METABOLIC ALTERATIONS AFTER TOTAL BODY DOSES OF X-RADIATION

II. INCORPORATION OF DEOXYCYTIDYLIC AND THYMIDYLIC ACID INTO PURIFIED DNA AND NUCLEI IN PRESENCE OF REGENERATING-LIVER SUPERNATANT

J. L. VAN LANCKER*

Departments of Pathology and Biochemistry University of Utah, Salt Lake City, Utah (U.S.A.)

(Received July 12th, 1960)

SUMMARY

The incorporation of [^{3}H]deoxycytidylic acid and [^{3}H]thymidylic acid into purified deoxyribonucleic acid was measured in the presence of regenerating liver supernatant obtained at various time intervals after partial hepatectomy. The animals were sacrificed either 6 h or 24 h after X-radiation. The results suggest that X-radiation interferes with the biosynthesis of the enzymes involved in the last steps of deoxyribonucleic acid synthesis. The effect of X-radiation on the nucleus was also investigated by studying the incorporation of [^{3}H]deoxycytidylic acid into the deoxyribonucleic acid of regenerating liver nuclei (48 h after partial hepatectomy) obtained from animals sacrificed 24 h after the administration of 1500 R *in vivo*. The incorporation of the precursor was found to be considerably reduced by X-radiation.

INTRODUCTION

In vivo X-radiation of partially hepatectomized rats inhibits incorporation of tritium labeled thymidine into DNA of homogenates[1]. X-radiation affects both the nucleus and the cytoplasm, but the nature of the alteration in each case remains unknown[2]. The aim of the present study was to investigate the effect of X-radiation on both the priming capacity of the nucleus and the activity of the cytoplasmic enzymes involved in the last steps of DNA synthesis in a system easier to control than homogenates. Therefore, the incorporation of [^{3}H] DCMP or of [^{3}H]TMP into purified DNA and into regenerating liver nuclei in presence of regenerating rat liver supernatant was measured.

Abbreviations: DNA, deoxyribonucleic acid; DCMP, deoxycytidylic acid; TMP, thymidylic acid; RNA, ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane.

* Present address Dr. J. L. Van Lancker, Department of Pathology University of Wisconsin Madison, Wisc. (U.S.A.).

METHODS

Sprague-Dawley rats weighing 180 g were used. The methods of irradiation and partial hepatectomy have been described[1]. The nuclei used for incubations were prepared by differential centrifugation of a 5 % homogenate at 600 × *g* for 10 min, the nuclear pellet was washed twice in sucrose and checked for mitochondrial or microsomal contaminants[2]. Regenerating liver supernatant was prepared by centrifuging a 5 % homogenate (in 0.25 *M* sucrose) at 105, 400 × *g* for 60 min in the model L Spinco centrifuge equipped with rotor No. 40. The supernatant was checked for mitochondrial and microsomal contaminants by the determination of cytochrome oxidase and glucose-6-phosphatase activity[3]. 2 % cytochrome oxidase and 12 % glucose-6-phosphatase activity remained associated with the latter fraction. Regenerating liver cell fractions, were prepared by differential centrifugation of the homogenate. The nuclei were separated at 600 × *g* for 10 min, the small and large mitochondria were sedimented in a single pellet by centrifuging at 25,000 × *g* for 10 min and the microsomal pellet was sedimented at 105,400 × *g* for 60 min. Each fraction and a sample of the total homogenate were analyzed for nitrogen, DNA, RNA and for cytochrome oxidase, glucose-6-phosphatase, acid phosphatase, deoxyribonuclease, β-glucuronidase activities. The methods of analysis were the same as those used in previous studies[3] except for deoxyribonuclease determinations which were done at pH 5 in presence of 0.05 *M* acetate buffer. The creatine kinase used for the incubations was prepared according to the method of NODA *et al.*[4]. The results of the incorporation studies were, however, similar when pyruvic kinase and pyruvic phosphate (purchased from Sigma) were used as high energy phosphate generators. [^{3}H]DCMP was obtained from Schwartz Laboratories (0.93 C/m mole).

