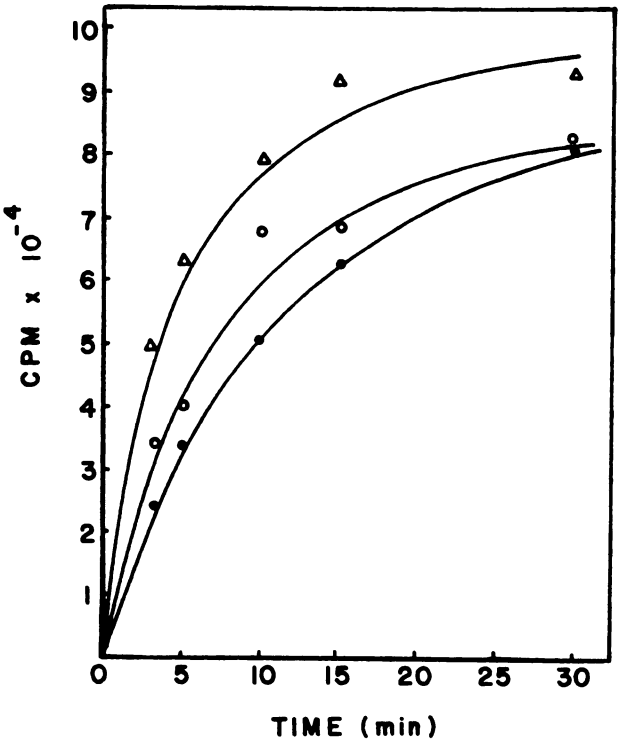

FIG. 2. *A plot of CPM vs time for monoazide bound photolytically to calf thymus DNA*

Effects of time of irradiation and variations in distance of target from the light source on photolytic binding of [^{14}C]ethidium monoazide to calf thymus DNA. The distances from light source to the samples were 2 (△), 10 (○), and 20 (●) cm. The DNA and ethidium monoazide concentrations were 50 μM and 10 μM, respectively. The lines are predicted curves calculated from equation (1) with time (instead of polymer concentration) as the independent variable and using m and I values obtained by non-linear least squares analysis of the data as described in the text.

concentration equal to the value of the parameter τ. Since there are three parameters in the logistic curve and since only six data points were available from the poly A data, there is considerable uncertainty about the estimated value of CPM_{max}. The photolytic efficiency obtained from this CPM_{max} is subject to the same uncertainty. However, the value obtained, 0.42, seems reasonable and is consistent with the other photolytic efficiencies listed in Table 1.

Because of the nature of the extrapolation of polymer concentration, it was important to compare covalent adduct formation to a macromolecule which did not show a specific binding affinity for ethidium. Thus, Fig. 3 also presents experiments done with calf thymus histones in comparison with poly A and RNA. Because of the charged nature of the histone and the drug, a strong non-covalent complex would not be anticipated. Extrapolation of histone concentration in the photolytic binding experiments to infinity permitted a calculation of the photolytic efficiency of only 0.06. This contrasted sharply with the photolytic binding to polymers which showed strong non-covalent interactions with the drug.

Binding studies were then extended to include nucleoids prepared from human lymphocytes. These experiments were done to gain information about binding to more complex structures. Figure 4 shows a binding curve obtained for the photolytic attachment of ethidium azide to nucleoids. Nucleoids are released when the cells are lysed in the presence of non-ionic detergents and high salt concentrations. These structures resemble nuclei and contain nuclear RNA and DNA, but are depleted of

