PART II

MECHANISMS UNDERLYING INCREASE IN RAT LIVER RNA SYNTHESIS FOLLOWING WHOLE-BODY X-IRRADIATION

GENERAL INTRODUCTION TO THE PART

Responses of animals to ionising radiation vary depending on the dose received (1) and a whole spectrum of signs and symptoms is exhibited as dose of exposure is increased. Three types of syndromes of radiation injury can be broadly classified on the basis of the mean survival time. These are (i) for survival ranging from days to weeks for exposures from a few hundred to thousand roentgens, termed as the bone-marrow syndrome; (ii) for survival times of three to four days duration in case of animals exposed to doses ranging from thousand to ten thousand roentgens, termed as gastro-intestinal syndrome; and (iii) for survival times of hours to a few days in respect of animals exposed to doses beyond ten thousand roentgens, termed as central nervous system syndrome (2). From the point of view of physiological regulation the most important doses are sub-lethal doses or the doses at which the animals die several days after radiation exposure (bone-marrow syndrome). Inhibition of DNA synthesis (3,4), changes in protein synthetic patterns (5,6), uncoupling of oxidative phosphorylation (7), release of lysosomal enzymes (5,8), impairment in gastro-intestinal functions (9,10), increased urinary excretion of amines (11,12) and fat infiltration (5,13) have been found to be some of the early biochemical effects after radiation exposure. It is not known as yet whether these effects reflect disturbances in regulatory mechanisms or are

manifestations of compensatory processes (such as repair of damaged DNA, tissue regeneration by cell replacement). Nevertheless, probes into the mechanisms underlying these perturbations promise to yield valuable clues for the elucidation of biochemical controls under normal conditions.

This part of the thesis will describe studies aimed at examining the mechanisms underlying enhancement in the liver RNA synthesis of rats. It is divided into three sections. Section 1 relates to the nature of changes induced in the activities of chromatin template and RNA polymerase. Section 2 incorporates studies which were aimed at examining whether the radiation-induced changes in transcription machinery are related to changes in chromatin structure and metabolism of its constituents. Section 3 covers studies on the neuro-endocrine mechanisms that may underlie the radiation-induced activation of the rat liver transcription apparatus.

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Part II

Section 1

Changes elicited by whole-body radiation exposure in the components of transcription machinery of the rat liver

INTRODUCTION

Some of the early signs of responses of mammals to ionising radiations (sub-lethal and lethal doses) are exhibited in significant changes in rates of synthesis of proteins in a number of organs (1-6). While protein synthesis is expected to decrease in animal tissues following such injury, rates of protein synthesis in certain tissues exhibit striking increases (1, 2, 6) because of compensatory and other indirect mechanisms. Interest in radiation-provoked changes in protein synthesis was generated by the reports (7, 8) that whole-body radiation-exposure of some animal species at sub-lethal and near-lethal doses steps up protein synthetic capacity of liver between 12-36 hr post-irradiation. This elevation in protein synthesis is found to be associated with increase in total ribosomal population and also with enrichment of polysomes at the expense of monosomes (7, 8). It has been reported that these changes are predominantly the consequence of a general stimulation in RNA synthesis (8, 9). Studies described in this section were directed to ascertain whether this stimulus in RNA synthesis stems from activation of RNA polymerase or increased transcriptive efficiency of liver chromatin or due to combination of these two effects. An evidence has been obtained to suggest that template activity is greatly amplified during 4 to 18 hr following exposure of rats to 1000 r x-irradiation. Further work has shown that the number of

available RNA chain initiation sites is also substantially increased in response to irradiation.

MATERIALS AND METHODS

Animals and radiation:

Male Wistar strain rats, each weighing between 150-160 g and fed on a laboratory stock diet, were used. They were fasted for 18 to 20 hr prior to killing.

Rats were given a single whole-body dose of 1000 r x-irradiation by housing them in groups of three in a three-place perspex container located at a distance 50 cm from 250 KV x-ray generator (Siemens, Stabilipan). The latter was operated at 15 mA tube current with an added filter of 2 mm Al. The dose rate was 100 r per minute.

Measurement of incorporation of ^{32}P-orthophosphate into liver RNA:

Rats received ^{32}P-orthophosphate (carrier free, 2 mCi/100 g body wt) in 1 ml saline by intraperitoneal injections 1 hr prior to sacrifice. RNA was extracted from livers by the procedure of Munro and Fleck (10). Briefly, the procedure involved addition of 0.6 N perchloric acid to the homogenate to precipitate macromolecules. The precipitate was twice washed with 0.2 N perchloric acid and suspended in 0.3 N KOH and kept for hydrolysis of RNA at 37° for 60 min. DNA and protein were precipitated by the addition of equal volume of cold 1.2 N perchloric acid to

the KOH hydrolysate. The supernatant obtained by centrifugation contained hydrolysed RNA. Aliquots of the RNA hydrolysate were taken for determination of RNA content by colorimetric analysis involving orcinol reaction (11) and for ^{32}P-radioactivity. The latter was determined by spotting aliquots (0.2 ml; ~0.05 mg) on Whatman No.3 filter paper strips (7 cm x 1.8 cm) which were dried by blowing hot air and then counted for radioactivity. The results are expressed as relative specific activity i.e. specific activity of RNA divided by specific activity of inorganic phosphate in liver homogenate.

Specific activity of inorganic phosphate

The specific activity of ^{32}P-orthophosphate in liver homogenates was determined after deproteinising with cold 10% TCA essentially by the procedure described by Ernster et al. (12). The method briefly involves formation of phosphomolybdic acid by ammonium molybdate in the presence of concentrated H_2SO_4 and extraction of the phosphomolybdic acid formed by a mixture of isobutanol-benzene (1:1). The formed cerulomolybdate was then determined colorimetrically after the additions of appropriate amounts of H_2SO_4 dissolved in ethanol and $SnCl_2$ solution to the drawn-off isobutanol-benzene phase. In order to determine radioactivity, an aliquot was taken directly from the isobutanol-benzene phase, pipetted into vials and counted for radioactivity.

Sucrose density gradient analysis of ^{32}P-labelled liver RNA:

Rats received ^{32}P-orthophosphate (carrier free, 2 mCi/100 g body wt, in 1 ml saline) by intraperitoneal injections 1 hr prior to sacrifice. The procedure followed for isolation of labelled RNA was that of Scherrer and Darnell (13). Livers were excised and homogenised in 5 volumes of cold acetate buffer (0.05 M acetate buffer pH 5.1, 0.14 M NaCl, 0.5% sodium dodecyl sulphate, 4 mg/ml bentonite, and 150 µg/ml polyvinyl sulphate). The homogenate was mixed with equal volume of phenol, saturated with acetate buffer, heated at 65° for 5 min and centrifuged at 10,000 x g for 15 min at 4°. The aqueous layer containing RNA was withdrawn carefully and the phenol treatment was repeated on this solution till no protein layer was visible in the interphase. RNA was precipitated by addition of 2 vol. of cold ethanol and kept in cold overnight. The precipitate was dissolved in the acetate buffer and passed through Dowex-1-formate column (1 cm x 5 cm) to remove inorganic phosphate and nucleotides. RNA was reprecipitated by addition of cold ethanol and then dissolved in the acetate buffer (determination of radioactivity in the cold TCA extract of an aliquot of the RNA solution revealed that the RNA was practically free from contamination by ^{32}P-containing oligonucleotides).

The RNA solution in acetate buffer (1 ml; 25 E_{260} units) was layered on buffered linear (10-40%) sucrose gradient (0.01 M acetate buffer pH 5.1, 0.1 M NaCl and 1 mM EDTA) and spun at 63,000 x g for 15 hr at 4° using the SW 25.1 rotor in a Beckman model L2-65B ultra centrifuge. After centrifugation fractions of 1 ml were collected from the gradients for the determinations of E_{260} and ^{32}P-radioactivity (as described below).

Assay of RNA polymerisation by liver nuclei

The procedure for isolation of liver nuclei and assays for Mg^{++}-activated and $Mn^{++}/(NH_4)_2SO_4$-activated RNA polymerase reactions were according to Widnell and Tata (14). In brief, livers were homogenized in 0.32 M sucrose containing 3 mM $MgCl_2$ and centrifuged at 600 x g for 10 min at 4° to pellet crude nuclei. The crude nuclei obtained were purified by suspending in 2.4 M sucrose containing 1 mM $MgCl_2$ and sedimenting at 40,000 x g for 1 hr at 4° using the Type 30 rotor in a Beckman L2-65B ultracentrifuge.

The assay system contained in a volume of 0.5 ml: 50 µmoles Tris-HCl buffer (pH 8.0); 2.5 µmoles $MgCl_2$ in Mg^{++}-activated reaction and 2 µmoles $MnCl_2$ and 0.4 M $(NH_4)_2SO_4$ in $Mn^{++}/(NH_4)_2SO_4$-activated reaction; CTP, UTP, GTP, 0.3 µmole each; 0.02 µmole ^{14}C-ATP (27.2 mCi/mmole) and nuclei equivalent to 0.3 mg DNA. Incubationsat 37° were carried out for 15 and 45 min for Mg^{++}-activated and $Mn^{++}/(NH_4)_2SO_4$-activated reactions, respectively. The reaction was stopped by the addition of 5 ml of 0.5 N perchloric acid (PCA) at 0°. Two mg RNA in aqueous solution (pH 7.0) was then added and the precipitate obtained was collected by centrifugation, washed twice with 4 ml of 0.2 N PCA and twice with a mixture of ethanol-ether (3:1). The resulting ^{14}C-RNA was extracted from the precipitate by two successive treatments with 10% NaCl containing 0.5 mg of carrier RNA per ml at 100° for 30 min. RNA was precipitated from the combined supernatant

by the addition of 2 vol of ethanol at 0°. The precipitate was collected, dissolved in liquid ammonia and counted for radioactivity.

Uptake of ^{3}H-UTP by isolated nuclei in vitro

The nuclei isolated as described above were suspended in 0.25 M sucrose. 0.1 ml aliquots of nuclear suspension (≡ 0.3 mg DNA) were incubated separately in the reaction mixtures (in 0.5 ml final volume) for assays of Mg^{++}-dependent and $Mn^{++}/(NH_4)_2SO_4$-dependent RNA polymerisation activity with the following changes: instead of GTP, CTP, UTP and ^{14}C-ATP, 0.3/umole of ^{3}H-UTP (50,000 cpm) and 15/ug actinomycin D were included in each of the reaction mixtures. Actinomycin D was included to suppress RNA synthesis, if any, from endogenous ribonucleoside triphosphates. The incubations were carried out at 37° for 10 min. The nuclei were collected by centrifugation at 1600xg, resuspended in 0.5 ml of 0.25 M sucrose containing 0.01M cold UTP, re-centrifuged and counted for radioactivity.

Assays of template activities of chromatin and DNA

Chromatin and DNA were isolated from rat liver and their template activities were assayed using exogenous RNA polymerase prepared from rat liver. The procedures were as follows:

Preparation of purified chromatin: The chromatin was isolated by the procedure of Marushige and Bonner (15). Liver tissue was homogenised (10%) in saline-EDTA (0.075 M NaCl, 0.024 M EDTA) pH 8.0 and centrifuged at 1500 x g for 15 min. The precipitate was washed once with saline-EDTA and then three times with Tris-HCl buffer (0.05 M, pH 8.0). The

resulting pellet was suspended in 0.01 M Tris-HCl buffer pH 8.0. Ten ml aliquots of this was layered on 40 ml of 1.7 M sucrose in 0.01 M Tris-HCl buffer, pH 8.0. The upper 2/3rd portion of the gradient was mixed and then centrifuged at 90,000 x g using the 8 x 50 ml rotor in an MSE Superspeed-50 ultracentrifuge. The pellet was suspended in 0.01 M Tris-HCl buffer pH 8.0, dialysed overnight against the same buffer in cold. The chromatin was sheared in a Virtis "45" homogeniser using the microhomogenising flask and stirred for 30 min. It was then centrifuged at 10,000 x g for 30 min in a Sorvall superspeed RC2-B centrifuge. The supernatant containing solubilised chromatin was used for the assay of template activity.

Preparation of DNA: DNA was extracted from rat liver nuclei by Marmur's procedure (16). An additional step included was treatment of isolated DNA with pronase to remove RNase. Before use, pronase was self-digested for 2 hr at 37°. This preparation was added at the level of 50 µg/ml to the solution of DNA in saline-citrate (SSC) buffer and the mixture incubated with a few drops of chloroform at 37° for 18 hr. Thereafter, the DNA was reprecipitated with ethanol and this procedure was repeated three times. The combined precipitate was suspended in 1/100 SSC and extensively dialysed and used for the assay of template activity.

Preparation of RNA polymerase: RNA polymerase was prepared from rat liver nuclei by the method of Roeder and Rutter (17) to the stage of their fraction IV. The purified nuclei were suspended in TSMD

(0.01 M Tris-HCl pH 7.9; 1.0 M sucrose; 0.005 M $MgCl_2$ and 0.005 M dithiothreitol). Ammonium sulphate (4.0 M adjusted to pH 7.9 with ammonia) was added to bring the concentration to 0.3 M. The viscous solution was sonicated in 20 ml aliquots for 1 min at 17-20 Kc with an MSE model 60 W ultrasonic disintegrator. The suspension was then rapidly mixed with two volumes of TGMED (0.05 M Tris-HCl pH 7.9; 25% V/V glycerol; 5 mM $MgCl_2$; 0.1 mM EDTA and 0.05 mM dithiothreitol). The precipitate was rejected by centrifugation for 1 hr at 105,000 x g. To the clear supernatant, solid ammonium sulphate (0.42 g/ml) was added and centrifuged for 1 hr at 105,000 x g. The precipitate was collected and suspended in TGMED buffer pH 7.9 and dialysed overnight against the TGMED buffer containing 0.05 M $(NH_4)_2SO_4$. The dialysate was centrifuged for 1 hr at 160,000 x g to remove the particles if any. The supernatant containing RNA polymerase was used for assay.