The labeled thymidylic acid used in these experiments was prepared from tritium labeled regenerating rat liver DNA. Partially hepatectomized animals were injected intraperitoneally with [^{3}H]thymidine 18, 23 and 26 h after operation and were sacrificed 6 h later. The nuclear pellet was prepared from liver homogenates[2]. After purification the extracted DNA[5] was treated with crystalline deoxyribonuclease and snake venom phosphodiesterase free of 5′ nucleotidase[6]. The nucleotides so obtained were then separated by the method of CANELLAKIS *et al.*[7]. Except when stated otherwise, the incubation methods were those described by MATSAVINOS AND CANELLAKIS[8]. The incubation was stopped by addition of perchloric acid to the mixture to reach a final concentration of 0.06 *M*. After repeated washings with cold 5 % perchloric acid, alcohol and Bloor's solution, the nucleic acids were extracted at 95° with a solution containing 10 % sodium chloride and 0.5 *M* Tris buffer. The DNA was separated from RNA by the method of SCHMIDT AND THANNHAUSER[9]. After precipitation of the nucleic acids with alcohol the contaminating RNA was hydrolysed by heating the nucleic acid preparation at 80° in 0.1 *N* sodium hydroxide. The DNA was then precipitated with 2 *N* hydrochloric acid and washed with alcohol. These procedures were repeated until the products of the sodium hydroxide hydrolysis were free of orcinol reacting substances. After several washings with cold alcohol, the purified DNA pellet was dissolved in 0.1 *M* ammonium hydroxyde and the concentration of deoxyribose was determined by the diphenylamine method[10]. Great care was taken to plate[1] identical amounts of DNA (0.08 to 0.1 mg when nuclear DNA and 0.06 to 0.07 mg when purified DNA was used). Therefore, only minor corrections

for self-absorption had to be introduced. Self-absorption curves for increasing concentration of [^{3}H] DCMP and DNA labelled with deoxycytidylic acid and thymidylic acid were prepared.

