NUCLEIC ACID INTERACTIONS

II. Chemical Linkage of the Carcinogen 3,4-Benzpyrene to DNA Induced by Photoradiation

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In the course of earlier investigations, it has been shown that hydrophobic forces and stacking interactions are of great importance to the structures and properties of nucleic acids.[1,2] Hydrophobic compounds which are biologically active but chemically inert may, therefore, be expected to interact with nucleic acids extensively. This has been shown to be the case for the steroid hormones and aromatic hydrocarbons[3]. The large binding constant of 3,4-benzpyrene to DNA reported in this communication also supports this deduction.

Physical binding of carcinogenic hydrocarbons to DNA is well recognized[4, 5, 6], although its biological significance remains unknown[7,8]. The physical effect of such molecules on a biological system is likely to be transient and to depend upon many environmental factors. If on the other hand, covalent linkages are formed between carcinogenic hydrocarbons and nucleic acids, intereference with biological function should be effective and permanent.

How might polycyclic aromatic hydrocarbons become covalently linked to DNA in aqueous medium at neutral pH and at room temperature? Radiation absorbed by the hydrocarbons but not by the DNA appears to be an effective way to supply energy to accomplish this end. In this communication, we report that chemical linkage of the carcinogenic 3,4-benzpyrene (BP) or its degradation products to denatured DNA results from radiation with wave lengths above 300 mμ, presumably at the absorption bands of BP. After a few hours of such radiation, over 50% of the benzpyrene-H^3 supplied was found to be linked covalently onto DNA.

Materials

DNA: Highly polymerized calf thymus DNA (Nutritional Biochemicals Corporation, Cleveland, Ohio) was redeproteinized and denatured at 100°C as described previously.[3] No sonication or shearing was applied to these DNA solution in 0.0025 M Na_2HPO_4, 0.005 M Na H_2PO_4 and 0.001 M ethylene diamine tetraacetic acid, pH 7.1 (HMP).

3,4-Benzpyrene (BP): Non-radioactive BP was from Nutritional Biochemical Corporation.

tritiated BP (448 mc/mM) was purchased from Nuclear Chicago Corp. Des Plaines, Ill. which was prepared by Radiochemical Centre, Amersham, Buckinghamshire, England. The radioactive BP was freeze-dried from a benzene methanol solution before use. Purity of the two BP preparations was rechecked by Radiochemical Centre and was found to be 96% and 99% respectively by chromatographic procedure. These samples were also chromatographed[9] by us and all radioactive counts were found to be at the blue fluorescence spot of authentic BP.

Enzymes: Pancreatic DNAase and venom diesterase were both from Worthington, Biochem. Corp., Freehold, N. J.

Instrumentation and Procedure:

Apparatus for Photoradiation:-

The optical components were borrowed from the monochromatometer built by Prof. M. Delbrück to whom we are grateful. The light souce was a General Electric, one kilowatt, air cooled, B-H6 mercury arc lamp. Two identical spherical mirrors (5.25 inches in diameter and 8.5 inches in radius of curvature) were placed 28 inches apart facing each other. The light source and the reaction vessel were placed at the focal point of the two mirrors.

Reaction Vessel:

The vessel was a 15 ml. tube with a surrounding water jacket, both made of pyrex glass. Measurements made with the Cary spectrophometer indicated that u.v. light is cut off by this vessel; i. e., 2 O. D. at 290 mμ and the absorbancy rises steeply at lower wave length. In some experiments, the cut off point was moved to 340 mμ (i.e. 2 O.D.) by pumping 0.1 M solution of sodium salicylate through the water jacket.

Measurement of Incident Light Energy on the Reaction Vessel:

A cuvette containing carbon black suspension in a known amount of water was placed inside the reaction vessel. The rise in temperature in the cuvette during radiation of a given duration was measured by a thermocouple connected to a potentiometer. Heat loss

due to conduction is negligible. The cuvette covered at least 75% of the illuminated area. Energy was received over the 3 cm^2 area of the cuvette at the rate of 6 watts. Only 3 watts were received when circulated with 0.1 M salicylate as filter. According to the technical bulletin of General Electric Co. (FN-412) the energy emitted from 320 mμ to 400 mμ is about 20% of the radiant energy transmitted by the vessel; namely, the radiation above 300 mμ. Therefore, the flux of energy at the wave lengths where the BP absorbs is estimated to be about 1.5 watts for the water-circulated vessel.

Radiation procedure

2-10 ml. of solution was placed in the vessel. Nitrogen saturated with water was bubbled through the solution vigorously 30 minutes before the radiation, as well as during radiation. The vessel was stoppered and gas was allowed to escape through a long chimney to ensure an nitrogen-saturated atmosphere. The temperature of the solution in the vessel was kept at 23^0C but when 0.1 M salicylate was circulated instead of with water, the temperature rose from 23^0C to 30^0C in four hours due to the limited volume of cooling liquid circulated.