The template activity of liver chromatin or DNA was determined essentially by the method of Church and McCarthy (18). The assay systems contained the following components (in a final volume of 0.5 ml): 20/umoles of Tris-HCl buffer (pH 8.0); 2/umoles of $MgCl_2$; 0.5/umole $MnCl_2$; 5.0/umoles of 2-mercaptoethanol; 0.2/umole each of CTP, UTP and GTP, 0.2/umole of ^{14}C-ATP (sp. act. 27.2 mCi/mmole), 20/ug of rat liver DNA and liver RNA polymerase preparation equivalent to 100 Mg protein · chromatin when added as a template was equivalent to 20/ug DNA. After incubation for 10 min at 37°, the reaction was stopped by the addition of 1.0 ml of 5% TCA containing 0.01 M sodium pyrophosphate at 0°. The acid-insoluble material was collected by filtration on Millipore membrane filters (0.45/u) presoaked in ATP

solution (0.01 M, pH 7.0), washed with four 5-ml portions of 2% TCA containing 0.01 M sodium pyrophosphate dried at room temperature and radioactivity was counted.

Isolation of E.coli RNA polymerase

E.coli RNA polymerase was prepared by the procedure as described by Burgess (19) up to his fraction IV.

Ribonuclease assay

The ribonuclease activity in chromatin was assayed as described by Dahmus and Bonner (20). The chromatin was incubated with labelled RNA in the standard mixture for RNA synthesis, minus the enzyme RNA polymerase and the nucleotide triphosphates, for 10 min at 37°. The reaction was stopped by adding cold TCA and the mixture was passed through the Millipore membrane filter. An aliquot of the filtrate was taken for measurement of radioactivity.

Assay of RNA polymerase binding site on the chromatin

The number of RNA polymerase binding sites (RNA chain initiation sites) on the chromatin was determined as described by Simpson (21) under low-salt reaction conditions which allow only RNA chain initiation followed by high-salt reaction conditions which allow only RNA chain elongation. The detailed experimental procedure is as follows: final volume (0.5 ml) of the reaction mixture contained : 50 μmoles Tris-HCl (pH 8.0); 1 mmole $MnCl_2$; 0.2 μmole each of ~~UTP~~ ATP, GTP and ~~^{14}C-ATP~~ ^{3}H-UTP; chromatin template equivalent to 20 μg DNA and RNA polymerase (100 μg protein) were incubated under low-salt conditions for 15 min at 37°.

At the end of this incubation period, $(NH_4)_2SO_4$ and $MgCl_2$ were added to a final concentration of 0.4 M and 5 mmoles respectively. A zero-time sample was withdrawn and the propagation reaction initiated by adding 0.2 µmole CTP. At the end of 10 min incubation at 37°, samples were precipitated with 10% TCA containing 0.01 M sodium pyrophosphate and were filtered through membrane filters (0.45 µ), washed thrice with 5 ml portions of cold 5% TCA, dried and counted for radioactivity. Frequencies of occurrence of polymerase binding sites were calculated from the transcription rates of respective templates under the conditions described above.

Measurement of radioactivity

Radioactive samples on membrane filters or Whatman No.3 filter paper strips were placed in glass vials containing 10 ml of the scintillator-0.5%, 2,5-diphenyl-oxazole (PPO) in toluene. For aqueous radioactive samples, which were placed directly in vials, 10 ml of the scintillator -0.5% 2,5-diphenyl oxazole (PPO), 10% naphthalene in dioxane-was used. The radioactivity was determined in a Beckman LS-100 liquid scintillation system. Counting efficiencies for ^{14}C and ^{3}H were respectively, 90% and 58%. Counting efficiency for ^{32}P ranged from 20-30%.

Chemical analysis

RNA was estimated by the orcinol reaction using yeast RNA as standard (11). DNA was estimated by the diphenylamine reaction using calf thymus DNA as standard (22). Protein was estimated by the method of Lowry et al. (23) using bovine serum albumin as standard.

Materials

GTP, CTP, UTP (Sigma Chemical Co., U.S.A.); ^{14}C-ATP (sp. act. 27.2 mCi/mmole, Schwartz Biochemicals, U.S.A.); ^{32}P-orthophosphate (carrier free) Generally labelled ^{3}H-ATP (40 mCi/mmole), ^{3}H-UTP (18.2 Ci/mmole) (Isotope Division of this Research Centre, Bombay) and Dowex-1-chloride (Sigma Chemical Co., U.S.A.) were used.

RESULTS

In Table 1 are shown results of the experiments on incorporation of intraperitoneally injected ^{32}P-orthophosphate into liver RNA following whole-body exposure of rats to 1000 r x-rays [rats of 100-150 g body weight succumb to this dose within 14-21 days presumably due to 'bone-marrow syndrome' (24)]. The rate of RNA synthesis in rat liver progressively increases from 4 hr following whole-body x-irradiation, the stimulation being much pronounced at 18 hr. Thereafter, the rate declines returning to normal by 36 hr.

After ascertaining that radiation significantly elevates RNA synthetic potential of the liver, studies were directed to examine whether synthesis of any specific RNA(s) was preferentially stepped up following the radiation exposure. For this purpose, liver RNA of irradiated rats and unirradiated controls was labelled by a 30 min pulse of intraperitoneally injected ^{32}P-orthophosphate, isolated and then analysed by sucrose density gradient centrifugation. The sedimentation profiles are shown in Fig.1. The RNA profiles from both unirradiated and irradiated groups appear similar. This indicates that the syntheses of all types of RNAs may have been stepped up and that irradiation may not

Table 1

Effect of whole-body x-irradiation on the labelling of liver RNA following ^{32}P-orthophosphate pulse

	Relative specific activity (*)	Per cent increase
Unirradiated	1360 ± 87	
Irradiated (1000 r)		
hr		
4	1988 ± 103	46.1
8	2165 ± 98	59.2
12	2360 ± 111	73.5
18	2606 ± 139	91.6
24	2243 ± 117	64.6
36	1450 ± 108	6.6

* $\frac{\text{cpm/mg RNA}}{\text{cpm/pmole of orthophosphate in liver homogenate}}$

Each rat received ^{32}P-orthophosphate (carrier free, 2 mCi/100 g body wt.) 1 hr prior to sacrifice. Other details are as in the text. Each value represent average of three independent experiments ± SEM.

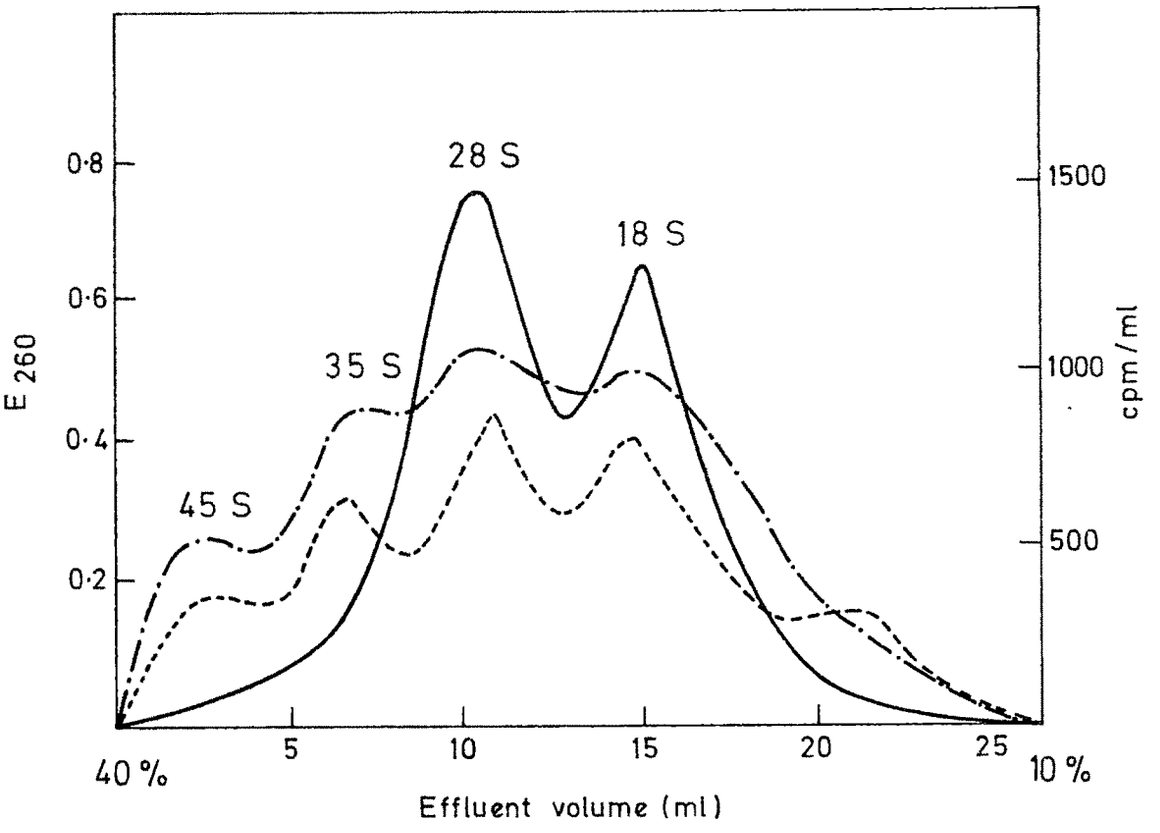

Fig. 1. Sedimentation profiles of ^{32}P-orthophosphate pulse-labelled liver RNA in rat at 18 hr after exposure to 1000 r.

Rats received ^{32}P-orthophosphate (carrier-free, 2 mCi/100 g body wt) 30 min before sacrifice. An aliquot of RNA solution in acetate buffer pH 5.1 (equivalent to 1.0 mg, E_{260} units 25) was layered on a 28 ml linear sucrose density gradient (10-40%), centrifuged at 63,000 x g for 15 hr and 1 ml fractions were collected for E_{260} and radioactivity determination.

———— optical density; - - - - - radioactivity, unirradiated; -.-.-.-.- radioactivity, irradiated.

have selective stimulatory action on the synthesis of any particular size of RNA.

It has often been found that changes in RNA synthetic patterns in eukaryotic cells are a direct reflection on the activities of cognate nuclei to polymerize RNA from nucleotide precursors in vitro (25, 26). Widnell and Tata (14) have demonstrated that in isolated rat liver nuclei, the enzyme activity can be resolved into two reactions, one is allowed in the presence of Mg^{++} ions and low ionic strength yielding a product similar to ribosomal RNA, while the other is predominant in the presence of Mn^{++} ions and high concentration of salt such as 0.4 M $(NH_4)_2SO_4$ the product of which is DNA-like RNA. The Mg^{++}-activated reaction seems to occur in the nucleolar region and is catalysed by Type I polymerase, whereas $Mn^{++}/(NH_4)_2SO_4$-activated reaction occurs in extranucleolar region being catalyzed by Type II RNA polymerase (27). Omata et al. (28) found that both Mg^{++}-activated and $Mn^{++}/(NH_4)_2SO_4$-activated RNA polymerase activities in liver nuclei undergo an increase by about 40% over the controls at 24 hr following 650 and 1000 r exposure. Hidvegi et al. (9) observed that in guinea pigs Mg^{++}-activated RNA polymerase activity is increased by 200% at 9 hr after exposure to 2000 r.

As seen in Table 2, total-body exposure to 1000 r x-irradiation causes marked elevation in both the RNA polymerase reactions. The maximum effect is discernible at 18 hr post-irradiation, being about 180-220% over unirradiated controls for Mg^{++}-activated reaction and about 36-37% over unirradiated controls for $Mn^{++}/(NH_4)_2SO_4$-activated reaction. The results corroborate very well with the data on the sedimentation analysis of ^{32}P-pulse-labelled RNA. illustrated in Fig.1 and support the contention that whole-body x-irradiation elicits stimulation in syntheses of all types of RNAs in the liver.

Table 2

Effect of whole-body x-irradiation on the two RNA polymerase reactions in isolated rat liver nuclei

	Amount of RNA synthesized	
	Mg^{++}-activated (*)	$Mn^{++}/(NH_4)_2SO_4$-activated (**)
Experiment 1		
Unirradiated	250	1080
Irradiated (1000 r)		
hr		
4	310	1155
12	415	1239
18	700	1470
36	280	1102
Experiment 2		
Unirradiated	270	1150
Irradiated (1000 r)		
hr		
4	390	1278
12	505	1372
18	865	1580
36	440	1260

* pmoles of AMP incorporated into RNA/15 min/mg DNA for Mg^{++}-activated reaction.