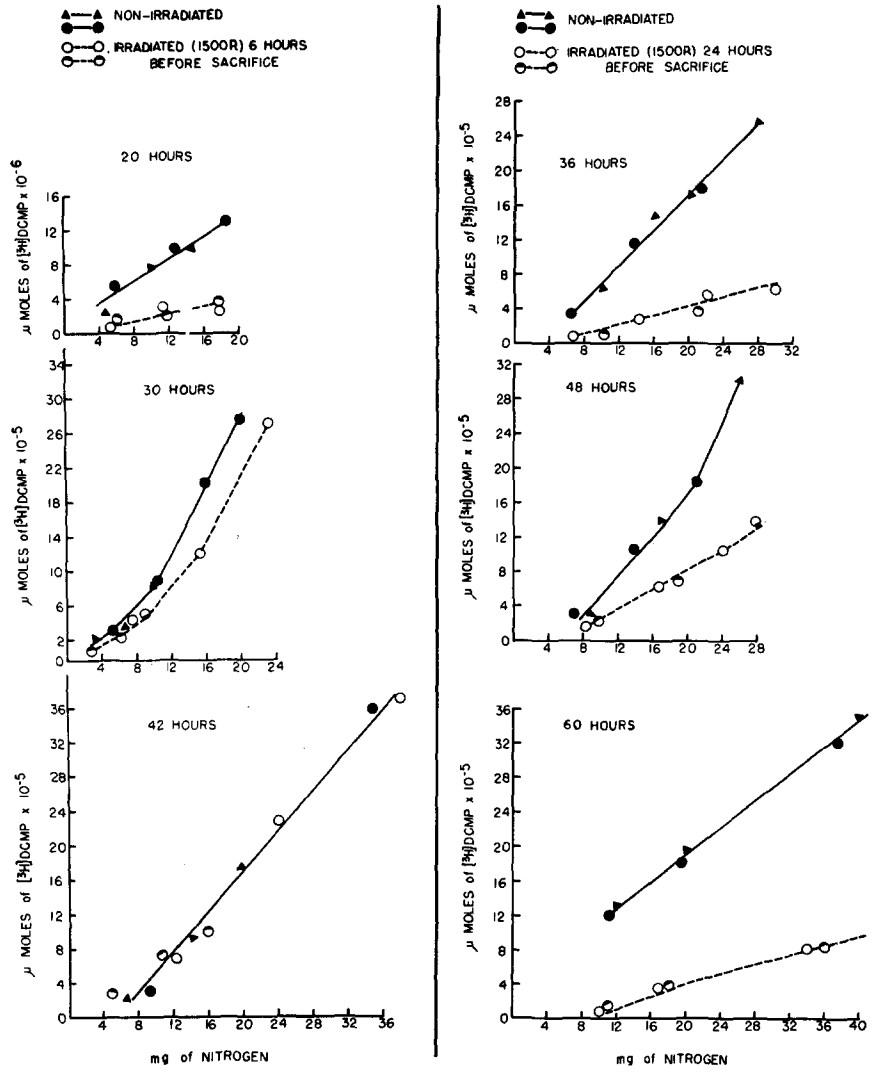

Fig. 1. The animals received 1500 R, 12, 14, 24, and 36 h after hepatectomy and were sacrificed 6 or 24 h later as indicated on the graph. Two separate experiments were done at each time sequence. The results of each experiment are plotted with different symbols. The time in hours refers, in each case, to the time interval between hepatectomy and sacrifice. For each experiment 3 normal and 3 irradiated livers were pooled separately. 10 ml of the incubation mixture B described by CANELLAKIS AND MATSAVINOS[8] was used for each test. The primer was Worthington DNA boiled for 10 min[17,18]. The preparation was incubated for 60 min at 37°. The data are expressed in μmoles of [^{3}H]DCMP incorporated /mg of nitrogen present in the regenerating liver supernatant added.

TABLE I

Two tissue fractionation studies were done at 20 and 36 h after partial hepatectomy. In each experiment tissue fractions of non irradiated animals (I, II) were compared with tissue fractions of irradiated animals (III, IV). The latter received 1500 R, 6 h before death when sacrificed 20 h after hepatectomy, or 24 h before death when sacrificed 36 h after hepatectomy. Two regenerating livers were pooled for each tissue fractionation study. The data are expressed in mg of nitrogen and RNA or in enzyme units present in two livers. The units of acid phosphatase, glucose-6-phosphatase and β-glucuronidase refer to the decomposition of 1 μmole of substrate /min under the conditions of the assay. The deoxyribonuclease unit corresponds to the increase in absorbancy of the acid soluble fraction at 260 mμ per 15 min incubation under the condition of the assay. The COOPERSTEIN-LAZAROW unit was used for cytochrome oxidase[19]. Recovery refers to the sum of the absolute values found in each individual fraction expressed in % of the absolute values in the homogenate. The nitrogen, RNA content, and enzyme activity in each fraction are expressed in % of the recovered values.

