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Induced formation of covalent bonds between nucleoprotein components. V. UV or bisulfite induced polynucleotide-protein crosslinkage in bacteriophage MS2.

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ABSTRACT.

UV (λ = 254 nm) irradiation of bacteriophage MS2 or its treatment with bisulfite induce covalent crosslinkage of the RNA to the coat protein. εN-(2-oxopyrimidyl-4)-lysine was found in the phage hydrolysates after either type of treatment. An equimolar mixture of O-methylhydroxylamine and bisulfite causes complete disappearance of the cross-links. This led to the conclusion that one of the factors responsible for the UV-induced polynucleotide-protein crosslinkage and the main factor in treatment with bisulfite is substitution of the exocyclic amino group of the activated cytosine nucleus by the lysine residue ε-amino group of the protein.

INTRODUCTION

The various types of non-covalent interactions between protein and nucleic acid components are decisive factors in the formation and stabilization of the specific nucleoproteins. Some of these interactions can pass over into crosslinkage when nucleoprotein components, in particular nucleic bases are appropriately activated. Thus, the formation of poly-nucleotide-protein covalent bonds in double-stranded DNA containing phages S_D /1-3/ and PM2 /4/, and in single-stranded RNA containing phage MS2 /5/ can be brought about by nucleophilic agents. A mechanism involves activation of the electrophilic center at the cytosine C4 atom by saturation of the C5-C6 double bond and subsequent substitution of the exocyclic amino group by the amino acid residue nucleophilic group as has been postulated and verified on model mixtures /6/:

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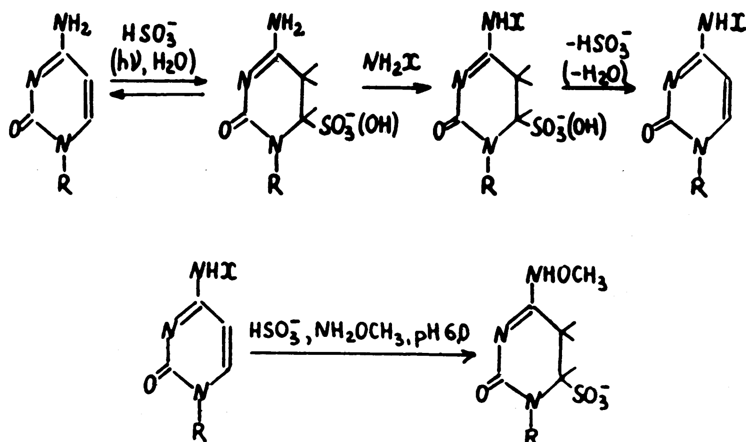

The polynucleotide-protein crosslinkage can also be induced by UV irradiation /7-15/. According to published reports uracil, thymine and possibly purine bases /16-22/ can take part in this process. The possibility for cytosine participation in crosslinkage has been demonstrated by model experiments in our laboratory (to be published elsewhere). In the latter case the reaction can proceed via photohydrate or by direct substitution of the exocyclic amino group in the excyted cytosine nucleus.

In this report it is shown that when polunucleotide-protein crosslinkage in the phage MS2 virion is induced by either UV light (254 nm) or bisulfite treatment, εN-(2-oxopyrimidyl-4)-lysine appears in the phage hydrolysates. This fact is supposed to be the proof that one of the factors causing photoinduced crosslinkage and apparently the main factor causing bisulfite induced binding is a reaction between an activated cytosine nucleus and the lysine ε-amino group of the protein according to the scheme presented above.

MATERIALS AND METHODS

N-(2-oxopyrimidyl-4)-alanine was kindly supplied by Dr. M.Yu.Lidak (Institute of Organic Synthesis, Riga). εN-(2-oxopyrimidyl-4)-lysine was synthesized according to

/6/. $Na_2S_2O_5$ was used after recristallization of the commercial reagent. Silochrome II (porous silica gel, pore size 1400 Å) was obtained from Reakhim (USSR), polyethyleneglycol, M.W. 6000 was from Merck (Darmstadt, West Germany), reagents for protein gelphoresis were obtained from Reanal (Hungary), all other chemicals used were commercial, grade chemically pure, from Reakhim (USSR).