** pmoles of AMP incorporated into RNA/45 min/mg DNA for $Mn^{++}/(NH_4)_2SO_4$-activated reaction.

Both Mg^{++}-activated and $Mn^{++}/(NH_4)_2SO_4$-activated assay systems contained in a volume of 0.5 ml: 0.02 µmole of ^{14}C-ATP (sp. act. 27.2 mCi/mmole) and nuclei equivalent to 0.3 mg DNA. Other details are as in the text. Each value is average of two determinations on nuclei from pooled livers (two rats per group).

The higher efficiency of liver nuclei derived from irradiated rats to polymerise RNA *in vitro* could be apparent. Liver nuclei could have become more permeable to nucleoside triphosphates following whole-body radiation exposure - this could have led to increased labelling of RNA in the nuclear RNA polymerase assayseven in the absence of accelerated rate of RNA polymerisation. The possible radiation-effect on the permeability of liver nuclei was tested by following uptake of ^{3}H-UTP. The results shown in Table 3 reveal that in Mg^{++}-dependent reaction system containing actinomycin D (added to prevent RNA polymerisation), liver nuclei from irradiated rats were efficient in ^{3}H-UTP uptake (approximately 2 times) than were those from unirradiated animals. In the presence of $Mn^{++}/(NH_4)_2SO_4$ the ^{3}H-UTP uptake by liver nuclei from irradiated rats is not significantly different from liver nuclei of control animals. This is possibly due to the fact that the high $(NH_4)_2SO_4$ concentration may have made nuclei more permeable *in vitro*, thus obliterating any differences in permeability occurring *in vivo* in liver nuclei derived from the two groups of rats. That ammonium sulphate increases permeability of nuclei to ^{3}H-UTP is clearly apparent from the comparison of the results obtained in the presence of Mg^{++} with those obtained in the presence of $Mn^{++}/(NH_4)_2SO_4$. Notwithstanding their increased permeability, nuclei of irradiated rats are more efficient in RNA polymerisation as compared to those from unirradiated controls. This is clearly seen from the Table 4, when the extent of RNA polymerisation by liver nuclei is expressed as the rate of RNA labelling (Table 2) divided by the rate of RNA uptake (Table 3).

Table 3

Effect of whole-body x-irradiation on the uptake of ^{3}H-uridine triphosphate by isolated liver nuclei *in vitro*

	^{3}H-UTP taken up by nuclei from reaction mixtures containing (cpm)	
	Mg^{++}-activated	$Mn^{++}/(NH_4)_2SO_4$-activated
Unirradiated	5775 ± 107	10760 ± 375
Irradiated (1000 r)		
hr		
18	11470 ± 179	11270 ± 450
36	6210 ± 97	10900 ± 125

Nuclei (equivalent to 0.3 mg DNA) were incubated at 37° for 10 min in reaction mixtures (final volume 0.5 ml) used for assaying Mg^{++}-activated and $Mn^{++}/(NH_4)_2SO_4$-activated RNA polymerisations, except that ^{3}H-UTP (0.3 μmole; 50,000 cpm) and actinomycin D (15 μg) were included in place of ^{14}C-ATP, GTP, UTP and CTP. Other details are as described in the text.

Table 4

Effect of whole-body x-irradiation on Mg^{++}-activated and $Mn^{++}/(NH_4)_2SO_4$-activated RNA polymerisations by liver nuclei

	Relative rate of RNA polymerisation	
	Mg^{++}-activated	$Mn^{++}/(NH_4)_2SO_4$-activated
Unirradiated	4.32×10^{-2}	9.99×10^{-2}
Irradiated (1000 r)		
hr		
18	6.10×10^{-2}	13.04×10^{-2}
36	4.50×10^{-2}	10.00×10^{-2}

Ratio of the results in Table 2 (experiment I) to the results in Table 3.

While interpreting the results obtained with intact nuclei, it may be borne in mind that the assay is based on the presence of endogenous chromatin template. Hence the observed increases in the rate of RNA polymerisation could be due to higher activity of RNA polymerase or increased activity of chromatin or the activation of both these components of transcriptional machinery.

Consequently, changes in enzyme activity cannot be distinguished from alterations in the efficiency of the template in this assay. Availability of a method for isolation of liver chromatin in a form similar to that found in vivo makes it possible to assess differences that may have occurred in the template efficacy of liver chromatin in animals exposed to radiation (15). Such changes in template activity of liver chromatin have been found to be brought about following administration of hormones such as hydrocortisone (20, 29), triiodothyronine (29), estradiol (18, 30), testosterone (31, 32), and drugs like 3-methylcholanthrene (33). The transcriptive ability of liver chromatin of rats at various times following whole-body x-irradiation was determined using exogenous RNA polymerase prepared from rat liver according to the procedure of Roeder and Rutter (17) (upto fraction IV). The characteristics of the soluble enzymes are summarized in Table 5. DNA-dependence of the enzyme is clearly established. The enzyme requires all the four ribonucleoside triphosphates for the full activity. It will also be seen from Table 5 that isolated chromatin supports RNA synthesis by added RNA polymerase, though at a much reduced rate compared to rat liver DNA. Results shown in Table 6 reveal that E.coli polymerase prepared by the method of Burgess (19)

Table 5

Assay of rat liver RNA polymerase

	pmoles AMP incorporated into RNA/10 min
Complete system	150
DNA omitted	18
Enzyme omitted	7
UTP, GTP and CTP omitted	12
DNA omitted and chromatin added	49

The complete system contained 0.2 μmole of ^{14}C-ATP (sp. act. 27.2 mCi/mmole) and 20 μg rat liver DNA in 0.5 ml final volume. Other components of the system and conditions of assay are as described in the text. Chromatin when added was equivalent to 20 μg DNA. Each value is an average of three independent determinations.

Table 6

Template activity of rat liver chromatin

by homologous and E.coli RNA polymerases

	AMP incorporated into RNA pmoles/10 min
E.coli RNA polymerase	46.9
Homologous RNA polymerase	33.2

The complete system contained 0.2/umole of ^{14}C-ATP (sp. act. 27.2 mCi/mmole), chromatin equivalent to 20/ug of DNA and RNA polymerase preparations equivalent to 100/ug enzyme protein in 0.5 ml final volume. Other details are as described in the text.

to the purification stage comparable to liver RNA polymerase preparation used here was more efficient to support RNA synthesis on rat liver chromatin than was rat liver RNA polymerase preparation. This seems to be due to the recognition of more number of RNA chain initiation sites by the bacterial enzymes[21]. Since this non-specific recognition was liable to lead to erroneous conclusion, it was decided to use homologous RNA polymerase throughout the studies described in the thesis.

Results on the rate of RNA synthesis as a function of template concentration are presented in Fig.2. At optimal concentrations, the template activity of chromatin is about 4.5 times less than that of rat liver DNA. It may also be noted that the template concentration for maximum reaction rate is nearly the same irrespective of whether the DNA is present as such or in the form of chromatin. Marushige and Bonner (15) have also found that although Vmax for the rate of RNA synthesis by E.coli RNA polymerase on rat liver DNA is approximately five times as higher as that for RNA synthesis on rat liver chromatin, the concentrations of template required to attain one-half Vmax are the same for the two systems. On the basis of these observations one can propose that the polymerase binds equally to DNA and to chromatin, but that in the latter case, transcription is reduced due to components such as histones complexing with DNA.

The results of Fig.2 also show that the template efficiency of chromatin obtained from rats at 18 hr post-irradiation is about 1.5 times higher than that from unirradiated rats.

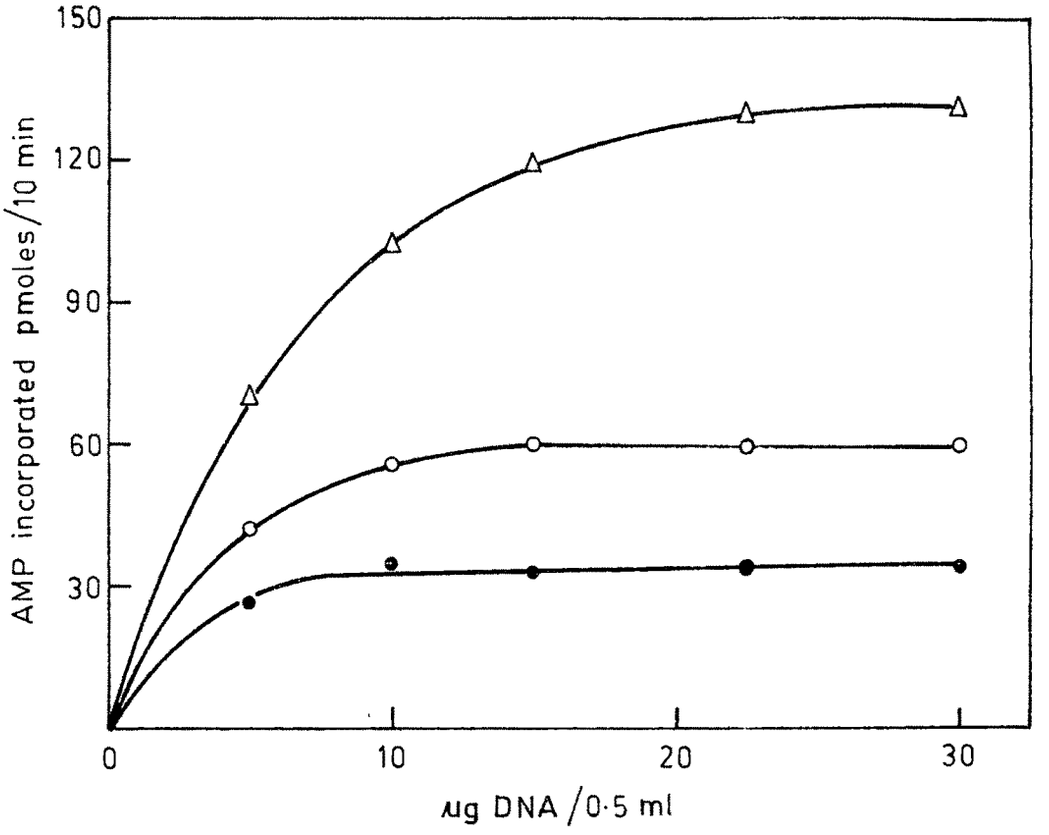

Fig.2. Rate of RNA synthesis as a function of template concentrations.

Reaction mixtures (0.5 ml) contained various amounts of DNA either as such or in the form of chromatin, liver RNA polymerase preparation equivalent to 100/ug protein and 0.2/umole of (^{3}H) ATP (40 mCi/mmole). RNA synthesis by enzyme alone (9.8 to 10.6 pmoles AMP incorporated) has been subtracted. Each point is an average of two determinations.

Δ——Δ , liver DNA from unirradiated rat;

●——● , liver chromatin from unirradiated rat;

○——○ , liver chromatin from irradiated rat (1000 r, 18 hr).

It is seen from Table 7 that RNase activity of liver chromatin, as determined by using ^{32}P-labelled rat liver RNA, is very little and hence could not have interfered with the template assay of chromatin (i.e. by degradation of RNA synthesised in the assay procedure). Further, there was no significant difference between chromatins from irradiated (18 hr) and unirradiated rats with respect to this enzyme activity. This would preclude the possibility that the observed changes in the rate of RNA synthesis in the assay of template activity are due to interference by contaminating RNase.

In Table 8 are shown data on template activity of rat liver DNA and chromatin at various times after whole-body exposure to 1000 r. At 4 hr post-irradiation, the template efficiency of chromatin is only slightly increased. It rises to about 24-25% higher than control at 12 hr post-irradiation and as high as 51-63% above control at 18 hr post-irradiation. The template activity of DNA at these time intervals is unchanged.

The fluctuations in template activities of chromatin could be related to change either in the number of RNA polymerase binding sites or in the rate of progression of RNA polymerase molecules along the template. The number of available initiation sites on chromatin were assayed by the method of Simpson (21). In this method, RNA chains are initiated on chromatin in the presence of exogenous RNA polymerase under the conditions that prevent elongation of RNA chains (low-salt minus CTP) and then only initiated chains are allowed to grow under the conditions that prevent initiation (high-salt plus all the four nucleoside triphosphates).

Table 7

RNase activity associated with purified liver chromatin isolated from normal and irradiated rats

	RNase activity (*)
Unirradiated	0.054 ± 0.003
Irradiated (1000 r)	
hr	
8	0.052 ± 0.002
18	0.058 ± 0.004

Reaction mixture contained standard reaction components minus RNA polymerase and nucleoside triphosphate. 50 µg of ^{32}P-RNA and chromatin corresponding to 30 µg of DNA per 0.5 ml reaction mixture were used.

* RNase activity is expressed as the fraction of initial radioactivity rendered acid-soluble after incubation at 37° for 10 min. The results are average of four independent experiments ± SEM.

Table 8

Effect of whole-body x-irradiation on template activity of purified liver chromatin and DNA

	pmoles of AMP incorporated into RNA	
	DNA	Chromatin
Experiment 1		
Unirradiated	133.0	35.0
Irradiated (1000 r)		
hr		
4	135.8	38.6
12	134.9	43.5
18	137.0	57.0
Experiment 2		
Unirradiated	146.8	44.2
Irradiated (1000 r)		
hr		
4	147.2	47.2
12	146.1	55.4
18	145.0	66.8

The assay system for template activity contained 0.2 μ mole of ^{14}C-ATP (sp. act. 27.2 mCi/mmole) and 20 μg of DNA either in the form of chromatin or purified as such in 0.5 ml final volume. Other details are as given in the text. Each value is an average of two independent determinations.