	20 h after hepatectomy				*36 h after hepatectomy*			
	Non irradiated		*Irradiated*		*Non irradiated*		*Irradiated*	
	I	*II*	*III*	*IV*	*I*	*II*	*III*	*IV*
Nitrogen								
Total	189.2	193.4	190.2	194.5	205.0	203.0	187.0	194.2
Recovery	98.0	94.5	96.0	97.0	89.5	97.0	96.0	89.1
Nuclei	15.6	16.4	13.6	15.0	16.4	18.2	18.8	31.5
Mitochondria	18.7	18.0	19.0	18.7	13.2	19.7	11.0	15.7
Microsomes	38.6	36.4	36.0	35.6	41.5	39.0	35.2	37.0
Supernatant	27.1	29.2	31.0	30.7	28.9	23.1	35.0	32.3
RNA								
Total	45.2	43.4	42.3	44.2	71.2	62.7	71.2	61.7
Recovery	97.5	91.5	102.0	99.0	99.0	97.1	98.0	96.2
Nuclei	12.4	13.5	13.0	9.0	16.8	21.0	16.8	16.2
Mitochondria	13.0	12.5	13.0	7.0	8.4	9.2	8.0	8.6
Microsomes	60.8	62.0	62.0	62.0	67.5	63.6	67.5	63.5
Supernatant	13.8	12.0	12.0	12.0	7.3	6.2	7.7	11.7
Cytochrome oxidase								
Total	180.0	170.2	167.2	172.1				
Recovery	95.0	93.0	90.5	84.0				
Nuclei	4.2	4.6	3.8	4.2				
Mitochondria	76.3	78.0	69.4	78.0				
Microsomes	19.5	17.4	26.8	17.8				
Supernatant	—	—	—	—				
Acid phosphatase								
Total	33.6	32.2	33.0	30.0	37.9	45.2	38.0	40.5
Recovery	94.0	95.0	92.0	96.0	92.1	94.0	96.1	89.2
Nuclei	4.1	4.2	4.5	4.1	6.7	11.4	7.6	7.8
Mitochondria	60.0	59.0	58.7	59.0	47.0	44.0	40.4	46.0
Microsomes	23.0	22.8	20.3	18.9	25.7	24.7	34.0	25.2
Supernatant	12.9	14.0	16.5	18.0	20.6	19.9	17.9	21.0
Glucuronidase								
Total	8.5	8.3	7.4	8.1	12.1	14.0	14.2	13.3
Recovery	94.0	91.0	92.0	105.0	93.0	94.0	97.1	96.3
Nuclei	3.1	4.2	5.6	5.2	4.2	4.5	3.9	5.6
Mitochondria	51.1	48.0	52.2	47.1	48.0	51.2	58.0	43.6
Microsomes	21.2	23.1	26.0	19.3	20.1	23.9	19.6	24.5
Supernatant	24.6	26.5	16.2	28.4	27.7	20.4	18.5	26.3
Deoxyribonuclease								
Total	35.4	37.6	36.1	37.0	56.0	58.2	49.4	57.0
Recovery*	120.0	110.0	115.0	125.0	124.0	125.1	120.	110.
Nuclei	3.3	4.3	2.6	6.5	4.1	2.4	5.2	3.4
Mitochondria	43.5	42.5	42.5	46.5	39.0	41.2	48.6	44.5
Microsomes	20.0	19.4	20.6	16.0	27.2	24.3	19.1	18.1
Supernatant	33.2	33.8	34.5	31.0	29.7	32.1	27.1	34.

* Those recoveries greater than 100% have been discussed previously[3].

Continued TABLE I

	20 h after hepatectomy				*36 h after hepatectomy*			
	Non irradiated		*Irradiated*		*Non irradiated*		*Irradiated*	
	I	*II*	*III*	*IV*	*I*	*II*	*III*	*IV*
Glucose-6-phosphatase								
Total	92.0	88.2	94.0	96.4	117.0	142.0	126.3	124.3
Recovery	96.1	91.5	94.5	99.0	96.0	94.0	93.0	98.0
Nuclei	5.1	3.3	3.7	3.4	2.6	2.0	2.1	4.5
Mitochondria	2.9	2.7	2.7	3.1	10.8	10.0	8.7	11.2
Microsomes	86.0	86.5	88.0	81.5	82.0	86.8	86.0	80.2
Supernatant	6.0	7.5	5.6	6.0	4.6	1.2	3.2	4.1

RESULTS

The incorporation of [^{3}H] DCMP into purified DNA was measured in presence of regenerating liver supernatant obtained from animals irradiated (1500 R *in vivo*) 14, 24, and 36 h after hepatectomy and sacrificed 6 or 24 h after X-radiation.