Bacteriophage MS2 was grown on E.coli AB 259 C 3000 /23/. In order to obtain [^{14}C] pyrimidine-labeled MS2 ten minutes before infection 2-[^{14}C]-uracil (Isotope, USSR, 57 mCi/mmole) was added into bacterial culture at a concentration of 1,7 μCi/ml. The phage was purified by polyethyleneglycol precipitation and subsequent centrifugation in a preformed CsCl density-gradient /5/. The infectivity of the phage suspension was determined by the standard method /23/. The phage preparations thus obtained (10^{14} p.f.u./ml, $O.D._{260} \sim 20$, radioactivity of labeled phage $1,74 \times 10^{16}$ cpm per optical density unit)were stored in a frozen state, the titer being constant under these conditions.

UV irradiation of MS2 phage. The phage suspension in 0,1 M NaCl, 0,01 M $MgCl_2$ was placed in the hermetic quartz cuvette (0,1 cm light path, optical density at 254 nm ~ 1) and irradiated by unfiltered light from a low pressure mercury lamp. The incident light intensity was determined by ferrioxalate actinometry to be $5,16 \times 10^{15}$ quanta/min/cm^2.

Treatment of phage MS2 with bisulfite. The bisulfite treatment was performed in two ways:

a). 1 ml of [^{14}C]-labeled phage suspension (21 o.d.u., 35×10^6 cpm) was mixed with an equal volume of 1 M $Na_2S_2O_5$ (pH 7,2) and the mixture was incubated in sealed polyethylene tubes at 30° for 4 hr. The phage was separated from the bisulfite by gel filtration on 1,5 x 1 5 cm Sephadex G-25 (fine) column, elution being carried out with 0,5 M NaCl. Then phage was precipitated by polyethyleneglycole /5/.

In order to determine the bisulfite irreversibly bound to the phage components, the unlabeled phage was modified by

1 M $[^{35}S]$ $Na_2S_2O_5$ (0,11 mCi/mmole, Isotope USSR) under conditions above described. The modified phage freed of excess reagent by gel filtration was dissociated by adding urea and sodium dodecylsulfate (final concentrations 7 M and 1% respectively) and heating for 10 min at 60°. The RNA was then separated from protein and the residual bisulfite by gel filtration on SilochromeII column in 0,1% sodium dodecylsulfate (Fig. 1). The radioactivity of the fractions was determined in a dioxane scintillator using SL-30 (Intertechnique, France) scintillation spectrometer.

b) The phage suspension was dialyzed firstly against 1 M $Na_2S_2O_5$ solution (pH 7,5), containing 0,02 M β-mercaptoethanol for 70 hr at room temperature /4/, then for 5 hr against 0,5 M NaCl made up to pH 8,5 with alkali and finally overnight against neutral 0,5 M NaCl. The resulting phage was precipitated with polyethyleneglycol.

The detection of polynucleotide-protein crosslinkage. In order to determine the number of pyrimidine nucleotides co-

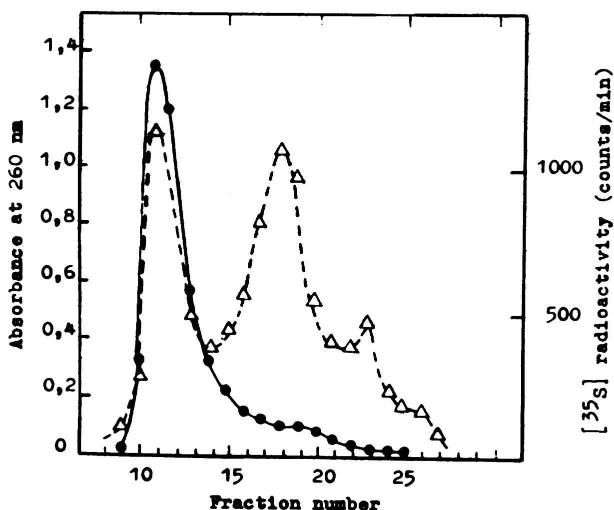

Fig.1. Separation of MS2 RNA from the proteins after treating of the phage with $[^{35}S]$ $Na_2S_2O_5$. Gel filtration on porous silica gel in 0,1% sodium dodecylsulfate; 1x80 cm column, elution rate 30 ml/hr, 2 ml fractions. —●—●— optical density at 260 nm; --△--△- $[^{35}S]$ radioactivity in counts/min/ml.