The results given in Table 9 clearly show that chromatin from irradiated rats (18 hr) has much greater number of RNA polymerase binding sites than those on the liver chromatin from unirradiated controls. The extent of increase is around 49% which implies that the amplification in template activity of chromatin is mainly due to increased number of RNA-polymerase binding sites.

It is possible that whole-body x-irradiation can also cause activation of RNA polymerase or increase in the level of enzyme protein. Data shown in Table 10 indicate that specific activity (as expressed by units/mg protein) of RNA polymerase preparation from liver nuclear suspension and also the level (as expressed/mg DNA of nuclear suspension) of this enzyme in liver at 18 hr post-irradiation are about equal to those in unirradiated rats. It may however be pointed out that the RNA polymerase solubilised from nuclear suspension was to the extent of 78%. Further the isolated enzyme is only partially pure and hence the possibility that irradiation, besides its action on template of chromatin, could also raise the level of RNA polymerase to some extent cannot entirely be ruled out.

Three RNA polymerases have been identified so far in rat liver nuclei. As mentioned before Type I RNA polymerase is believed to catalyse synthesis of ribosomal RNA and Type II that of Hn RNA. The third enzyme, Type III enzyme - which is present in much smaller amounts - may be involved in the synthesis of 5 S and tRNAs (27). Tata and coworkers (34) have devised an assay system to distinguish between Type I (which may also include Type III) and Type II polymerase activities on the basis of the fact that the fungal toxin, α -amanitin, inhibits specifically

Table 9

Number of initiation sites available on rat liver chromatin : effect of whole-body x-irradiation

Source of chromatin	Available polymerase binding sites per 10^6 nucleotide pairs
Unirradiated	11.3
Irradiated (1000 r) hr 18	16.8

The number of polymerase binding sites were determined as described in the text, under the low-salt reaction conditions that allow only RNA chain initiation followed by high-salt reaction conditions which allow only RNA chain elongation in the presence of ^{3}H-UTP to label growing RNA chains. The calculations were based on the results which indicate that rat liver chromatin contains one site per 88,500 nucleotide pairs i.e. 11.3 sites per 10^6 nucleotide pairs.

Table 10

Effect of whole-body x-irradiation on liver RNA polymerase activity

	Total activity (units/mg nuclear DNA)	Specific activity (units/mg protein)
Unirradiated	1746	1520
Irradiated (1000 r)		
hr		
18	1735	1572

RNA polymerase was isolated from nuclear suspension of pooled livers (two rats per group) up to fraction IV of the method as described in the text.

The assay mixture contained (in 0.5 ml): 20/ug rat liver DNA, liver RNA polymerase preparation equivalent to 100/ug protein, 0.2/umole of ^{3}H-ATP (40 mCi/mmole). Other constituents are as described in the text. One unit of activity is defined as that amount which catalyses the incorporation of 1 pmole of AMP per 10 min. Each value is an average of 3 independent experiments.

Type II polymerase. This procedure was used to see if whole-body irradiation affected the relative proportions of Type I and Type II polymerases. From the results given in Table 11, it will be seen that both Type I and Type II activities remain unaltered in the liver of irradiated rats.

DISCUSSION

The results presented here indicate a striking effect of whole-body radiation exposure on the RNA synthetic rate in the rat liver. The changes correspond well with those in the ability of isolated liver nuclei to polymerise RNA *in vitro*. The observation is in consonance with several reports (14) indicating an interlink between the rate of RNA synthesis in eukaryotic cells and *in vitro* RNA polymerising ability of their nuclei.

As already discussed in 'Results', the RNA polymerisation by nuclei is the resultant effect of both activities of endogenous RNA polymerases and of endogenous chromatin template. Interestingly, neither activities of Type I nor Type II RNA polymerases isolated free of endogenous template are significantly altered in response to irradiation. Only the template activity of chromatin seems to be considerably enhanced after the radiation stress. It appears from this finding and many others reported in the literature that there be a variety of ways by which transcription machinery can be activated. For example, administration of growth hormone (35) results in enhancement in RNA polymerase activity but not in template activity of chromatin. Studies described in Part III indicate that in the case of liver regeneration,

Table 11

Activity of RNA polymerase (in presence and absence of α-amanitin) in the rat liver following total-body x-irradiation

	pmoles of AMP incorporated into RNA/10 min	
	without α-amanitin	with α-amanitin
Unirradiated	1460 ± 29.3	845 ± 30.10
Irradiated (1000 r)		
hr		
4	1385 ± 36.2	832 ± 35.9
8	1415 ± 26.9	860 ± 28.0
18	1492 ± 28.3	880 ± 31.9

The assay mixture contained (0.5 ml) 0.2/umole of ^{14}C-ATP (sp.act. 27.2 mCi/mmole), 20/ug rat liver DNA with or without 2/ug of α-amanitin, and 100 Mg of RNA polymerase enzyme protein. Other details are as described in the text. Each value represents average of three independent experiments ± SEM.

the activation in transcription is related both to increase in RNA polymerase activity and amplification in template activity of chromatin. Estradiol likewise causes in the uterus stimulation in template activity of chromatin and in RNA polymerase activity (36).

Whole-body x-irradiation therefore provides a useful system to study regulation of template activity of chromatin. The template activity of chromatin (as assayed in vitro using exogenous RNA polymerase) is mainly governed by two factors: (i) number of promoter regions on chromatin available for binding to RNA polymerase and (ii) rate of progression of RNA-polymerase molecules along the template. The results show that chromatin derived from irradiated rats has much increased number of available initiation sites as compared to that from unirradiated rats. This poses the question: whether the new binding sites created on chromatin after irradiation reflect de-repression of some normally repressed cistrons and in turn, induced synthesis of new RNA species. The profiles of ^{32}P-orthophosphate pulse-labelled RNAs, seem to be identical and do not therefore suggest any qualitative differences in RNAs synthesised. No doubt, for a better qualitative assessment of RNA synthetic patterns, the technique such as determination of nucleotide sequence homologies by hybridisation with DNA will have to be resorted to.

It would however be difficult to envisage a substantial de-repression of cistrons in an animal tissue which may be reflected in induction of synthesis of a great variety of new proteins. The plausible alternative interpretation for the observed phenomenon are: (i) Many of the meaningful cistrons may have more than one copy in the genome and

not all of these are in active form in normal conditions. A stimulus such as radiation stress could lead to de-repression of these quiescent cistrons; (ii) More cistrons may be de-repressed in response to radiation stress, but their RNAs may not be translated. Some of these RNAs may be confined to the nucleus and have regulatory roles such as postulated in the model of transcription proposed by Britten and Davidson (37); (iii) It is possible that there are more than one RNA polymerase binding sites in a promoter region of a eukaryotic cistrons, not all of them may be active normally. Activation of chromatin could then mean capability of promoter regions to bind increased number of RNA polymerase molecules; or (iiia) If, as postulated by Szybalski (38) for certain cistrons in λ phage, there is a drift region between promoter site and RNA chain initiation site, under the conditions of RNA chain initiation assay described here, more number of RNA polymerase molecules may be bound to initiating region (promoter site + drift region). Activation of chromatin could then mean capability of initiating regions to accept increased number of RNA polymerase molecules at a time.

It may be mentioned here that the *in vitro* assay employed here for determination of the number of available RNA chain initiation sites involves the use of saturating levels of exogenous RNA polymerase. Such a situation may not be present *in vivo*. Nevertheless the assay gives a fairly good measure of the extent to which the number of RNA polymerase binding sites may have been increased.

Finally, an interesting finding emanating from this study may be discussed. A significant change is discerned in permeability of liver nuclei towards nucleoside triphosphate precursors as a result of

irradiation. It may be asked ~~for~~ whether the radiation-induced changes in permeability of nuclei to nucleoside triphosphates are linked to radiation-induced stimulation of RNA polymerisation or whether the two radiation effects are independent phenomena. It is tempting to suggest that the two phenomena may be interrelated. For, both the rate of RNA polymerisation by nuclei and the uptake of ribonucleoside triphosphates by nuclei in vitro are elevated and declined in parallel fashion during the 36 hr-period subsequent to whole-body irradiation to 1000 r.

The results quite clearly indicate that the transcriptional changes elicited by whole-body radiation exposure stem from modulations at the level of non-DNA components of liver chromatin since the template activity of liver DNA remains unaffected. The involvement of non-DNA components of chromatin in radiation-induced amplification in chromatin function is the subject-matter of the next section. Studies on the mechanism by which irradiation brings about activation at the level of transcription are described in Section 3 of this part.

SUMMARY

Exposure of rats to 1000 r x-irradiation leads to enhancement in synthesis of RNA in the liver during 4 to 24 hr following irradiation. At these post-irradiation times, both Mg^{++}-activated and $Mn^{++}/(NH_4)_2SO_4$-activated reactions by isolated liver nuclei are accelerated. Template activity of isolated liver chromatin is considerably amplified during this post-irradiation period (53 to 61% increase over control at 18 hr post-irradiation). Similar striking

increase is also discernible in the number of RNA chain initiation sites on chromatin (49% increase over control at 18 hr post-irradiation). Activity in the liver RNA polymerase (as examined in the enzyme preparation isolated free of DNA) however remains unaltered in the whole-body irradiated rat. The results strongly suggest that the known increase in liver protein synthesis in mammals in response to whole-body x-irradiation may mainly be the consequence of the stimulus at transcription level, specifically radiation-caused elevation in chromatin template efficiency.

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Part II

Section 2

Radiation-induced changes in structure and turnovers of chromatin constituents in the rat liver

INTRODUCTION

Studies reported in the literature indicate an intimate involvement of non-DNA components of chromatin in the regulation of transcription function (1-10). Thus chemical modifications in histones such as acetylation (11), phosphorylation (12), methylation (13), and thiolation (14) have been found to be associated with gene activation in organs of animals subjected to various kinds of stimuli or stresses (14,15). Turnovers or chemical modifications of different nonhistone chromosomal proteins have also been implicated in gene activation (16,17).

In the previous section, evidence has been presented to suggest that whole-body x-irradiation considerably activates transcription machinery in rat liver and that this effect is due to amplification of template activity of chromatin. The radiation-provoked changes seem to be elicited at the level of non-DNA components of the chromatin since the template activity of liver DNA was not altered in response to total-body radiation exposure. The radiation-provoked amplification in chromatin template activity hence provides a useful system to elucidate regulatory mechanisms of transcription function of chromatin especially to test the postulated roles of non-DNA components in these mechanisms.

The studies described in this section have attempted to examine changes in the metabolism of non-DNA components of liver chromatin which may occur in conjunction with radiation-provoked amplification of liver chromatin template activity.

MATERIALS AND METHODS

Animal experiments

As described in Section I.

Isolation of liver chromatin

As described in Section I.

Absorption spectra of liver chromatin

Chromatins isolated from the livers of unirradiated and irradiated rats were solubilised in 0.01 M Tris-HCl buffer pH 8.0 at the concentrations of 23/ug DNA/ml and the absorption spectra were determined in a Perkin-Elmer 124 double beam ratio spectrophotometer.

Thermal melting profiles of liver chromatin

The procedure of Mandel and Marmur was employed for the measurement. Liver chromatins derived from unirradiated and irradiated animals were solubilised in dilute saline-citrate buffer (0.015 M NaCl and 0.0015 M sodium citrate, pH 6.0) at the concentrations adjusted to 0.2 O.D. at 260 nm at 25°. The solutions of chromatins were taken in quartz cuvettes and nitrogen was bubbled through them gently to expel dissolved oxygen. The cuvettes were finally stoppered and kept in the cuvette chamber of Shimadzu spectrophotometer, the chamber being heated by passing hot glycerol through the thermospacers. For measuring temperature of the solutions, copper-constantine thermocouple whose one end was heated in the buffer along with the samples and other end being kept in ice was used. The temperature was recorded

in a potentiometric recorder. Heating was done at the rate of one degree rise per 2 min. While heating was done, the O.Ds. of the samples were taken in every two minutes till no rise in O.D. was observable. The relative absorbance was calculated and plotted against temperature to obtain melting profiles. Tm, the temperature at which half the maximum rise in O.D. was observed, was calculated from the thermal melting profiles.

Measurement of incorporation of ^{32}P-orthophosphate into histone and nonhistone protein fractions

^{32}P-Sodium orthophosphate was injected intraperitoneally (carrier free, 2 mCi/100 g body wt) to rats 1 hr before killing. Purified nuclei were obtained from the livers as described in Section 1. The nuclei were washed with saline-citrate solution four times. The washed nuclei were treated with 0.25 N HCl for 60 min to separate histones and nonhistone proteins. To the extract (supernatant), 6 volumes of cold acetone were added and the mixture was kept overnight to precipitate total histones. The precipitated histones were then dialysed and lyophilised.

The nonhistone proteins were extracted from acid washed pellet with 0.1 M Tris-HCl buffer pH 8.4 containing 0.01 M EDTA and 0.14 M 2-mercaptoethanol and ^{32}P-radioactivity incorporated into these protein fraction was counted in a liquid scintillation system.