Preparation of DNA - Benzpyrene Solution

Finely divided BP-H^3 was placed in buffer solutions of DNA (2-5 mg/ml) and the suspension was shaken for 3-4 days in the dark, at 23^0C, before being filtered through fine sintered glass funnels.

Counting Procedure

The BP-H^3 was counted in a Packard Tricarb liquid scintillation spectrophotometer as described previously.[3] The counting efficiency as determined by a tritiated water standard was approximately 25%. Together with the specific activity cited for the BP-H^3, a conversion factor of 2.5×10^{14} cpm/mole BP was established.

DNA and DNA-BP Photoproduct Precipitation Procedure

For precipitation, amonium acetate was first added into the DNA solution to a final

concentration of 0.4 M and 3x volume of cold 100% Ethanol then added. The polymer was allowed to precipitate at 5^0C for 30 minutes, and then washed thrice with 100% cold ethanol and twice with ether before vacuum drying.

This procedure removes 99.8-99.9% of the physically bound BP from the DNA.

Extraction of the BP-H^3 from a DNA solution by three volumes of cyclohexane and heptane removed only ca. 14% of the radioactivity. Four times extraction with three volumes of these solvents still left 50% of the BP in the DNA solution. Extraction once with benzene (3 x volume) removed about 80% of BP-H^3. The procedure of solvent extraction is much less efficient and hence less satisfactory for removal of the physically bound BP from the DNA as compared to the ethanol precipitation procedure.

DNAase hydrolysis

DNA (1 mg/ml) was hydrolyzed at 37^0C in 0.01 M $MgCl_2$, 0.01 M Tris pH 7.5 for 20 to 30 hrs. with DNAase (10γ/ml).

Sucrose gradient centrifigation and electrophoresis:

Gradient centrifugation was performed with 5-20% sucrose in HMP.

Gradient electrophoresis was performed by Mr. Thomas Burke with the instrument built by Prof. N. Davidson[10], the gradient being 5-20% sucrose in 0.01 M NaCl, 0.001 M Tris pH 7.5.

Other Instruments

Analytical ultracentrifugation was performed in a Model E ultracentrifuge, Spinco Division, Beckman Instrument Inc. with ultraviolet absorption optics. Patterns on the films were traced with a Double-Beam Recording Microdensitometer, Joyce Loebl Co., Newcastle-upon-Tyne, England. All the sedimentation coefficients reported were corrected to 20^0C but not for solvent viscosity and density.

Absorption spectra were obtained with both Cary 11 and Cary 14 spectrophometers, Applied Physics Corporation, Monrovia, Calif.

Measurement of Physical Binding

Changes in solubility of BP in the presence of denatured DNA were measured by a

procedures similar to those employed for phenanthrene[3, 11], the solid BP-H[3] being shaken at 5°C and at 15°C with denatured DNA at 3 concentration levels (0.0015 to 0.005 M) for three days in HMP (with or without 0.5M NaCl) before filtration. For the determination of nk where n is number of binding sites and k is the number average equilibrium constant calculated on a per nucleotide basis[3], both the DNA concentration and the total BP concentration in the presence of DNA can be reliably determined for a given sample of DNA. The problem was in the determination of the free BP in solution; i.e., the solubility of BP in buffer. Experimental data was found to vary from 10,000 to 200,000 cpm/ml. The only dependable procedure found to measure the free BP was the removal of the DNA-BP complex through sedimentation. After centrifugation for 16 hours at 39,000 rpm in the Spinco Model L centrifuge with a SW 39 rotor the supernatant at the top was found to contain 0.66 $O.D._{260\ m\mu}$ and 7600 cpm/ml of the original solution of 50 O.D. and over 10^6 cpm/ml. This number, 7600 cpm/ml, equivalant to 7.5 µg/ml, is higher than the value 4 µg/liter determined by nephelometric method reported by Davis et al[12] and was adopted for the calculation of the nk for various experiments. Calculation of the amount of BP dissolved in solution of 5 mg/ml denatured DNA from its spectrum between 3200-3900 Å and its extinction coefficient agreed well with the radioactivity measurement.