valently bound to protein, the polyethyleneglycol precipitates of the bisulfite treated phage were centrifuged for 30 min at 0° (5000 rpm), suspended in a small volume of 0,5 M NaCl and divided into two parts, to one of which calculated amounts of glacial acetic acid and concentrated HCl were added (the ultimate concentrations were 66% and 1 M respectively). The mixture was incubated for 2 hr at 60° in a sealed tube and then proteins were separated from the RNA hydrolisate by gel filtration on 0,8 x 105 cm Sephadex G-25 (fine) column, equilibrated with 66% acetic acid, elution being carried out with the same solution at a rate 0,25 ml/min. Radioactivity of fractions was determined with scintillation spectrometer (Fig. 2).

The other portion of phage was treated with an O-methylhydroxylamine-bisulfite mixture (1 M both in $CH_3ONH_2 \cdot HCl$ and $Na_2S_2O_5$). The mixture was incubated at 37° for 5 to 6 hr and then dialized overnight against 0,5 M NaCl. Then the phage was precipitated with polyethyleneglycol and hydrolyzed

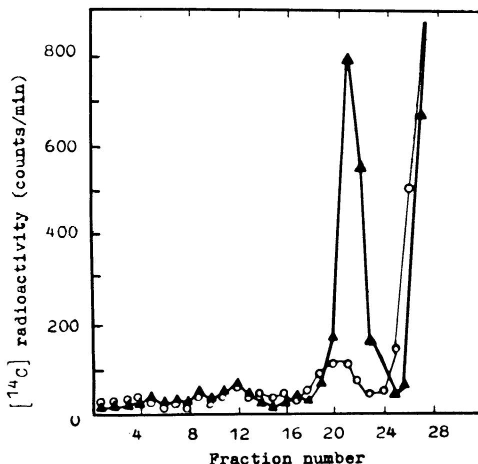

Fig.2. Separation of MS2 proteins from the acid (1N HCl) hydrolysis products of RNA by gel filtration on Sephadex G-25 (fine) in 66% acetic acid. MS2 labeled with ^{14}C in the pyrimidine rings: -o-o- before irradiation, -▲-▲- after absorbing 5×10^4 quanta/virion.

with 1N HCl in 66% acetic acid. Subsequent treatment was as described above.

The UV irradiated phage was treated analogously with the exception of the O-methylhydroxylamine-bisulfite procedure.

After gel filtration of the phage hydrolysate and determination of the fractions radioactivity, the protein-containing fractions were concentrated by evaporation and analyzed as described below.

Polyacrylamide gel electrophoresis of phage proteins. The protein-containing samples were incubated at 60° for 15 min in 8 M urea, 1% sodium dodecylsulfate and 0,1% β-mercaptoethanol solution. Then samples were electrophoresed on 10% polyacrilamide gel /24/ (6 v/cm, 7-8 ma per 6 mm i.d. tube). After electrophoresis, the gels were stained with Coomassie Blue. For analysis of the radioactivity distribution gels