The H_1 histone fraction was extracted from the liver nuclei by the method 1 of Johns (19). Washed nuclei were extracted with 5% (W/V) perchloric acid ($HClO_4$) to selectively isolate H_1. To the $HClO_4$ extract trichloroacetic acid was added (final concentration 20%) to precipitate the H_1 histone fraction, which was dissolved in water and reprecipitated

with TCA (20%, W/V). The precipitate was again solubilized in water, dialyzed against water and lyophilised. The lyophilised total histones and H_1 fractions were re-dissolved in water to appropriate concentrations for the determination of ^{32}P-radioactivity. ~~in them.~~

Measurement of incorporation of labelled leucine into liver chromosomal nonhistone proteins

Irradiated and unirradiated rats (150-160 g) were injected with 600/uCi of ^{3}H-DL-leucine and 83/uCi of ^{14}C-DL-leucine respectively and sacrificed 1 hr later. The livers from both the irradiated and control rats were immediately removed and homogenised together in 0.025 M citric acid. Nonhistone proteins were isolated from the mixed homogenate according to the procedure described by Shelton and Allfrey (20) as outlined below. The purified nuclei were extracted with 0.15 M NaCl, to remove all soluble proteins. The extracted chromatin pellet was re-extracted with 0.2 N HCl to remove histones. The remaining pellet (acid-washed chromatin) was dispersed in 0.1 M Tris-HCl buffer pH 8.4 containing 0.01 M EDTA and 0.14 M 2-mercapto-ethanol (TEM). The suspension was mixed with an equal volume of cold phenol saturated with TEM buffer and centrifuged at 10,000 x g for 10 min. The aqueous phase was collected, re-extracted with an equal volume of phenol and centrifuged as before. The phenol layers were pooled and dialysed overnight against 100 volumes of 0.1 M acetic acid containing 0.14 M 2-mercaptoethanol and then overnight against 0.05 M acetic acid containing 9.0 M urea and 0.14 M 2-mercaptoethanol. The dialysis was continued further for few hours against 0.1 M Tris-HCl pH 8.4 containing 8.6 M urea, 0.01 M EDTA and 0.14 M 2-mercaptoethanol. This dialysis

procedure brought the phenol-soluble nonhistone proteins back into aqueous solution which made them amenable for electrophoretic analysis. The proteins thus obtained were resolved by electrophoresis in polyacrylamide gels. The procedure used was based on the separation of protein-sodium dodecyl sulphate (SDS) complexes (20, 21) under conditions in which the distance of migration of individual proteins are correlatable with their molecular weights (22-24). The aqueous solution of proteins was further dialysed overnight against 0.01 M sodium phosphate buffer pH 7.2, containing 0.1% SDS and 0.14 M 2-mercaptoethanol and later against 0.01 M sodium phosphate buffer pH 7.2 containing 1% SDS and 0.14 M 2-mercaptoethanol for 24 hr. The nonhistone chromosomal proteins were lyophilised and then used after dissolving in the same buffer to a suitable concentration.

The polyacrylamide gels were composed of 10% acrylamide 0.2% N, N'-methylene bis acrylamide, dissolved in 0.1 M sodium phosphate buffer pH 7.2 containing 0.1% SDS. Polymerization was carried out by the addition of 0.3 ml of 10% ammonium persulphate (W/V) and 0.03 ml of N,N,N',N'-tetramethylene diamine to 40.0 ml of acrylamide solution. After polymerization, the gels were pre-electrophoresed at 4.5 volts per cm for 30 min with 0.1 M sodium phosphate buffer pH 7.2, 0.1% SDS on both upper and lower electrode compartments. An aliquot of the solution of nonhistone proteins (0.1 ml containing 300 µg proteins) ~~solution~~ containing tracking dye (bromophenol blue) and 10% (W/V) sucrose was then applied to the gels. Electrophoresis was carried out at 4.5 volts per cm for 7 hr. The gels were removed from the tubes and dipped for ½ hr in 20% sulphosalicylic acid to fix the separated protein bands which were then localised by staining

with Coomassie Brilliant Blue. For radioactivity determination of fractionated proteins, unstained replicate gels were used. The gels were sliced transversely into discs of 2 mm thickness. These were dissolved in 0.6 ml of H_2O_2 at 60° and radioactivity in each slice was determined in a liquid scintillation system.

In one set of experiments, administration of radioactive amino acids was reversed - the unirradiated controls rats received ^{3}H-leucine and irradiated ones ^{14}C-leucine. The pooled livers from the animals were processed as above for the analysis of the labelling patterns of nonhistone chromosomal proteins.

Measurement of incorporation of labelled lysine into chromosomal nonhistone proteins.

Irradiated and unirradiated rats (150-160 g) were injected with 400/uCi of ^{3}H-L-lysine and 50/uCi of ^{14}C-L-lysine respectively and sacrificed 1 hr later. Nonhistone chromosomal proteins from the mixed liver homogenates were isolated, separated and their labelling patterns analysed by the procedures as detailed above.

Determination of molecular weights of electrophoretically separated proteins

This was done by the method of Shapiro *et al.* (22) using proteins of known molecular weights, viz., bovine serum albumin, alcohol dehydrogenase, myoglobin and cytochrome C.

Amino acid analysis of nonhistone chromosomal proteins

For amino acid analysis, the chromosomal nonhistone protein fraction was dissolved in 6.0 N HCl in a pyrex glass tube. The tube was sealed under vacuum and was kept for hydrolysis in an oven at

110° for 22 hr. The contents of the tube were then diluted with a mixture of citrate buffer of pH 5.28 and pH 2.2 in the proportion of 4:1. The amino acid analysis was then carried out by a Beckman Unichrome amino acid analyser. Amino acid composition of the fraction shown in Table 1 is similar to those of nonhistone chromosomal proteins reported by others (20, 25).

In vitro phosphorylation of liver chromatin

The capacity of liver chromatin to phosphorylate its own proteins by the transfer of phosphate group in the gamma position of ATP was assayed with little modifications by the procedure of Kamiyama et al. (26). In brief, the procedure is as follows: The complete system, in 0.5 ml final volume, contained: 50 µmoles of Tris-HCl buffer pH 7.5, 2.5 µmoles of ATP-γ-^{32}P (sp. act. 1 x 10^6 cpm/µmole) 12.5 µmoles of $MgCl_2$ and chromatin equivalent to 100 µg of DNA. It was incubated at 37° for 15 min and stopped by the addition of 10% TCA. The chromatin was collected on a Millipore filter paper and washed thrice with 0.02 M Tris-HCl pH 7.5 to remove the free ATP-γ-^{32}P and the ^{32}P-radioactivity retained on the filter representing phosphorylated chromosomal proteins was counted in a liquid scintillation system.

Assay of phosphokinase activity associated with nonhistone chromosomal proteins

The enzymatic activity catalysing transfer of gamma-phosphate group of ATP to proteins was assayed according to the procedure described by Kamiyama et al. (26). The reaction mixture (0.25 ml) contained 20 µmoles of Tris-HCl pH 7.5, 2.5 µmoles of ATP-γ-^{32}P,

Table 1

Amino acid composition of phenol-soluble nonhistone chromosomal proteins from rat liver chromatin

Amino acids	moles/100 moles total amino acids
Aspartic acid	10.22
Threonine	3.72
Serine	5.27
Glutamic acid	13.16
Proline	5.99
Glycine	9.72
Alanine	7.86
Valine	6.24
Methionine	1.62
Isoleucine	4.11
Leucine	7.89
Tyrosine	3.15
Phenylalanine	4.14
Lysine	6.21
Histidine	2.3
Arginine	5.8

Results are average values for three preparations of the phenol-soluble nonhistone proteins of rat liver chromatin.

5 μmoles of $MgCl_2$, 25 μmoles of NaCl and 40 μg of nonhistone chromosomal protein preparation. The incubation was carried out at 37° for 10 min and was stopped by the addition of 10% TCA. The acid-insoluble material was collected on a Millipore filter paper and washed thrice with 2% TCA and ^{32}P-radioactivity due to phosphorylated proteins retained on the filter was counted in a liquid scintillation system.

Preparation of ATP-γ-^{32}P

Adenosine triphosphate labelled with ^{32}P in the phosphate group at the gamma position was prepared essentially according to the method of Glynn and Chappell (27) in the enzymatic reaction of ATP, 3-phosphoglycerate, muscle glyceraldehyde 3-phosphate dehydrogenase, yeast phosphoglycerate kinase and neutralised solution of ^{32}P-orthophosphate. After incubation at 26° for 1 hr, the reaction mixture was treated with alcohol, concentrated and passed through Dowex-1-formate (10% cross linked) column for the recovery and purification of ATP-γ-^{32}P. The radioactivity in β-phosphate group of the labelled ATP, as determined by the method of Berenblum and Chaim (28), was found to be negligible.

Measurement of acetylation and methylation of nonhistone chromosomal proteins in liver

Rates of acetylation and methylation of nonhistone chromosomal proteins in the liver were studied by injecting intraperitoneally to rats ^{14}C-sodium acetate (80 μCi/100 g body wt) and ^{14}C-methionine (80 μCi/100 g body wt) respectively. The rats were sacrificed 1 hr later, livers were removed, nonhistone chromosomal proteins were isolated as described above and ^{14}C-radioactivity incorporated into the proteins was counted in a liquid scintillation counting system.

Chemical analysis

Histones and nonhistone chromosomal proteins were separated from chromatin with 0.25 N HCl at 4° and centrifuged. The supernatant liquid containing histones was carefully removed and neutralized and analyzed for protein content. The pellet containing nonhistone chromosomal proteins was dissolved in 1 N NaOH in a boiling water bath and analysed for protein content. DNA, RNA and proteins were estimated as described in Section I.

Measurement of radioactivity

As described in Section I.

Materials

^{32}P-orthophosphate (carrier free), ^{3}H-DL-leucine (sp. act. 5 Ci/mmole), ^{14}C-DL-leucine (sp. act. 0.25 mCi/mmole), ^{3}H-L-lysine (sp. act. 1.25 Ci/mmole), ^{14}C-L-lysine (sp. act. 30 mCi/mmole)(Isotope Division, this Research Centre), ATP (Sigma), 3-phosphoglycerate, muscle glyceraldehyde 3-phosphate dehydrogenase, yeast phosphoglycerate kinase (Boehringer and Sohne GMBH), ^{14}C-sodium acetate (sp. act. 23.35 mCi/mmole), ^{14}C-methionine (sp. act. 13.4 mCi/mmole) (Isotope Division, this Research Centre) were used.

RESULTS

Chemical and structural characteristics of liver chromatin from irradiated rats

In the initial experiments, effect of whole-body radiation exposure on the composition of liver chromatin was studied. The

results are presented in Table 2. The levels of histones and nonhistone chromosomal proteins (in relation to DNA) did not show any detectable alterations following whole-body x-irradiation (18 hr after 1000 r). The RNA content likewise remained unaltered in response to total-body radiation exposure.

No significant structural differences could be discernible between liver chromatins from unirradiated and irradiated rats in respect of their absorption spectra (Fig.1) and thermal melting profiles (Fig.2). It is quite conceivable that the methods used in the experiments described above are not sensitive enough to detect any structural and compositional changes that may have been elicited by radiation exposure.

Radiation-elicited changes in metabolism of chromosomal proteins

Further studies were therefore directed to ascertain whether radiation exposure could evoke any metabolic changes in chromosomal constituents. Unlike chromatin, template activity of DNA from livers of irradiated ratsdoes not exhibit any change and hence it may be expected that radiation stress rather than bringing about changes in DNA structure may provoke some changes in metabolism or structure of non-DNA chromatin constituents.

Phosphorylation of liver histones following total-body irradiation: Histones have been thought to act as repressors of transcription functions of DNA (1,2,5,29-31). A plausible mechanism for radiation-induced gene activation could hence be minimisation of this repressive effect through modifications either in histone turnover or in their structure. Preliminary studies indicated (results not shown) that whole-body radiation exposure

Table 2

Macromolecular composition of rat liver chromatin

Component	Relative proportions	
	Unirradiated	Irradiated*
DNA	1.00	1.00
Histone	0.72	0.76
Nonhistone protein	0.54	0.55
RNA	0.035	0.038

* 18 hr after 1000 r.

Values given are averages of four independent experiments.