Results and Discussion

Physical Binding

Solubilities of BP in various nucleic acid solutions were measured in the determination of nk values as described above. The nk values (M^{-1}), for the binding of BP to heat denatured thymus DNA are: 5.4×10^4 in HMP and 4.0×10^4 in HMP plus 0.5 M NaCl at 5°C, and 3.8×10^4 in HMP and 2.6×10^4 in HMP plus 0.5 M NaCl at 15°C. There are considerable uncertainties in these values which arise from the problem of measurement of the solubility of BP in buffer and from the large influence of secondary structure of DNA on nk. In agreement with our previous finding , binding of BP onto the native DNA is about 25-30 fold less than that onto the denatured DNA. Furthermore, we feel that to a large degree the small and varible binding of BP to the native DNA may reflect the extent of denatured material or region present in the sample. Also in agreement with the studies

of testosterone binding to denatured DNA at various temperatures [3], the binding of BP to DNA has negative values for both enthalpy and entropy changes. It should be emphasized that in these experiments the ratio of DNA concentration/free BP concentration is very large because of the insolubility of BP. Thus, there is no reason to believe that the binding sites of the DNA are being saturated. These measurements, therefore, can only provide the value of nk and not the number of binding sites present in DNA. [5]

Radiation-induced Linkage of BP to denatured DNA as studied by extraction and precipitation procedure:

BP physically bound to DNA can be removed by precipitation and extraction. By the procedures described above, DNA can be quantitatively reisolated from a solution of 5 mg/ml containing 2.9×10^6 cpm of BP/ml with 99.8% of the radioactivity removed. BP chemically linked to DNA, however, will not be removed by these procedures. Photo radiation for four hours, results in 53% of the BP-H^3 accompanying the DNA precipitate after the precipitation and washing. This large amount of radioactivity ($\simeq 3 \times 10^5$ cpm/mg of DNA) could not be removed by further extraction with ethanol, ether, cyclohexane, heptane or benzene. The DNA precipitate linked with the BP-H^3 or the degradation products of BP-H^3 will be termed DNA-BP which was readily redissolved in aqueous buffer for further study.

A supersatured BP solution in HMP containing 2×10^5 cpm/ml was radiated for seven hours, after which, the solution was found to lose about 2/3 of its radioactivity. The remaining radioactivity in HMP was washed away by the same extraction and precipitation procedure as used for the DNA-BP solution. Furthermore, when DNA was added to this irradiated BP solution no radioactivity could be found in the DNA on reisolation.

The dependence of DNA-BP formation on time of irradiation was investigated by the precipitation extraction procedure. (Fig. I) After irradiation of a solution containing 100 $O.D._{260\ m\mu}$ of DNA and 1.5×10^6 cpm of BP for one hour, there was a 5% loss of total radioactivity. No further loss was found upon further radiation up to the seventh hour. After the first hour of radiation, 30% of the total BP-H^3 counts in solution was found

to be precipitated with the DNA, (Fig. I) while after four hours the plateau was reached with 52% of total BP-H^3 in solution linked with the DNA.

Insert Fig. I

Characterization of DNA-BP

A DNA-BP sample, precipitated after four hours of irradiation, was centrifuged in a sucrose gradient. (Fig. 2a) In another tube of the rotor, BP which remained in solution after irradiation for seven hours (see above paragraph) was also centrifuged as a control. In Fig. 2a the $O.D._{260\ m\mu}$ curve for DNA may be seem to be superimposible on that of the radioactivity of BP-H^3. Fig. 2b, indicates that the radioactivity of the irradiated BP-H^3 simply diffused from the top of the tube downward during the 20 hours of sedimentation. These results clearly indicate that in the DNA-BP sample the radioactivity of the BP-H^3 was linked to the DNA.

Insert Fig. 2

The same DNA-BP sample was also analyzed by sucrose-gradient electrophoresis. Fig. 3 shows again, that the $O.D._{260\ m\mu}$ curve of the DNA is closely followed by the radioactivity of the BP-H^3. This also indicates that the radioactivity of the BP-H^3 is linked to the DNA.

Insert Fig. 3

This DNA-BP preparation was incubated with DNAase at 37° C for 16 hours. The solution was then centrifuged for 16 hours at 39,000 rpm in the SW 39 rotor of the Spinco Model L Ultracentrifuge and contents of the centrifuged tube (4 ml) fractionated into four equal portions. The results of Table I show that before the DNAase treatment, the radioactivity of the BP-H^3 sedimented with the DNA to the bottom of the tube. After the enzymatic hydrolysis, the radioactivity of BP-H^3 was found in the supernatant together with the nucleotides and oliogonucleotides. The hydrolyzed DNA-BP was precipitated with ethanol and washed as described. Half of the radioactivity and optical density was found in the precipitates and half in the supernatant. The nonhydrolyzed control on precipitation and washing yielded about 90% of the radioactivity and the optical density in the precipitates.

Insert Table I

These experiments clearly show that before enzymatic hydrolysis of the DNA-BP, the counts of BP-H^3 followed the DNA; while after the hydrolysis, the counts of BP-H^3 now followed the nucleotides and oligonucleotides.