The relationship between the incorporation of the precursor and the amount of supernatant present in the system is not strictly linear; therefore, the incorporation was, in each case, studied at various concentrations of enzymes. The results of two separate experiments at various time sequences are presented in Fig. 1. Inhibition of incorporation was observed in all cases where the animals were sacrificed 24 h after X-radiation. It makes little difference under those conditions whether the animals were irradiated 14, 24, or 36 h after hepatectomy. In contrast, marked inhibition

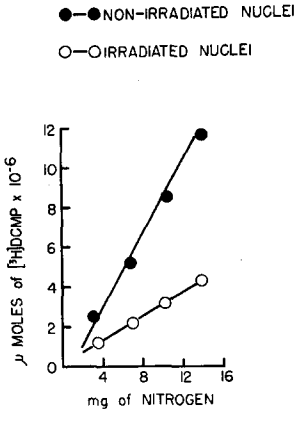

Fig. 2. The animals received 1500 R. 24 h after partial hepatectomy and were sacrificed 24 h later. The incubation mixture was the same as for Fig. 1. The nuclei of 3 non-irradiated and 3 irradiated regenerating livers were pooled separately. The DNA content of the nuclear preparation used for each test was 0.6 mg when nonirradiated nuclei and 0.58 mg when irradiated nuclei were used. The preparation was incubated at 34° for 25 min. The data is expressed as in Fig. 1.

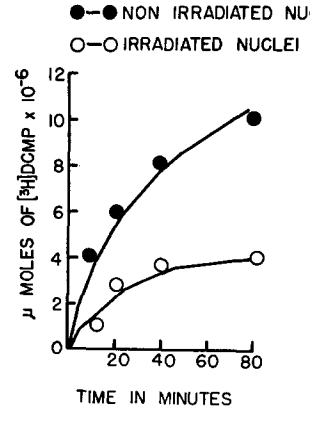

Fig. 3. The conditions of irradiation and incubation were the same as in Fig. 2 except that the enzyme concentrations were maintained constant (7.8 mg of regenerating liver supernatant nitrogen per incubation flask). The amount of the nuclear preparation was increased and the data are expressed in μmoles of [^{3}H]DCMP incorporated /mg of DNA added to the incubation flask.

was only observed in those animals irradiated 14 h after hepatectomy when the animals were sacrificed 6 h after irradiation.

The incorporation of [^{3}H]TMP into purified DNA in presence of regenerating liver supernatant obtained from animals irradiated 14 and 24 h after hepatectomy and sacrificed 6 h after X-radiation was also investigated. The results were identical to those obtained with [^{3}H]DCMP and therefore they are not reported in detail here.

The incorporation of [^{3}H]DCMP into non-irradiated and irradiated (1500 R *in vivo* 24 h after hepatectomy) regenerating liver nuclei was determined in presence of regenerating liver supernatant under conditions where the concentration of enzyme, the concentration of DNA or the duration of incubation were altered. In these experiments the animals were sacrificed 24 h after X-radiation. The results are plotted in Figs. 2, 3 and 4, and they clearly demonstrate that irradiated nuclei incorporate less of the precursor than non-irradiated.

The results of two tissue fractionation studies of regenerating liver homogenates obtained from animals irradiated 12 h after hepatectomy and sacrificed 6 or 24 h after X-radiation are presented in Table I. They show that X-radiation has no effect on total quantity and intracellular distribution of the various components investigated.

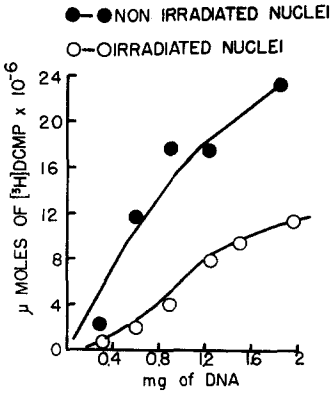

Fig. 4. The conditions of irradiation and incubation were the same as those of Figs. 2 and 3. The DNA (0.6 mg) and the regenerating liver supernatant concentration (7.8 mg of regenerating liver supernatant nitrogen per incubation flask) were maintained constant. Only the duration of the incubation is varied. The data are expressed in μmoles of [^{3}H]DCMP incorporated after various incubation times.