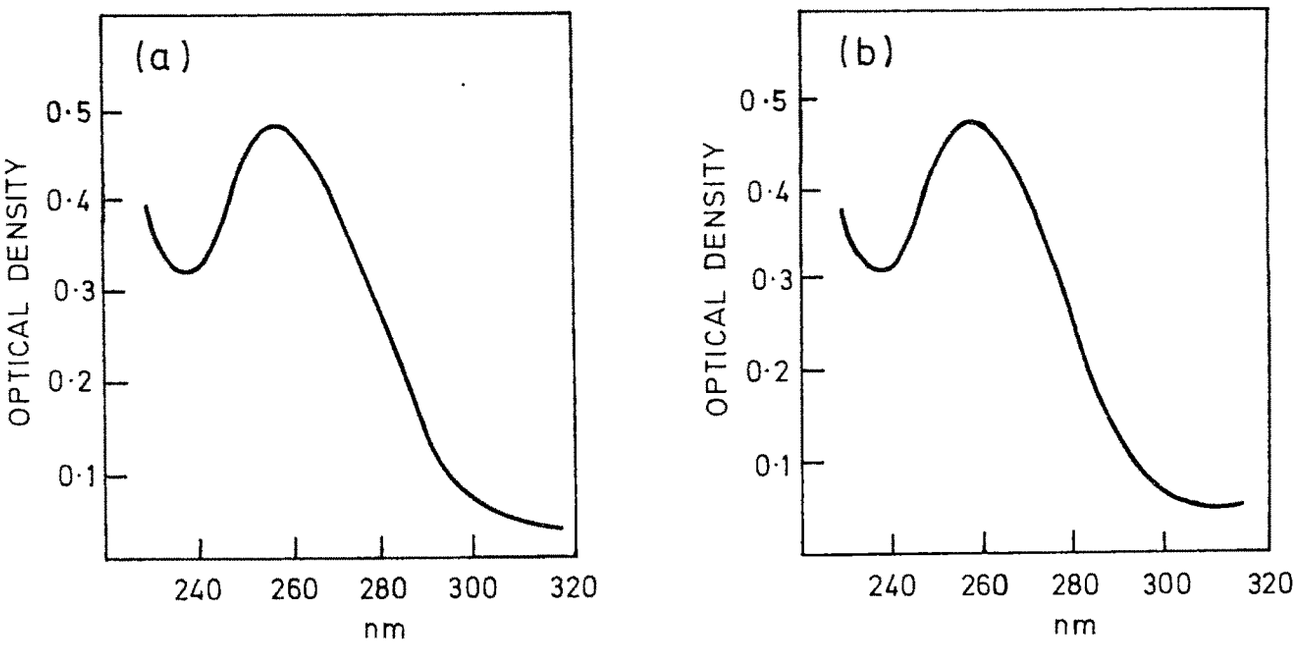

Fig. 1. Absorption spectra of liver chromatin from unirradiated (a); and irradiated (b); rats.

Determinations were made in 0.01 M Tris-HCl buffer, pH 8.0 at the concentration of chromatin equivalent to 23 µg of DNA per ml.

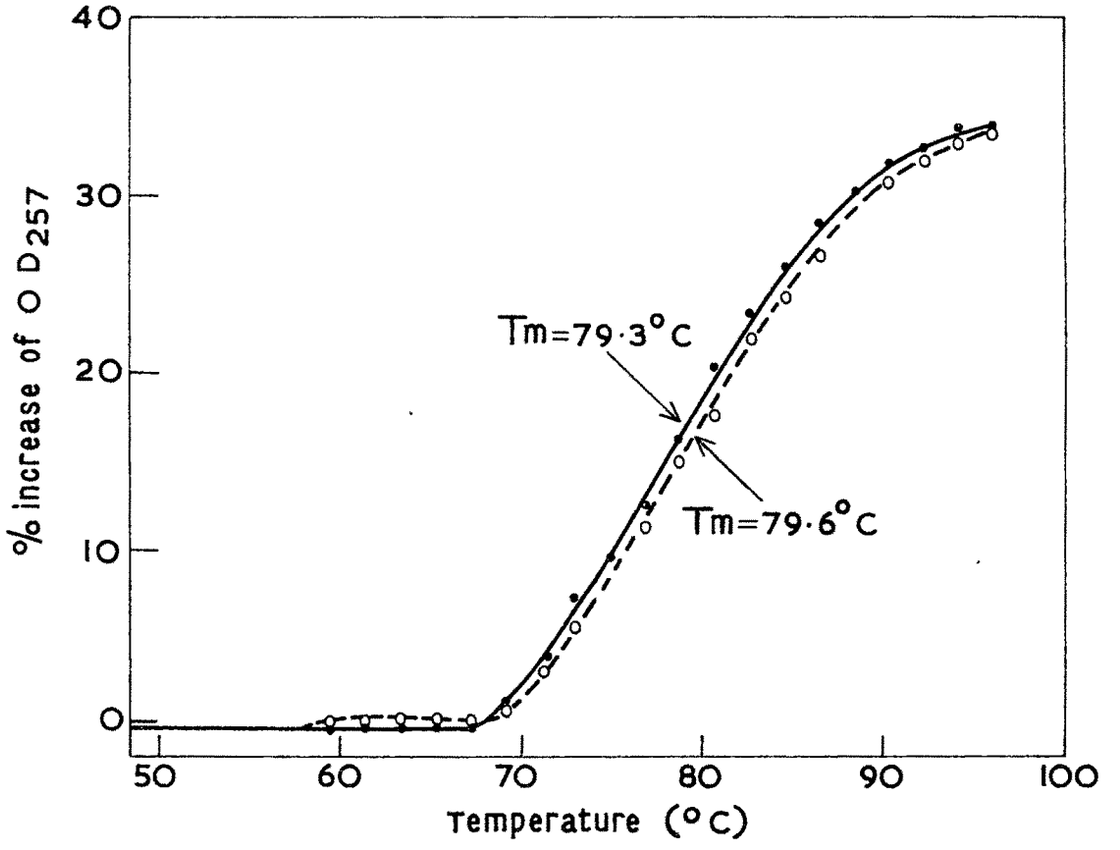

Fig.2. Melting profiles of liver chromatins from unirradiated and irradiated rats (16 hr, 1000 r). Determinations were made in dilute saline-citrate buffer (0.015 M NaCl and 0.0015 M sodium citrate, pH 6.0). Concentration of chromatin was adjusted to 0.2 O.D.257 at 25°C. •————•, liver chromatin from unirradiated rat; o- - - - -o, liver chromatin from irradiated rat.

of rats had no effect on the synthesis or degradation of liver histones. Murthy et al. (32) arrived at similar conclusions in respect of hydrocortisone-induced amplification of template activity of rat liver chromatin. These workers however demonstrated a significant stimulatory effect by the hormone on phosphorylation of histones (predominantly at serine and threonine residues). Experiments were therefore carried out to see whether whole-body radiation exposure has any influence on histone phosphorylation. As seen in Table 3, irradiation caused a marked increase in phosphorylation of both total histones and H_1 histone fraction.

Radiation-induced changes in turnovers of nonhistone chromosomal proteins: Selective turnovers of nonhistone chromosomal proteins are increasingly implicated in regulation of transcription in recent years (20,33,34). Hence studies were directed to examine whether these changes also occur in radiation-induced activation of transcription in the liver.

In the initial experiments, it was found that unlike labelling of histones (^{14}C-leucine) that of nonhistone chromosomal proteins is stimulated to some extent. At 18 hr post-irradiation, the increase is about 40-50% above control.

To determine whether any specific alterations in synthesis of chromosomal nonhistone chromosomal proteins occur in response to radiation exposure, a procedure similar to that described by Shelton and Allfrey (20) was adopted (see Materials and Methods). At various times following whole-body x-irradiation, rats were injected with ^{3}H-leucine and sacrificed 1 hr later. The livers from irradiated rats

Table 3

In vivo phosphorylation of histones in rat liver following whole-body x-irradiation

	Relative specific activity (*)	
	Total histones	H_1 histones
Unirradiated	2670 ± 88	1784 ± 47
Irradiated (1000 r) (hr)		
8	2895 ± 92	1946 ± 62
18	3320 ± 79	2252 ± 56

* $\frac{\text{cpm/mg histone}}{\text{cpm/pmole of orthophosphate in liver homogenate}}$

Rats were injected with ^{32}P-orthophosphate (2 mCi/100 g body wt.) 1 hr prior to killing. Other details are as described in the text. Each value represents average of four independent experiments ± SEM.

were pooled with those from unirradiated control animals which had been injected with ^{14}C-leucine 1 hr prior to sacrifice. Chromosomal nonhistone proteins isolated from the mixed homogenates were subjected to gel electrophoresis as described under Methods and Materials.

Radioactivity for each of the transversely cut 2 mm gel slices was determined and the results are illustrated in Fig.3. The graphs shown in Fig.3a represent ^{14}C counts and ^{3}H counts in electrophoretically migrated proteins derived from unirradiated and irradiated (18 hr post-irradiation) rats, respectively. The $^{3}H/^{14}C$ ratios (Fig.3b) reflect relative rates of labelling of individual protein components from irradiated and control animals. These ratios are more or less uniform along the gel excepting for the proteins located at slice numbers 12-13 and 19-20 where the ratios are higher than the rest. This would indicate that the syntheses of the proteins are selectively stimulated in irradiated animals. Alternatively, rates of degradation of the proteins could have been specifically suppressed resulting in relative enhancement in their labelling.

A reverse experiment in which control rats received ^{3}H-leucine and irradiated rats (18 hr post-irradiation) ^{14}C-leucine was also carried out. As seen in Fig.4, there was a decrease in the ratios of $^{3}H/^{14}C$ of the protein components belonging to the same regions, viz., slice numbers 12-13 and 19-20. Thus the observed effect is not an artefact arising from counting anomalies. In another set of experiments, labelled lysine instead of leucine was used and the results are illustrated in Fig.5. Again, the proteins migrated to positions 12-13

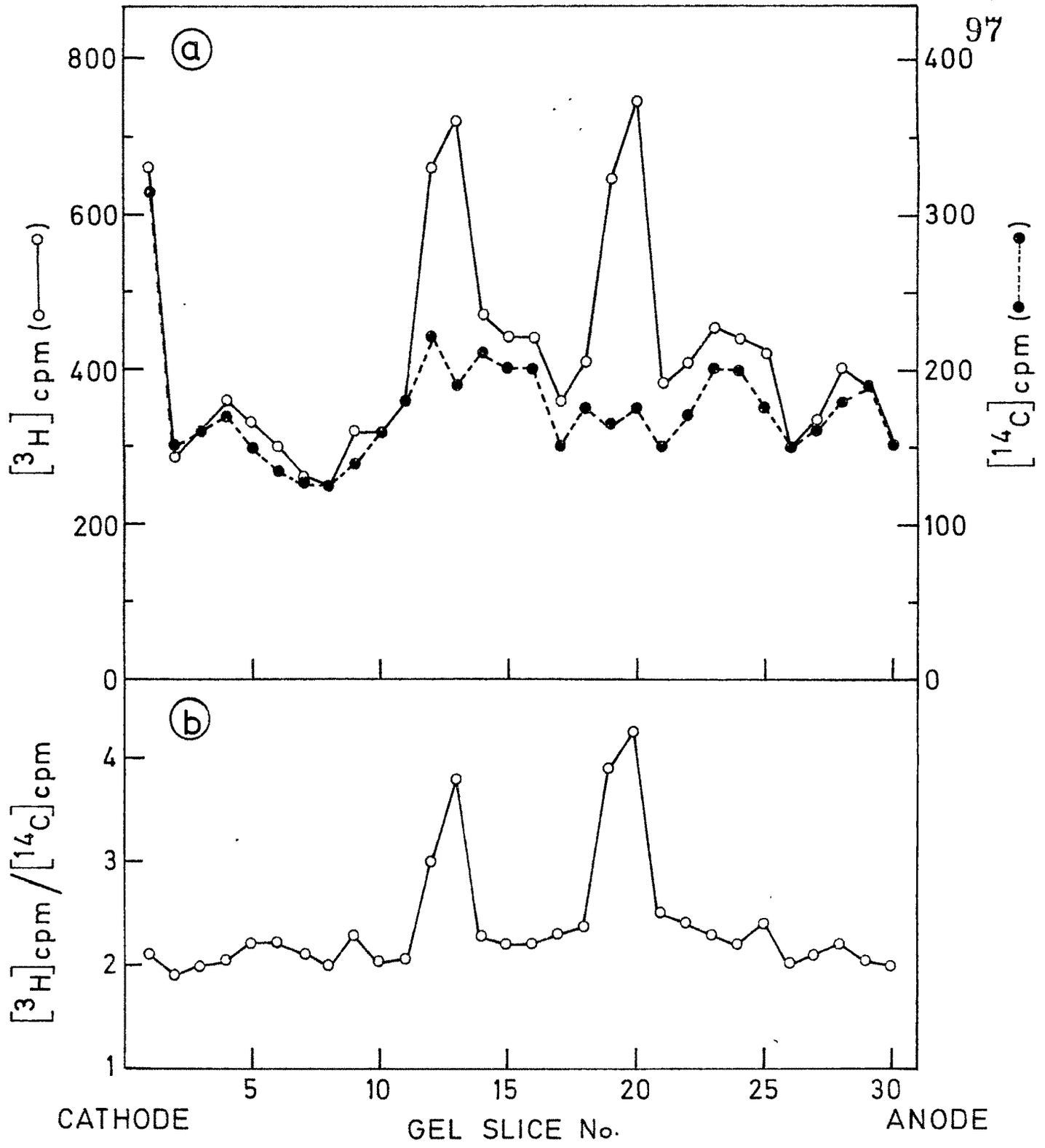

Fig. 3. Labelling patterns of electrophoretically separated non histone chromosomal proteins in the livers of irradiated rats (18 hr after 1000 r; injected with ^{3}H-leucine) and unirradiated controls (injected with ^{14}C-leucine).

(a) Radioactivity (absolute counts) in the nonhistone proteins from unirradiated rats (●- - - -●) and irradiated rats (o——o).

(b) Relative rates of labelling (^{3}H cpm/^{14}C cpm) of the nonhistone proteins from irradiated and unirradiated rats.