Formation of DNA-BP by photoradiation with wavelengths above 340 mμ

Experiments were done to ascertain whether the radiation absorbed by the DNA or that by the BP is mainly responsible for the formation of DNA-BP. As described in above sections, solutions irradiated in a vessel circulated with 0.1 M salicylate had the cut off point moved from 290 mμ to 340 mμ, and received only half of the amount of energy as compared to that in vessel circulated with water. Under these conditions, radiation absorbed by the DNA is filtered completely off before it reaches the sample. The sedimentation coefficient, $S_{20,\ 50\%}$ 6.35 of DNA so irradiated for five hours is little different from that of the non-radiated sample.

DNA in BP solution (same as that used in Fig. I) was irradiated for four hours under these conditions. After precipitation and extraction, the DNA-BP contained 400 cpm/$O.D._{260\ m\mu}$ half of that reported in Fig. I for the same duration of radiation. This is only to be expected since the solution received about half the amount of energy. Sucrose gradient sedimentation analysis of this DNA-BP sample also showed that the radioactivity of BP-H^3 followed the $O.D._{260\ m\mu}$ of the DNA.

This experiment indicates that the photoradiation at wave lengths above 340 mμ into the absorption band of BP is responsible for the formation of DNA-BP and that the possible leakage if any of radiation of wave legth below 290 mμ into the DNA absorption band should not be significantly involved.

Effect of radiation on the sedimentation of DNA

The sedimentation patterns of this commericial redeproteinized denatured DNA were broad. However, on prolonged centrifugation, some distinct components appeared to emerge. The $S_{20,\ 50\%}$ was observed to be around 5-6.5 S. Upon radiation for five hours, the pattern became somewhat sharpened and the $S_{20,\ 50\%}$ value decreased to 4-4.5 S. In the

presence of BP, essentially the $S_{20,\ 50\%}$ value, 4.9 S, was obtained. As reported above, the effect on the S value was minimized by circulating 0.1 M salicylate instead of water. The basic mechanism of this effect is not clear at present. Undoubtedly, it is related to the small but not zero photo absorption of DNA at wavelength near or above 290 mμ.

Spectral Studies of DNA-BP

The u.v. absorption spectrum of BP in ethanol is given in Fig. 4 which is identical to the spectrum of BP dissolved in 50% ETOH-50% water, cyclohexane and hexane. From a supersaturated solution at 98°C, the spectrum of BP in water was obtained which is also similar to that in ethanol. In DNA solution, the spectrum of BP has a very noticeable red shift (Fig. 5) and it is interesting to note that such shifts have been observed in aqueous caffeine solution.[4, 5, 13] A similar shift in the u.v. BP spectrum can also be obtained simply by using benzene as solvent (Fig. 4). These results indicate that the photoexcited state of BP is stablized in preference to the ground state of BP in benzene as well as in aqueous solution of DNA and caffeine. These data further suggest that interaction with the π-electron system is responsible for this preferential stabilization. This electronic interaction between the excited BP and DNA may be relevant to the finding in this communication.

Insert Fig. 4 and 5

Upon photoradiation, the absorption peaks between 350-395 mμ were gradually diminished, (Fig. 5) while absorption at 310-340 mμ and less markedly at 410-430 mμ was enhanced. The change in the spectrum was essentially complete after four hours in agreement with the BP-H^3 binding curve of Fig. I. The solution turned slightly yellowish upon prolonged irradiation. It should be emphasized that since the radiation was not monochromatic, photoproducts having absorption at wavelength above 300 mμ (cut off point of the vessel) were continuously being irradiated.

The amount of BP dissolved in DNA solution at 8°C is about 1 BP/1000 nucleotides and after radiation, the amount of radioactivity linked onto DNA was equivalent to 1 BP/2000 nucleotides. A BP and DNA solution was photoradiated in the usual manner, but after four

hours, more solid BP-H^3 was added to increase the total amount of BP-H^3 in solution prior to another four hours radiation. This was repeated three times. After the precipitation extraction procedure, the DNA-BP preparation was yellowish brown and had the ratio 1 BP/800 nucleotides, the u.v. spectrum of this material is very similar to the three hours radiated curve shown in Fig. 5 except it has a slight shoulder at 400 mμ. Upon centrifugation for 24 hours at 39,000 rpm in the SW 39 rotor, the tube contained a clear solution with a yellowish brown precipitate on the bottom which contained essentially all the the counts, and all the material which absorbs at 260 mμ and 320-400 mμ.