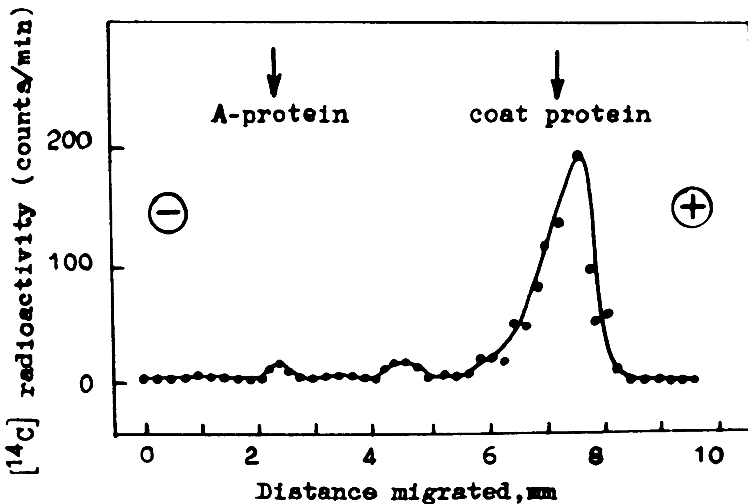

Fig. 3. Polyacrylamide gel electrophoresis of MS2 proteins. Distribution of radioactivity after electrophoresis of proteins from UV-irradiated ($\sim 3\times10^4$ quanta/virion) phage [^{14}C]-labeled in pyrimidine rings. Arrows point out the positions of the A-protein and the coat protein from the untreated phage.

were successively dispersed in Slicer Gel Divider (Savant, USA), the disintegrated gel being eluted with 0,1% sodium dodecylsulfate. Fractions corresponding to 2-3 mm gel length were collected into dioxane scintillator and counted. The results are presented in Fig. 3.

Thin-layer chromatography on cellulose. In order to detect and identify the minimal sized crosslinked fragments, the protein-containing fractions (see above) were made 6N in HCl and incubated in sealed tubes at 110° for 48 hr. The hydrolysates were then repeatedly evaporated with water and spotted on 15 x 15 cm cellulose thin layer plates. Non-radioactive uracil, cytosine, N-(2-oxopyrimidyl-4)-alanine and εN-(2-oxopyrimidyl-4)-lysine were added to the sample as markers, and two-dimensional chromatography was performed firstly in 86:14 (v/v) butanol-water system (I) (twice in one direction) and then in the perpendicular direction in isopropanol-conc. HCl-H_2O (65:16,7:18,3, v/v, system II). The UV-absorbing spots were marked, the rest plate surface was divided at will into small areas, cellulose layers of each area were fixed with a cellulose acetate solution in aceton-ethanol (2:1 v/v, cf. /25/) and the radioactivity of all areas was counted in toluene scintillator. The results of a typical experiment are presented in Fig. 4 and in Table 1. The εN-(2-oxopyrimidyl-4)-lysine spot was eluted from the cellulose with 0,01N HCl and respotted on FND-16 paper (Filtrak, DDR) for electrophoresis in 7% acetic acid (8 v/cm, 4 hr).

RESULTS

To detect the polynucleotide-protein covalent bonds the following approach was made use of. The [^{14}C]-pyrimidine-labeled phage MS2 (cytosine and uracil nuclei were approximately equally labeled) subjected UV irradiation or bisulfite treatment was hydrolized with 1 N HCl at 60° and the phage proteins were separated from RNA hydrolisate by gel filtration. The number of pyrimidine units covalently bound to the phage proteins was estimated by the amount of label in the

Table 1 Radioactivity Distribution (in per cent of total) in the Two-dimensional TLC on Cellulose of Protein Fraction (Peak I, Fig. 2) Hydrolysates (see Fig. 4).

	Untreated phage	UV-irradiated phage*	Bisulfite treated phage**
Uracil	52	30	40
Cytosine	48	20	35
εN-(2-oxopyrimidyl-4)-lysine	0	50	25

* /^{14}C/ radioactivity in the protein peak after gel filtration was 0.025% of that applied.