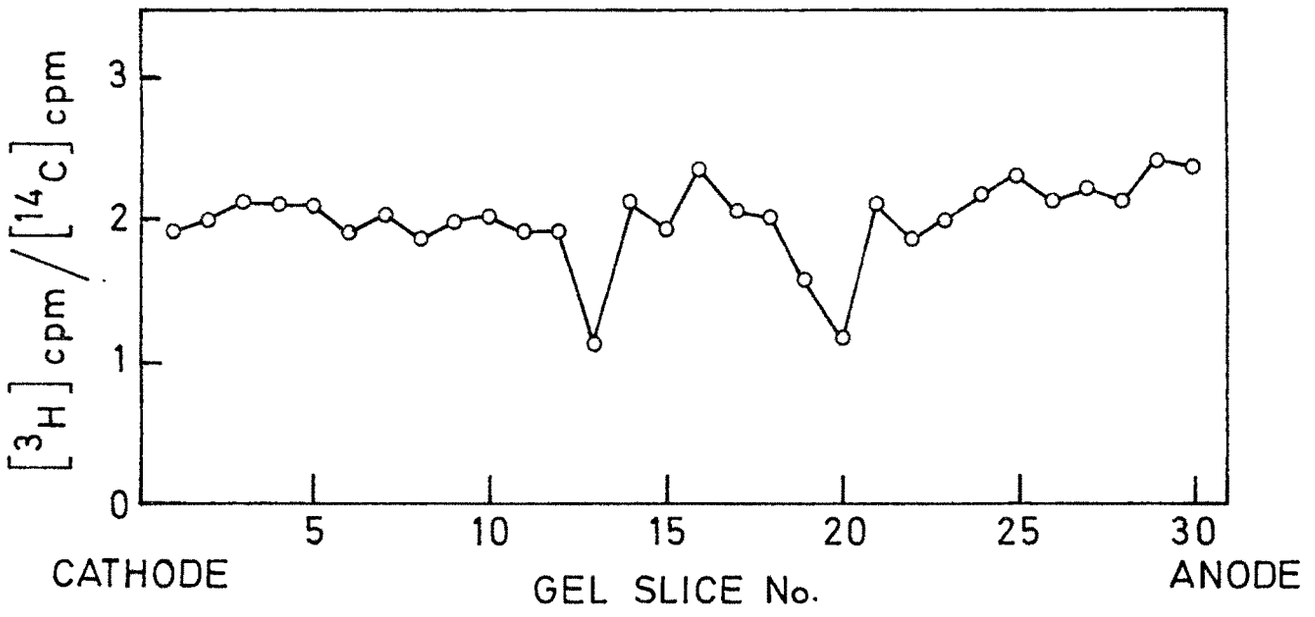

Fig. 4. Relative rates of labelling of electrophoretically separated nonhistone chromosomal proteins in the rat liver at 18 hr following whole-body exposure to 1000 r.

Irradiated rats received (^{14}C)-leucine and unirradiated rats, (^{3}H)-leucine. Total counts placed on the gel: 10,300 cpm of ^{3}H and 5340 cpm of ^{14}C; slice No. 13: 310 cpm of ^{3}H and 360 cpm of ^{14}C; slice No. 20: 310 cpm of ^{3}H and 350 cpm of ^{14}C.

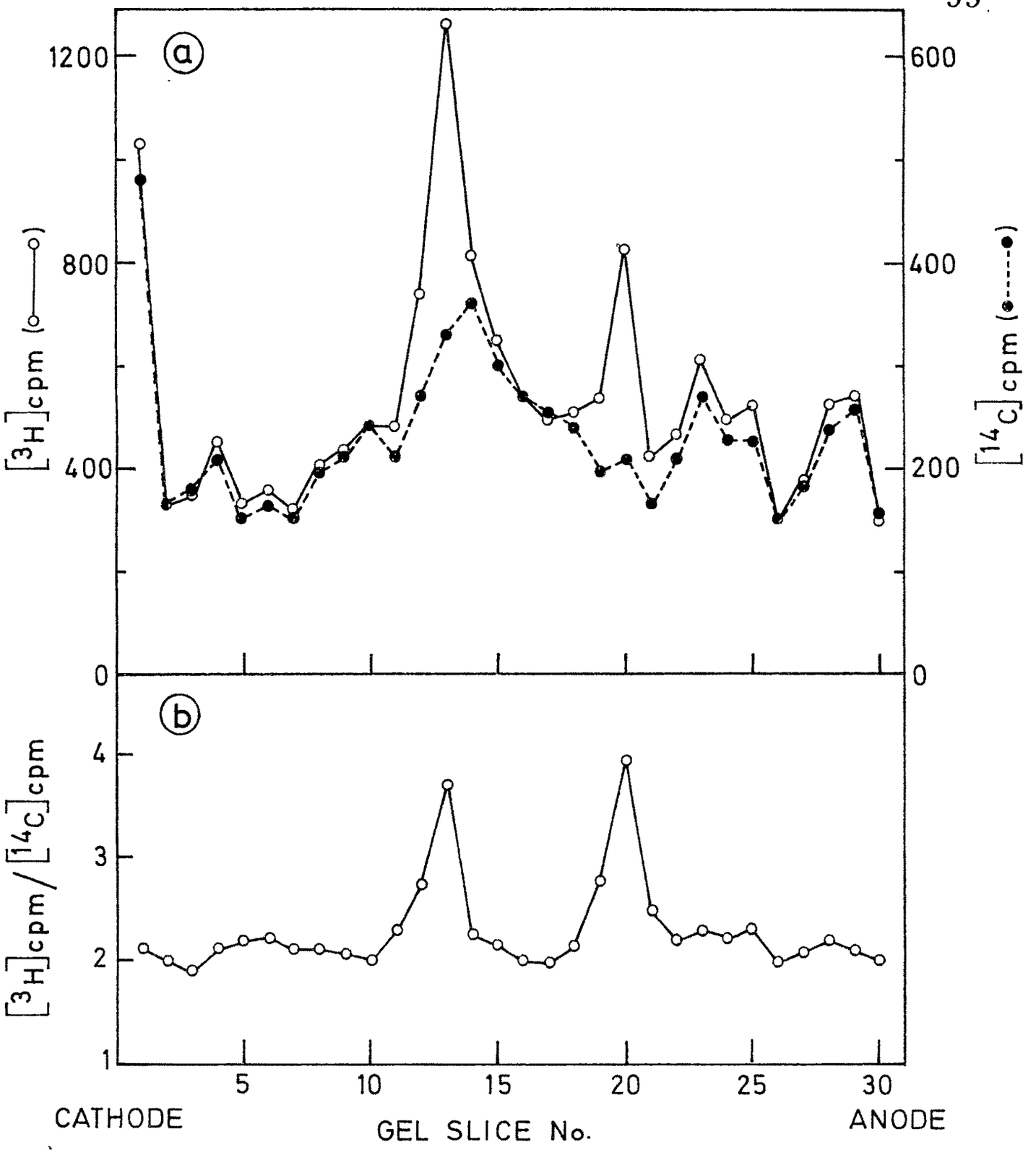

Fig. 5. Labelling patterns of electrophoretically separated nonhistone chromosomal proteins in the livers of irradiated rats (18 hr after 1000 r; injected with ^{3}H-lysine) and unirradiated controls (injected with ^{14}C-lysine).

(a) Radioactivity (absolute counts) in the nonhistone proteins from unirradiated rats (●- - - -●) and irradiated rats (o————o).

(b) Relative rates of labelling (^{3}H cpm/^{14}C cpm) of the nonhistone proteins from irradiated and unirradiated rats.

and 19-20 of the gel have shown specific enhancement in the relative rates of labelling as a result of whole-body x-irradiation. This may eliminate the possibility that the observed effects could arise from differences in specific radioactivities of the amino acids in liver soluble pools of control and irradiated rats.

As illustrated in Fig.6, the effects are apparent even at earlier times of 4 and 8 hr post-irradiation though not as much pronounced as at 18 hr post-irradiation. At 18 hr, labelling of the proteins is about 130% higher than that in unirradiated control (Fig.3). Similar analysis at 24 hr post-irradiation revealed that the magnitude of increase is much smaller being only 22% higher than normal. From the relationship of electrophoretic mobilities versus molecular weights of standard proteins, the molecular weights of the proteins for slice numbers 12-13 can be approximately estimated in the range of 57000-53000 and for slice numbers 19-20 in the range 34000-31000.

Phosphorylation of nonhistone chromosomal proteins in x-irradiated rat: In the next set of experiments, effect of whole-body radiation exposure on chemical modifications of nonhistone liver chromosomal proteins was investigated. Nonhistone chromosomal proteins have been known to contain phosphoproteins (35,36). It has been shown that there is a rapid incorporation of injected radioactive phosphate into these molecules. Like histone phosphorylation, phosphorylation of nonhistone chromosomal proteins of chromatin is also supposed to be intimately involved in the modulation of transcriptory function of chromatin (16,35). As seen in Table 4, there was a striking acceleration in the rate of phosphorylation of chromosomal nonhistone proteins in the rat liver at 18 hr post-irradiation.

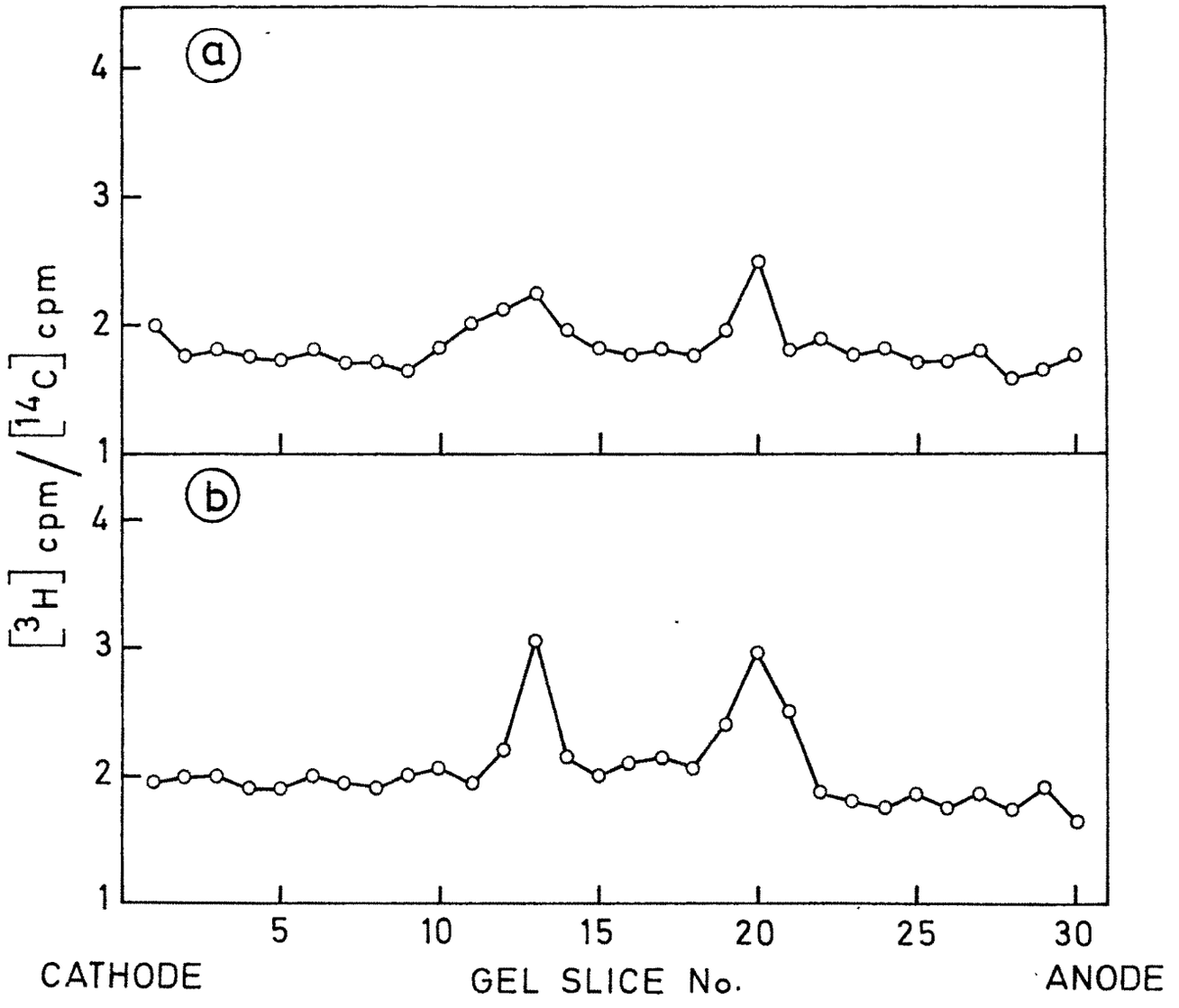

Fig. 6. Relative rates of labelling of electrophoretically separated nonhistone chromosomal proteins in the rat liver at 4 hr (a) and 8 hr (b) following whole-body exposure to 1000 r.

Irradiated rats received (^{3}H)-leucine and unirradiated controls (^{14}C)-leucine. Radioactivities of protein components (absolute counts) are as follows: (a) total counts placed on the gel: 5975 cpm of ^{3}H and 4970 cpm of ^{14}C; Slice No. 13: 365 cpm of ^{3}H and 175 cpm of ^{14}C. (b) total counts placed on the gel: 6295 cpm of ^{3}H and 4885 cpm of ^{14}C; Slice No. 13: 555 cpm of ^{3}H and 125 cpm of ^{14}C; Slice No. 20: 505 cpm of ^{3}H and 170 cpm of ^{14}C.