Studies of the enzyme-hydrolytic Products of DNA-BP[17]

The DNA-BP with high BP/DNA ratio just described above was hydrolyzed by DNAase and venom diesterase in 0.1 M Tris pH 8.0, 0.1 M $MgCl_2$ for 26 hours at 37°C. Paper electrophoresis experiments indicate that the products were essentially in form of nucleosides, since both the u.v. absorbing material and the radioactivity remained at the origin under a strong field gradient of 46 volts/cm for 75 minutes. This was presumably due to the action of the contaminating monoesterase in the venom diesterase. The product was also spotted on paper and eluted with benzene by ascending chromatography. Over 95% of the radioactivity together with the u.v. absorbing material was found to be retained at the origin. As a control, BP-H^3 shaken with denatured DNA but without exposure to photoradiation was spotted on paper. Elution of the control with benzene resulted in removing over 95% of the radioactivity from the origin to the solvent front. Repeated experiments of this nature clearly indicated that chemical linkage had been formed between BP-H^3 and DNA.

Comment and Conclusion:

The linkage between BP-H^3 or the degradation products of BP-H^3 and denatured DNA has been studied by experiments with precipitation and extraction, sedimentation, electrophoresis, spectroscopy, enzyme hydrolysis and paper chromatography of the hydrolyzed products. The results show vigorously that covalent linkage between BP-H^3

and DNA can be generated by photoradiation at the absorption band of BP. In the present experimental condition the "yield" of the photoproduct is high, reaching 50% after four hours radiation.

This reaction distinctly differs from photodynamic action[14,15] as it takes place in the absence of oxygen and the DNA is not destroyed. The major portion of the BP which absorbs the radiation forms covalent linkage with DNA.

To review and speculate about the implication of these findings with the carcinogenesis of aromatic hydrocarbons and its relationship with radiation lies beyond the scope and space allowed for this communication. Two salient ideas, however, will be discussed.

Certain classes of chemical carcinogens, such as, the mustards and imines are chemically very reactive. It is not difficult to conceive that these compounds many interact with the vital informational biopolymers in the cell. For the chemically inert carcinogenic hydrocarbons, an activated species must be generated if linkage to informational macromolecules is essential for its action. The current hypothesis is that these molecules may be functionalized, thereby activated, through the physiological oxidation or detoxication process. This process is likely to take place a distance away from the genetic apparatus of the cell, therefore, it may be difficult for the activated molecules to find their way to the genetic center without reacting with other less essential molecules on route. Polycyclic hydrocarbons, owing to their extensive π-electron system, are apt to form relatively stable, though reactive, free radicals or bi-radicals upon radiation.[16]

The affinity of denatured DNA for BP is 15 times higher than to phenanthrene, 10^3 times higher than to adenine and 10^4-10^5 times higher than to thymine. As mentioned above and in previous publications, these compounds all prefer the coil form of nucleic acids more than the helical form for binding. Furthermore, in agreement with the results on bindings of naphthalene and phenanthrene[3] the binding of BP onto DNA is much more greater than that onto RNA.[17] Thus, upon penetrating the nucleus, BP is most likely to interact physically with the DNA in the uncoiled or uncoiling stage; i.e., DNA which is replicating

or synthesizing RNA. If the BP is activated by radiation while it is physically bound, the results in this communication show that a covalent linkage between the DNA and BP can be formed.

Summary

The physical binding of benzpyrene-H^3 with coil form of thymus DNA has been studied. Covalent linkage between the benzpyrene-H^3 or the degradation products of benzpyrene-H^3 and DNA was generated by photoradiation at the absorption band of benzpyrene with the "yield" of photoproduct around 50%. This finding has been conclusively supported by experiments with precipitation and extraction, sedimentation, electrophoresis, spectroscopy, enzymic hydrolysis, and chromatography of the hydrolytic products.

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TABLE I

DISTRIBUTION OF THE OPTICAL DENSITY AND RADIOACTIVITY

OF THE DNA-BP IN CENTRIFUGE TUBES BEFORE AND AFTER DNAase TREATMENT*

Samples	Fraction in Tube		Optical Density at 260 mμ	cpm/ml	cpm/O.D.
I. DNA-BP	top	1	0.3	6,290	20,900
		2	0.3	5,880	17,600
		3	0.3	5,720	19,100
	bottom	4	7.7	96,500	12,500
II. DNA-BP treated with DNAase	top	1	2.2	18,800	10,300
		2	2.0	18,700	11,300
		3	2.0	18,800	11,300
	bottom	4	2.0	19,100	11,500
III. DNA with DNAase control	top	1	3.5	-----	-----
		2	3.2	-----	-----
		3	3.1	-----	-----
	bottom	4	3.0	-----	-----

* Solutions centrifuged for 16 hours at 39,000 rpm in SW-39 rotor, Spinco Model L ultracentrifuge.