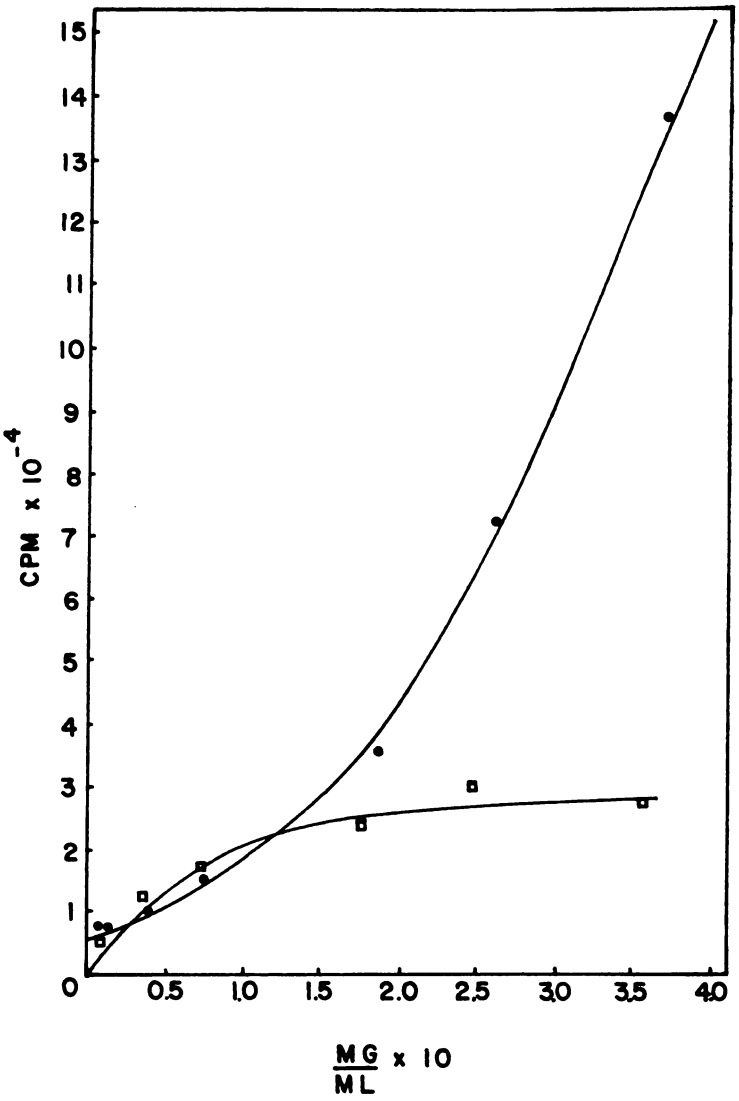

FIG. 3. *The effects of polymer concentration on photolytic binding of [^{14}C]ethidium monoazide to poly A* (●), *and calf thymus histones* (□)

For the histones the line is a predicted curve calculated from equation (1) with the values of the parameters I and m listed for the histones in Table 1. The line drawn through the poly A data is a predicted curve calculated from the following form of the logistic curve:

$$cpm = (cpm_{max}) \left\{1 + e^{-\beta\left(\frac{mg}{ml} - \tau\right)}\right\}^{-1}$$

Values of the parameters ($cpm_{max} = 2.46 \times 10^5$, $\beta = 10.08$ ml/mg, $\tau = .3476$ mg/ml) were obtained by non-linear least squares analysis of the data as described in the text.

nuclear proteins. Thus, while many targets in the nucleoids may be fully accessible to the azide for binding, such binding is subject to some of the same constraints for DNA binding as found in intact cells. The computed value for the efficiency of photolytic attachment of the ethidium-nucleic acid complex at infinite nucleoid concentra-

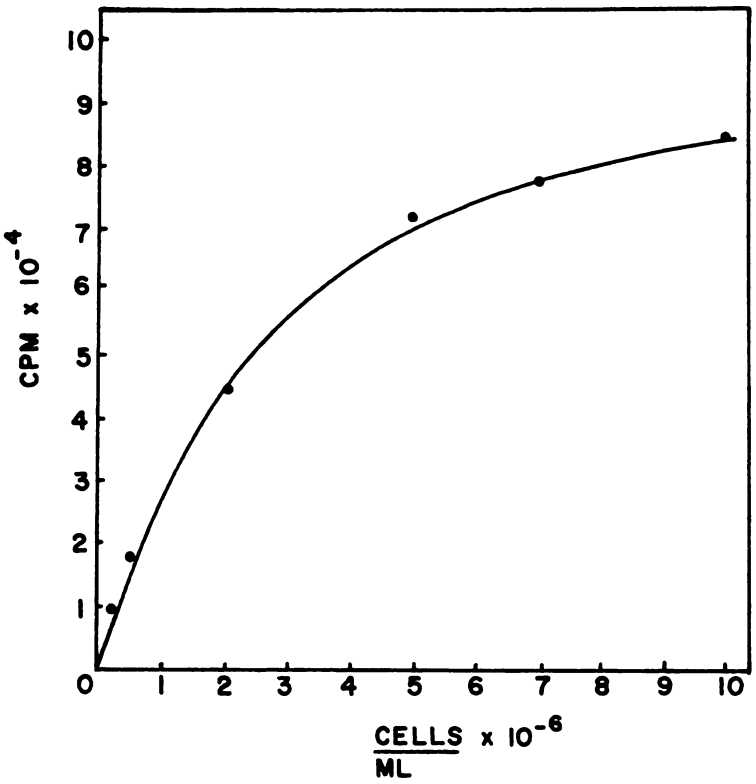

FIG. 4. *Binding of [^{14}C]ethidium azide to nucleoids prepared from human lymphocytes: effects of nucleoid concentration*

The line is a predicted curve calculated from equation (1) using the values of the parameters m and I listed for the nucleoids in Table 1.

tion was 0.19 as compared with 0.23 for native DNA free in solution.

The present experiments have shown that photolytic conversion of the noncovalent complex of the ethidium monoazide analogue and nucleic acids to a covalent adduct proceeds with an efficiency between 19 and 45%, depending on the physical state of the nucleic acid. The finding that the light intensity was not limiting, even with the low intensity lamp used, suggests the utility of photolytic studies in intact cells as a means of probing drug binding in relation to biological effects.

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