** /^{14}C/ radioactivity in the protein peak after gel filtration was 0.012% of that originally applied.

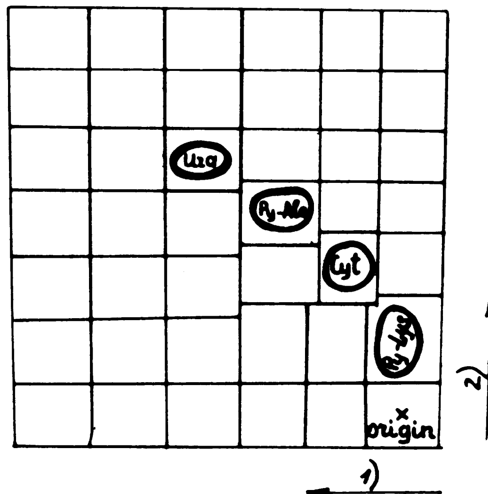

Fig.4. Two-dimensional thin layer cellulose chromatography of the hydrolysis (6N HCl, 110°, 48 hr) products of the proteins from UV-irradiated or bisulfite treated phage MS2. The heavy lines show the boundaries of the UV-absorbing zones; the fine lines, the boundaries of the sections extracted for the radioactivity determination. Cyt - cytosine, Ura - uracil, Py-Lys - εN-(2-oxopyrimidyl-4)-lysine, Py-Ala - N-2(oxopyrimidyl-4)-alanine. 1) and 2) - the first and the second chromatographic directions in the systems I and II, respectively (see Materials and Methods).

protein fractions.

This approach enables firstly to observe the cross-links resulting from the pyrimidine residues of the RNA exclusively and secondly to detect only those being stable under conditions of RNA acid hydrolysis. To awoid aggregation of the phage proteins, the hydrolysis and gel filtration were carried out in 66% acetic acid.

As one can see from Fig. 2, when the phage MS2 hydrolysate (1 N HCl, 60°, 2 hr) is gel filtered on Sephadex G-25, clear-out separation of the protein-bound label from the RNA hydrolysate occurs. The presence of a small amount of radioactivity (<0,01% of the label applied to the column) in protein peak on gel filtration of untreated phage hydrolysate (Fig. 2) is apparently due to nonspecific adsorption and provided the background correction when estimating the crosslinkage of the treated preparations. The covalent binding to protein of one pyrimidine unit per virion should lead to increase in radioactivity of the protein peak up to 0,06% (of the 3500 nucleoside units in the phage MS2 RNA about half are pyrimidines/26/).

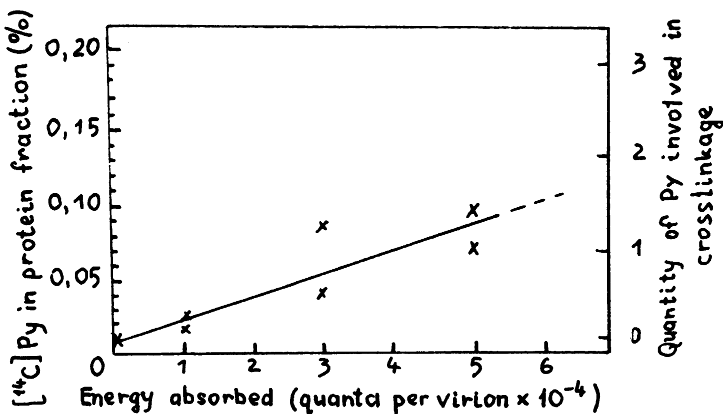

Fig. 5. Dependence upon the energy (λ = 254 nm) absorbed of the amount of pyrimidines remaining bound to proteins after the hydrolysis of the irradiated phage with 1N HCl and separation of the proteins from RNA hydrolysis products.

Crosslinkage induced by UV irradiation. As one can see from Fig. 5 the radioactivity in the protein containing fractions increases in proportion to the energy absorbed. Analysis of these fractions by polyacrylamide gel electrophoresis has shown the radioactivity caused by the presence of [^{14}C]-pyrimidines to be concentrated mainly in the band due to the coat protein (Fig. 3). These data bear witness to the fact that it is mainly this protein that takes part in the formation of the polynucleotide-protein cross-links of MS2.

Crosslinkage induced by bisulfite treatment. When phage MS2 is treated with 0,5 M $Na_2S_2O_5$ (pH 7,2) for 4 hr at 30° /5/ about 0,02% of the label is found in the protein fraction of the acidic hydrolysate. In treatment with 1 M $Na_2S_2O_5$ (pH 7,5) in the presence of β-mercaptoethanol for 70 hr, 0,045% of the label is found in the protein fraction. After the modified phage being treated with an equimolar O-methylhydroxylamine-bisulfite mixture, pH 6,0, the label content in the protein fraction falls to 0,006%,i.e. to the level characteristic of untreated phage. The fact that the O-methylhydroxylamine-bisulfite mixture acted as "decross-linking" agent is evidence that crosslinkage most likely proceeds according to the scheme presented above (cf./6/).