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17. We wish to acknowledge the assistance of Dr. A. E. Smith and Mr. M. P. Schweizer in these experiments.

LEGENDS

1. Formation of DNA-Bp after varying duration of photoradiation. Assayed by counts of BP-H^3 linked to DNA after precipitation and extraction.
2. A. Sucrose gradient sedimentation of DNA-BP. $O.D._{260\ m\mu}$ (---), C.P.M. (——)
   B. Sucrose gradient sedimentation of radiated BP. Centrifuged in SW-25 rotor, Spinco Model L, at 25,000rpm for 20 hours at 5^0C.
3. Sucrose gradient electrophoresis of DNA-BP. $O.D._{260\ m\mu}$ (---), C.P.M. (——) in 0.1 M NaCl, 0.001 M Tris. pH 7.5 for six hours with voltage gradient of 30 volt/cm.
4. u.v. spectrum of 3,4-benzpyrene dissolved in ethanol (——) and in benzene (----) spectra of benzpyrene dissolved in 50% ethanol-50% water, hexane, and cyclohexance is identical to that in ethanol.
5. u.v. spectrum of 3,4 benzpyrene dissolved in DNA solution (5 mg/ml, HMP). Control with no radiation (——), radiated for one hour, (---) for two hours, (-·-·-) for three hours, (-··-) and for four hours (····).