DISCUSSION

These experiments confirm previous findings *in vivo*[11,12] and *in vitro*[12,13] where it was demonstrated that X-radiation inhibits DNA synthesis in regenerating rat liver. Thus, the present work establishes a continuous base line of information between the *in vivo* situation and a simple *in vitro* system. It was also established that inhibition of DNA synthesis is due, altogether, to a decrease of the activity of the cytoplasmic enzymes involved in the last steps of DNA biosynthesis and to a decrease of the priming capacity of irradiated nuclei[2]. The reduced incorporation of [^{3}H]DCMP in presence of purified DNA and regenerating liver supernatant obtained from irradiated animals sacrificed 24 h after irradiation (Fig. 2) reflects a loss of enzyme

activity. Indeed, under those conditions the concentration of enzymes is rate limiting. It is improbable that this reduced activity is due to cellular death, because the rate of synthesis of total nitrogen, RNA and several enzymes (Table I) is not altered under those conditions. In addition, in regenerating liver X-radiation does not lead to the release of hydrolytic enzymes from cytoplasmic granules, a phenomenon which constitutes one of the earliest manifestations of autolysis[14]. The reduced enzyme activity observed after X-radiation must therefore be due either to the formation of enzyme inhibitors, to denaturation of the enzymes or to interference with biosynthesis of the enzymes. Experiments which will be published in detail later where irradiated cytoplasm was recombined with non-irradiated cytoplasm seem to exclude the existence of inhibitors. It is doubtful that the relatively small doses of X-radiation used *in vivo* denature the enzyme. The comparison of the results obtained 6 or 24 h after X-radiation also tend to exclude such a mechanism. Indeed, in absence of significant reduction of the cell population, denaturation would (in those animals irradiated 24 or 36 h after hepatectomy), lead to a similar drop in enzyme activity whether they are sacrificed 6 or 24 h after irradiation.

The results obtained in those animals irradiated 14 h after hepatectomy and sacrificed 6 h later suggest that X-radiation interferes with the biosynthesis of the enzyme involved in the last steps of DNA biosynthesis. Indeed, under those conditions, the animals are irradiated when no enzyme activity is measurable and sacrificed when DNA synthesis is very active. While this study was in progress two abstracts appeared describing experiments where partially hepatectomized rats were irradiated before the occurrence of DNA synthesis in the regenerating liver and sacrificed 6 h later[15,16]. Although these experiments were done under different conditions and only at one concentration of enzymes, the results are in agreement with those presented here. A recent report of BOLLUM *et al.*[13], suggests that reduced incorporation of DNA precursors after X-radiation results from interference with the activity of several enzymes involved in the last step of DNA synthesis. It is not astonishing that little or no effect of X-radiation could be measured when animals irradiated 24 or 36 h after hepatectomy are sacrificed 6 h later. Indeed, under those conditions large amounts of enzymes are already present in the regenerating liver at the time of irradiation, and, therefore, significant changes in activity will become manifest only if sacrifice occurs later (24 h, for example) after irradiation.

The reduced incorporation of [^{3}H]DCMP into irradiated nuclei in presence of regenerating rat liver supernatant confirms our previous observations in homogenates[2]. This may result either from direct effect of X-radiation on DNA or from interference with the mechanism that makes nuclear DNA available as primer. The nature of that effect needs to be investigated with an enzyme preparation free of deoxyribonuclease activity.