To investigate the effect of bisulfite on phage proteins, non-labeled phage MS2 was treated with 0,5 M [^{35}S] $Na_2S_2O_5$ at 30° for 4 hr. This led to incorporation of 0,9 moles of radioactive bisulfite per mole of the coat protein. Analysis of the proteins from the bisulfite treated phage by means of gel electrophoresis showed that the electrophoretic picture of the phage proteins had undergone no change.

Identification of RNA-protein crosslinked fragments. In order to detect the crosslinkage fragments of type "nucleic base-amino acid" the protein fractions after separation from RNA hydrolisate were hydrolyzed to amino acids under standard conditions. The covalent bonds formed by substitution of the cytosine amino group by the free amino group of the protein amino acid residue are quite stable under these conditions

/6/. As already mentioned it is just such bonds that should predominantly be formed in the bisulfite treatment and in the UV irradiation - alongside other types of crosslinkage.

Taking this into account we undertook the identification of the amino acid derivatives of cytosine in the phage protein hydrolysate of the modified phage preparation.

Since in the phage MS2 coat protein it is the lysine and N-terminal alanine residues that have the free amino groups /26/, non-radioactive N-(2-oxopyrimidyl-4)-alanine and εN-(2-oxopyrimidyl-4)-lysine, cytosine and uracil were added as markers to the protein hydrolysate in two-dimensional chromatographic analysis on cellulose thin layer (Fig. 4). In the case of the control experiment (untreated phage) radioactivity was detected only in the cytosine and uracil spots.

In both the UV-irradiated and the bisulfite treated phage samples a considerable amount of the radioactivity was revealed in the spot corresponding in position to εN-(2-oxopyrimidyl-4)-lysine but not N-(2-oxopyrimidyl-4)-alanine (Table 1, Fig. 4). The radioactivity of the other sections of the cellulose layer did not surpass that of the background. The electrophoretic mobility of the radioactive compound eluted from the corresponding spot of chromatogram was the same as for the known sample of εN-(2-oxopyrimidyl-4)-lysine (0,6 with reference to cytosine). The data obtained bear witness to the identity of the compound isolated from irradiated or bisulfite treated phage with the εN-(2-oxopyrimidyl-4)-lysine.

DISCUSSION

When nucleoproteins are exposed to UV light it is mostly the nucleic bases that become excited. The quantum yield of the purine phototransformation is considerably less than that of the pyrimidines /27-29/, although for the deoxyadenosine unit in the polymer structure /30,31/ and the deoxyguanosine unit even at the monomer level /32/ this difference is not so great as it is usually taken to be.

The direction and rate of both the primary photoreactions and subsequent conversions of the products are highly

dependent on the concentration and/or mutual orientation of the excited base and its neighbouring molecules (or macromolecular segments).

For instance, in aqueous solution, uridine undergoes largely photohydration, whereas if the bases are brought together (in frozen solutions or in oligonucleotides) the predominant reaction is dimerization /27-29/. When 5-bromouracil replaces thymine in DNA, the primary photoreaction, homolytic cleavage of the C-Br bond, is accompanied by breakdown of the deoxyribose moiety in the neighbouring nucleoside unit /33/.

Evidently in the case of nucleoproteins with fixed quaternary structure, the rate and mechanism of the primary photoreactions and the subsequent conversions of their products will be controlled by the orientation and nature of the groups comprising the environment of the base in the complex. In the case of TMV, where each base is, as it were, submerged in the protein, participants of the photoreaction could be water molecules hydrating the bases and the protein amino acid residues under contact with them. As a result no pyrimidine dimers are formed at all in the UV irradiation of TMV while the main cause of the photoinactivation is the photoinduced hydration of the pyrimidines and possibly the formation of polynucleotide-protein cross-links /34-36/.