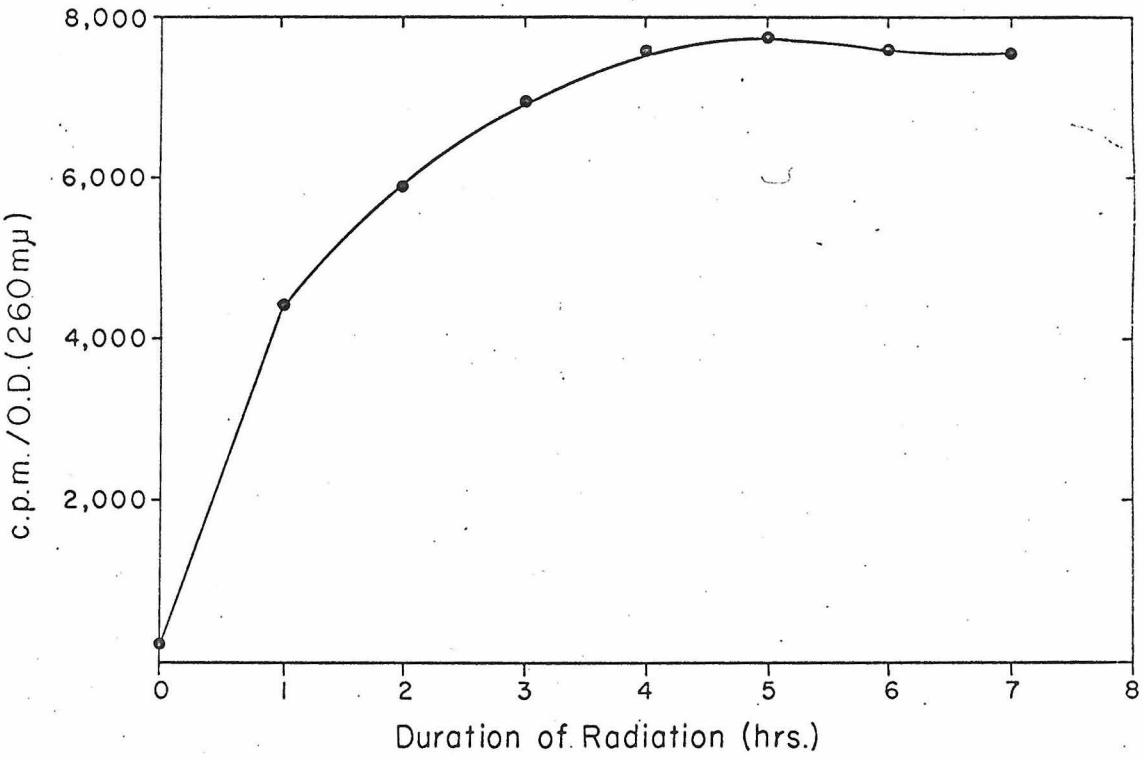

Fig. 1

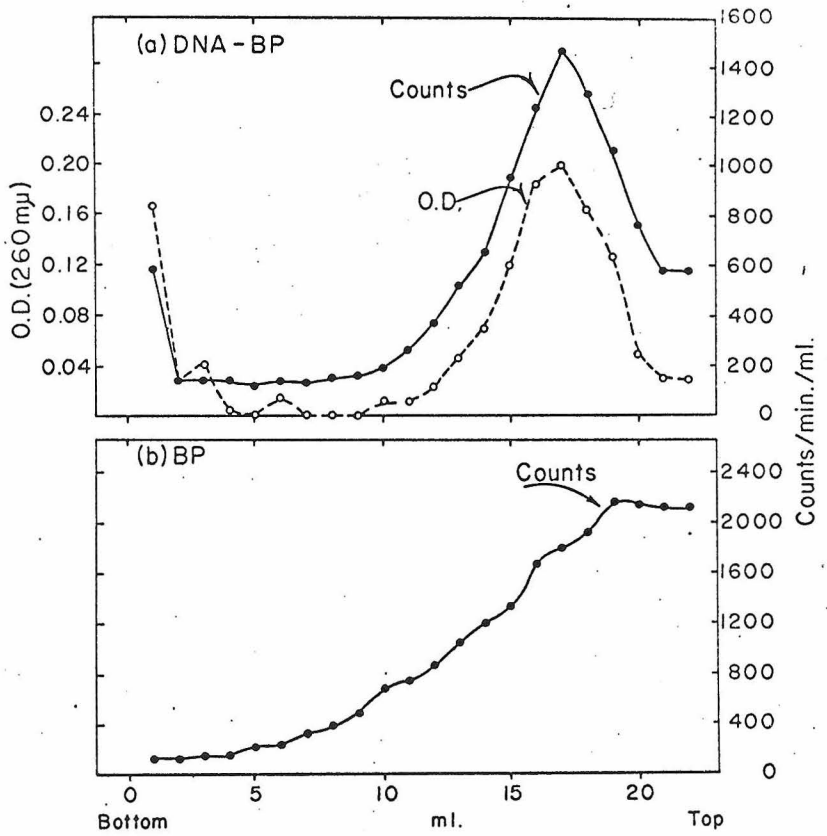

Fig. 2

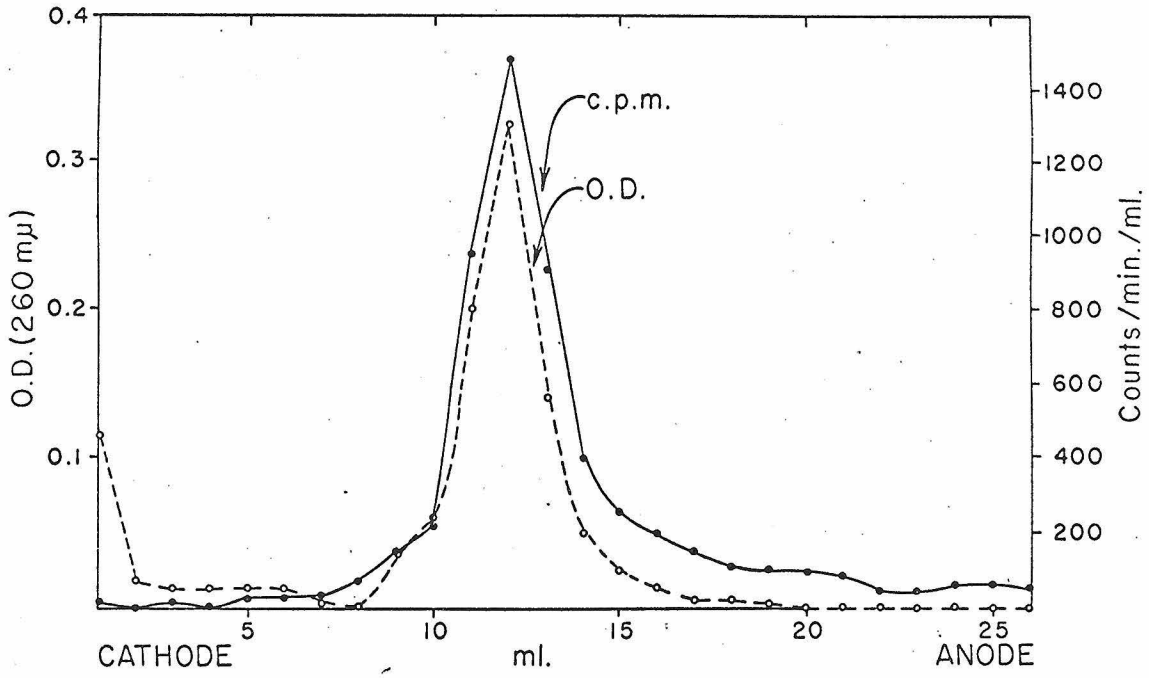