ACKNOWLEDGEMENTS

The author is grateful to the Department of Radiobiology which provided the X-ray machine used in these experiments. The skillful technical assistance of E. MICHELS and J. T. DOUGLAS is also gratefully appreciated. This investigation was supported by Public Health Service Research Grant (C-3873) from the National Cancer Institute Public Health Service and by a Senior Research Fellowship (SF-224) from the Public Health Service.

REFERENCES

[1] J. L. VAN LANCKER, *Biochim. Biophys. Acta*, 33 (1959) 587.
[2] J. L. VAN LANCKER, *Biochim. Biophys. Acta*, in the press.
[3] J. L. VAN LANCKER AND R. L. HOLTZER, *J. Biol. Chem.*, 234 (1959) 2359.
[4] L. NODA AND H. LARDY, *Methods in Enzymology*, Vol. II, New York, 1955 p.605.
[5] E. CHARGAFF, *The Nucleic Acids*, Vol. I, Academic Press Inc., New York, 1955, p. 324.
[6] R. O. HURST AND G. C. BUTLER, *J. Biol. Chem.*, 193 (1951) 91.
[7] E. S. CANELLAKIS AND R. MATSAVINOS, *Biochim. Biophys. Acta*, 27 (1958) 643.
[8] R. MATSAVINOS AND E. S. CANELLAKIS, *J. Biol. Chem.*, 234 (1959) 628.
[9] G. SCHMIDT AND S. THANNHAUSER, *J. Biol. Chem.*, 161 (1945) 83.
[10] Z. DISCHE, *Mikrochemie*, 8 (1930) 4.
[11] V. R. POTTER, J. L. VAN LANCKER AND R. E. BELTZ, *Abstract of the Scientific Proceed, Am. Assoc. Cancer Research*, 1956, p. 140.
[12] R. E. BELTZ, J. L. VAN LANCKER AND V. R. POTTER, *Cancer Research*, 17 (1957) 688.
[13] F. S. BOLLUM, J. W. ANDEREGG, A. B. MC ELYA AND V. R. POTTER, *Cancer Research*, 20 (1960) 138.
[14] J. L. VAN LANCKER AND R. L. HOLTZER, *Am. J. Pathol.*, 35 (1959) 563.
[15] R. E. BELTZ AND R. L. APPLEGATE, *Biochem. Biophys. Research Communs.*, 1 (1959) 298.
[16] S. OKADA AND C. M. WHEELER, *Federation Proc.*, 12 (1960) 351.
[17] I. H. LEHMAN, *Ann. N. Y. Acad. Sci.*, 81 (1959) 745.
[18] F. J. BOLLUM, *J. Biol. Chem.*, 234 (1959) 2733.
[19] S. J. COOPERSTEIN AND A. LAZAROW, *J. Biol. Chem.*, 189 (1951) 665.

Biochim. Biophys. Acta, 45 (1960) 63–70

INACTIVATION OF ENZYME FORMATION BY ULTRAVIOLET LIGHT

I. ACTION SPECTRA AND SIZE OF THE SENSITIVE UNIT

G. W. RUSHIZKY*, MONICA RILEY, LOUISE S. PRESTIDGE AND ARTHUR B. PARDEE

Virus Laboratory and Department of Biochemistry University of California, Berkeley, Calif. (U.S.A.)

(Received April 2nd, 1960)

SUMMARY

The action spectra for loss of ability to form the inducible enzymes β-galactosidase and tryptophanase by *Escherichia coli*, and also for colony formation, resemble the absorption spectra of nucleic acids. A minimum size of the sensitive unit for enzyme formation of 300,000 molecular weight units is computed. This suggests that the sensitive unit is either high molecular weight RNA or DNA and not soluble RNA. On the basis of previously reported quantum yields, the size of the target is estimated to be somewhat larger than the minimum, perhaps of mass 700,000. This result is in fair agreement with present estimates of the size of a functional gene or of the RNA of a ribonucleoprotein particle. These data do not permit a choice between RNA and DNA as target materials.

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

* Present address: NCI, NIH, Bethesda 14, Md. (U.S.A.).