In the UV irradiation of nucleoproteins, wherein only part of the bases are engaged in interaction with the protein (ribosomes, spherical viruses, DNA-RNA polymerase and DNA-repressor complexes etc.) dimerization of the pyrimidines takes place, as well as their photohydration /27-29/, and moreover the formation of cross-links at the sites of contact between the nucleic acid and the protein /7-15,37/.

The genetic consequences of the UV irradiation of spherical bacteriophage MS2 and related phages have been investigated earlier.

The UV irradiation of RNA-containing viruses and infectious RNA causes only inactivation but no mutation /29/. Basing on the published earlier data /8,38,39/ it is not difficult to calculate, that the dose of UV irradiation, res-

ponsible for one lethal hit in the case of the phages MS2 and f2 is only 4-10 times lower, than that responsible for hindering the penetration of phage RNA into the cell. Apparently the penetration is hindered by the formation of any kind of polynucleotide-protein linkage. Hence for these phages 10-25% of UV-induced inactivation are brought about by the polynucleotide-protein cross-links formation.

According to the data presented in this report the UV irradiation of phage MS2 causes the formation of covalent bonds between the coat protein and the phage RNA, in particular, between the cytosine nuclei and the lysine residues, one cross-link forming at a dose of $4x10^4$ quanta per virion (corresponding to about 8 lethal hits). In other words in the UV irradiation over 10% of the inactivation is caused by the formation of covalent bonds between the cytosine nuclei of RNA and the lysine residues of the coat protein. Cross-linkage occurs on a background of insignificant changes in the RNA (less than 0,5% of the RNA bases are modified at the moment of the formation of one cross-link per virion), i.e. it occurs on a practically intact, from the standpoint of higher structure, nucleoprotein.

The method used in the present study do not permit detection of other type of crosslinkage, such as crosslinkage with purine nuclei, uracil nuclei with cysteine residues, cross-links formed by substitution of the cytosine amino group by another grouping, such as seryl or treonyl hydroxyls, because most likely such cross-links do not withstand the vigorous conditions of acidic hydrolysis of the RNA or protein. We can estimate only very approximately, from account of the blocking of phage RNA penetration into the cell /8,38, 39/ that crosslinkage of the cytosyl-lysine type ($4x10^4$ quanta/virion/cross-link) comprises about 30% of all possible damages hindering the penetration ($1,4x10^4$ quanta/virion/damage).

As has been shown earlier, polynucleotide-protein cross-links in a bacteriophage MS2 virion can be also induced by bisulfite /5/.

We have shown here that one may isolate from bisulfite-

treated phage εN-(2-oxopyrimidyl-4)-lysine and also that the bisulfite induced crosslinkage completely disappears when the phage is reacted with an equimolar mixture of O-methylhydroxylamine and bisulfite. This leads to the inference that the formation and rupture of covalent bonds between RNA and the coat protein is in accord with our scheme presented above. The fact that εN-(2-oxopyrimidyl-4)-lysine can also be isolated from UV-irradiated phage is evidence in favor of at least partial crosslinkage according to this scheme also in the case of UV irradiation.

One can see quite clearly that both methods of inducing crosslinkage have their advantages and limitations. Thus, bisulfite induces only a single type of cross-links, namely substitution of accessible cytosine exocyclic amino groups by nucleophilic groups of amino acid residues. UV irradiation apparently causes variegated crosslinkage, the type, but not rate of the crosslinkage depending on the environment of the base. Moreover, the irradiation can be carried out over a wide range of conditions including those conductive to maximum stability of the relatively labile nucleoprotein complexes. It is to be stressed that in any case crosslinkage occurs only in preexistent complexes /9,11,14,15/.

The success achieved in the application of either method /4,5,9-15,37/ gives grounds to foresee that the induction of crosslinkage by UV irradiation or by nucleophilic agents will become a powerful tool in structural studies of the interacting nucleoprotein segments and in studies of the nature of the forces responsible for nucleic acid-protein recognition.

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