Fig 3

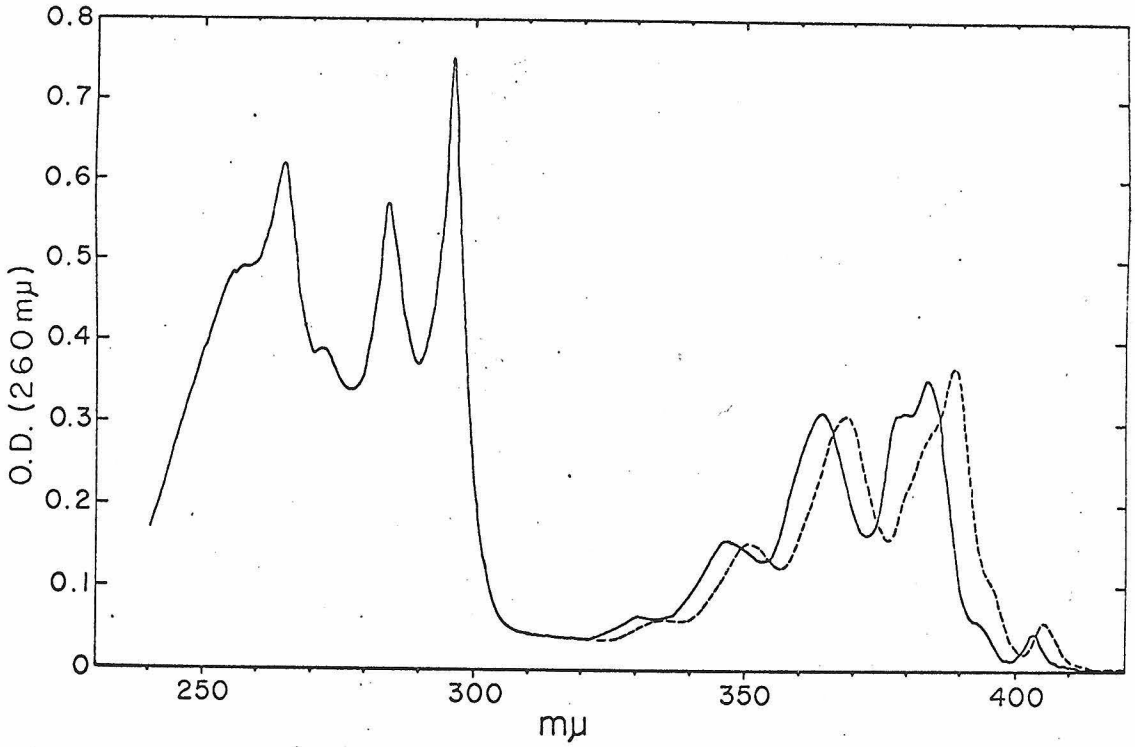

Fig. 4

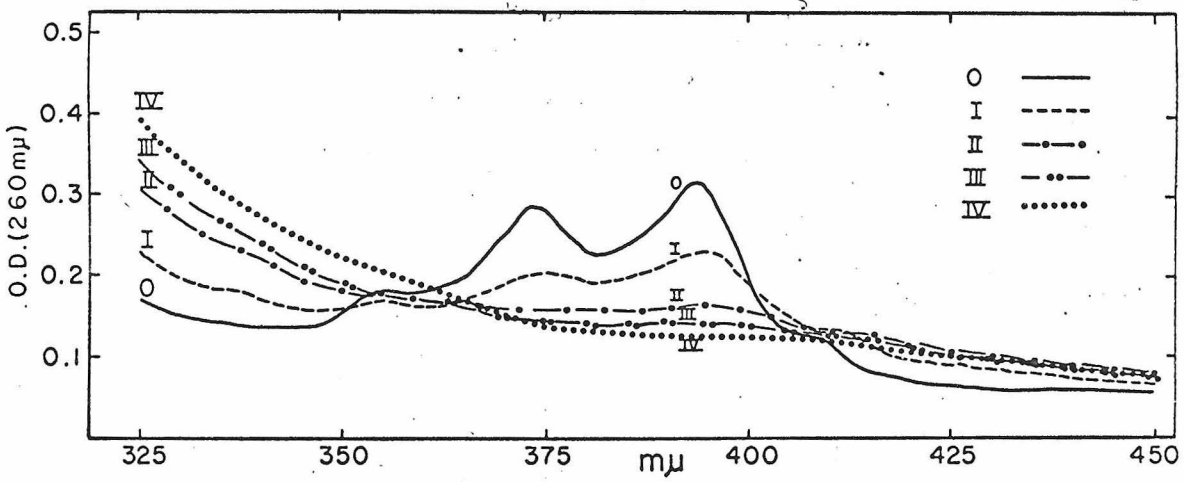

Fig. 5