Table 4

Incorporation of ^{32}P-orthophosphate into rat liver chromosomal nonhistone proteins following whole-body x-irradiation

	Relative specific activity (*)
Unirradiated	6860
Irradiated (1000 r) (hr)	
12	8715
18	10990

* $\frac{\text{cpm/mg nonhistone proteins}}{\text{cpm/pmole of orthophosphate in liver homogenate}}$

Rats were injected with ^{32}P-orthophosphate (2 mCi/100 g body wt.) and sacrificed 1 hr later. Other details are as in the text. Each value represents average of three independent experiments.

Effect of whole-body radiation exposure on phosphokinase activity associated with liver chromatin: Chromatin has been shown to be associated with a phosphokinase activity that can catalyse the introduction of the phosphate group in gamma position of ATP into chromosomal proteins (in serine and threonine residues) (37). Presumably phosphorylation of histones and nonhistone proteins may have been mediated by this enzymatic activity. Methods are available for assessing the phosphokinase activity and one such method uses gamma-labelled ATP to study the phosphate incorporation into proteins (26).

Since whole-body radiation exposure results in stimulation in the phosphorylation of histone and nonhistone proteins of the liver chromatin, it was thought of interest to assess the radiation effect on phosphokinase activity associated with liver chromatin. The results shown in Table 5 indicate a significant stimulation in the phosphokinase activity (expressed on the basis of equivalent amounts of DNA) as a result of whole-body radiation exposure.

The phosphokinase activity of chromatin is known to be associated solely with nonhistone proteins (38-41). In one set of experiments, phosphokinase activity in nonhistone proteins in livers of irradiated and control groups of rats was studied. Results in Table 6 show that the phosphokinase activity in nonhistone chromosomal proteins extracted from the livers of irradiated rats (expressed per unit weight of proteins) also record a significant increase.

Acetylation and methylation of nonhistone proteins of the liver in irradiated rats: Influence of irradiation on other chemical modifications

Table 5

In vitro phosphorylation of liver chromatins isolated from unirradiated and irradiated rats

	^{32}P incorporated into protein (cpm/10 min)	% increase
Unirradiated	18,590	-
Irradiated (1000 r) (hr)		
12	25,915	39.4
18	30,170	62.4

The complete system in 0.5 ml final volume contained, 50/umoles of Tris-HCl buffer pH 7.5, 2.5/umoles of ATP-γ-^{32}P, 12.5/umoles of $MgCl_2$ and chromatin equivalent to 100/ug of DNA was incubated at 37° for 15 min and stopped by the addition of 10% TCA. Each value is average of two independent experiments.

Table 6

Assay of phosphokinase activity associated with nonhistone chromosomal proteins isolated from unirradiated and irradiated rat livers

	^{32}P-incorporated into protein (cpm /10 min)
Unirradiated	3570
Irradiated (1000 r) (hr) 18	5280

The reaction mixture (in 0.25 ml) contained 20 μmoles of Tri-HCl, pH 7.5, 2.5 μmoles of ATP-γ-^{32}P, 5 μmoles of $MgCl_2$, 25 μmoles of NaCl and 40 μg of nonhistone protein preparation. The incubation was carried out at 37° for 10 min and was stopped by the addition of 10% TCA. Each value represents average of three independent experiments.

of nonhistone proteins, viz., acetylation and methylation - which have also been implicated in gene activation (15, 16)—was ascertained in further studies. Both the rates of acetylation and methylation of nonhistone chromosomal proteins in the liver were significantly enhanced following total-body radiation exposure (Table 7).

DISCUSSION

Whole-body radiation-elicited amplification in liver chromatin activity in the rat provides a unique system to study various facets of the transcription mechanism. Firstly, the changes brought about are in response to a stress involving indigenous neuro-endocrine triggering processes (see also the following section) and hence the stimulatory effect is not unphysiological. This gives an opportunity to assess quantitative correlation between the changes in template activity and associated changes. Secondly, there is a graded increase in template activity which is perceptible at about 4 hr after irradiation and becomes maximum at about 18 hr post-irradiation. No change could be discerned in the liver chromatin structure or composition during 18 hr after whole-body irradiation. This is in consonance with similar results obtained in studies on hormone-induced gene activation (42,43).

In a number of studies (15,16) reported in the literature, gene activation has been shown to be associated with diverse types of metabolic changes in chromosomal proteins. However, in most of these cases, only a single metabolic change has been studied (15,16). It is hence not possible to ascertain whether in a condition of gene activation, chromosomal proteins necessarily exhibit all possible kinds of metabolic

Table 7

Effect of whole-body x-irradiation on acetylation and methylation of nonhistone chromosomal proteins isolated from rat liver

	Acetylation	Methylation
	(cpm/mg protein)	
Unirradiated	1810 ± 27	975 ± 16
Irradiated (1000 r) (hr)		
18	2635 ± 35	1215 ± 23

Rats were injected with ^{14}C-sodium acetate (80/uCi/100 g body wt.) and ^{14}C-methionine (80/uCi/100 g body wt.) respectively 1 hr prior to sacrifice. Each value represents average of three independent experiments ± SEM.

changes (viz. in biosynthesis, phosphorylation and aceylation, etc.) or only a specific few. In the present experiments an attempt has been made to examine a number of known metabolic changes in chromosomal proteins together under a single condition of gene activation. It was of interest to find that a variety of changes, viz., phosphorylation of histones, phosphorylation, acetylation and methylation of nonhistone chromosomal proteins are associated with amplification of template activity of liver chromatin brought about by whole-body radiation exposure. Further the magnitudes of these modifications, at least in the case of phosphorylation on nonhistone chromosomal proteins, seem to be directly related to the degree of amplification of chromatin template activity. The present findings indicate that the underlying mechanism for increased phosphorylation of chromosomal proteins is probably due to enhancement of phosphokinase activity.

The most interesting observation that has come out of the present study is that the whole-body radiation stress has elicited a selective stimulus in the synthesis of two nonhistone proteins. This stimulus is also progressive, barely observable at 4 hr but becoming quite pronounced at 18 hr post-irradiation. Several workers have recently reported that conditions which lead to gene activation are associated with specific enhancement in synthesis of chromosomal nonhistone proteins in animal cells (33,34). Thus in rats, selective stimulation in the synthesis of nonhistone chromosomal proteins has been observed in the liver following administration of glucagon (33) and hydrocortisone (20) in the liver and uterus in response to estradiol (44). Interestingly, the chromosomal nonhistone proteins involved are different for

different conditions. For instance, hydrocortisone elicits increased formation of a nonhistone chromosomal protein in the liver of 41000 daltons molecular weight, whereas glucagon causes selective stimulation in the synthesis of two nonhistone chromosomal proteins in the liver of 84000 and 60000 daltons molecular weights. In the present experiments, whole-body x-irradiation resulted in enhanced formation of yet other nonhistone chromosomal proteins in the liver - of molecular weights 53000 and 31000 daltons.

A similar analysis for chemical modifications of individual chromosomal proteins may reveal whether there also occurs any selective stimulation in chemical modifications of certain chromosomal proteins.

Further study is essential to find out whether the alterations in chromosomal protein metabolism evoked by radiation stress are the primary events responsible for gene activation or the secondary changes consequent to gene activation. In this context, it may be mentioned that certain chemical modifications in chromosomal proteins which have been reported to be associated with gene activation were found to be independent of prior synthesis of either protein or RNA (35,38,45,46).

Finally, it may be asked: what are the signals which reach the transcription apparatus in the liver? Are the effects related to radiation action directly on the liver itself or they are consequences of indirect physiological reactions? Studies described in the next section have attempted to seek answers to these queries.

SUMMARY

Structural changes in liver chromatin and metabolism of its non-DNA components were studied in whole-body irradiated rat to elucidate the mechanisms underlying the observed elevation in the chromatin template efficiency in response to the radiation exposure (Section 1). No significant physico-chemical changes (namely, in chemical composition, UV absorption spectrum and thermal melting profiles) were apparent in the isolated liver chromatin of the irradiated rat. Turnovers of histones were also not significantly altered. Phosphorylation of both total histones and H_1 histone fraction was however found to be markedly increased after irradiation. Further experiments involving analysis of electrophoretically separated radioactive leucine labelled nonhistone chromosomal proteins revealed that during 18 hr period after exposure of rats to x-irradiation, syntheses of two nonhistone proteins were selectively enhanced. The molecular weights of these proteins were estimated as 53000 and 31000 daltons. Besides these changes, increases could also be discerned in methylation, acetylation and phosphorylation of the nonhistone chromosomal protein fraction. Chromatin from the irradiated rat exhibited increased phosphokinase activity—which may explain the increases in phosphorylation of histone and nonhistone proteins of chromatin. The magnitude of the changes in the metabolism of chromosomal proteins were strikingly correlatable with the extent of amplification in chromatin template efficiency as occured at various times post-irradiation. These results indicate that changes in the distribution and chemical modifications of chromosomal proteins may partly govern transcription functions of chromatin in eukaryotic cells.

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Part II

Section 3

Abscopal mechanisms underlying radiation-induced activation in the transcription machinery of the rat liver

INTRODUCTION

Studies described in the previous sections indicate that whole-body x-irradiation of rats leads to a considerable activation of transcription machinery in the liver and this change seems to be intimately interlinked with a variety of metabolic alterations in the chromosomal proteins. To get clearer insights into these radiation effects in particular and overall regulatory aspects of transcription in mammalian system in general, it is essential to ascertain whether the radiation-provoked changes in the transcription apparatus in the liver are similar to those brought about by the administrations of hormones (1-4) and drugs (5-6), or whether these alterations are elicited by altogether different mechanisms. Specifically it must be known if the observed changes are due to effects of irradiation directly on the transcription apparatus of the liver itself or if these are the consequences of events occurring elsewhere in the body. Studies described in this section were aimed at distinguishing between these two possible modes. The results clearly reveal that whole-body irradiation-induced activation of transcription machinery in the liver is solely the result of radiation-provoked neuro-endocrine reactions involving hypothalamus-pituitary-adrenal axes.

MATERIALS AND METHODS

Animal experiments

Bilateral adrenalectomy was performed on the rats via the dorsal route. Thereafter, they were maintained on physiological saline instead of water.

Irradiation was carried out as described in Section I. Shielding of liver and head during irradiation were achieved by covering the portions of the body containing liver and head appropirately by lead plates.

Measurement of rate of incorporation of ^{32}P-orthophosphate into liver RNA

Rats were given ^{32}P-orthophosphate (2 mCi/100 g body wt.) intraperitoneally 1 hr prior to sacrifice. The procedure ~~involved~~ employed for RNA extractions was as described in Section I.

Assay of template activities of chromatin and DNA

The procedures for isolation, purification and assays of RNA polymerase, chromatin and DNA were as described in Section I.

Chemical analysis

DNA, RNA and proteins were estimated as described in Section I.

Measurement of radioactivity

As described in Section I.

Materials

^{32}P-sodium orthophosphate (carrier free, Isotope Division of this Research Centre) and GTP, CTP, UTP (Sigma Chemical Co., USA), ^{14}C-ATP (specific activity, 27.2 mCi/mmole; Schwartz Biochemicals, USA) were used.

RESULTS

In order to ascertain whether the effects of whole-body radiation exposure on liver RNA synthesis are the result of radiation effects directly on the liver itself or whether they are due to abscopal or other

neuro-endocrine mechanisms, the liver of the rat was shielded during total-body irradiation. The results of this experiment are incorporated in Table 1. It is apparent that shielding of the liver has no effect at all on radiation-evoked stimulation in RNA synthesis. The findings therefore clearly indicate that the radiation effect on RNA synthesis in the liver is mediated by indirect mechanisms.

It has been suggested in a number of reports that irradiation leads to increased secretion of adrenal steroids (7,8) and these may have a role in enhancing the efficiency of the transcription apparatus in the liver. In order to investigate whether or not adrenals are involved, experiments were carried out on rats whose adrenals were removed prior to radiation exposure. The results of these experiments are given in Table 2. It is seen that the removal of adrenals leads to somewhat lower level of RNA synthesis. Whole-body radiation exposure accelerates liver RNA synthesis in sham-operated rats but in adrenalectomised rats no such stimulatory effect is discernible.

The Table 3 gives data on the template activity of chromatin in the liver of adrenalectomised rats after exposure to total-body irradiation. Although irradiation amplifies template activity of chromatin in sham-operated rats, no such elevation is seen in adrenalectomised rats. These results also strengthen the supposition that the increased RNA synthesis in the livers of irradiated rats could be related to amplification of template activity of liver chromatin.

The effect mediated through adrenals could be either due to direct radiation action on adrenals themselves or could be indirect.

Table 1

Liver RNA synthesis in x-irradiated rats : effect of shielding of the liver during irradiation

	RNA synthetic rates (*)	
	Liver unprotected	Liver protected
Unirradiated	1480 ± 103	1432 ± 91
Irradiated (hr after 1000 r)		
8	2351 ± 116	2260 ± 107
18	2795 ± 120	2713 ± 112

* $\frac{\text{cpm/mg RNA}}{\text{cpm/pmole of orthophosphate in liver homogenate}}$

Rats received ^{32}P-orthophosphate (2 mCi/100 g body wt.) 1 hr prior to sacrifice. Each value represents average of four independent experiments ± SEM.

Table 2

Rates of RNA synthesis in the liver after whole-body x-irradiation : effect of adrenalectomy

	RNA synthetic rates (*)	
	Sham-operated control	Adrenalectomized
Unirradiated	1107 ± 78	925 ± 82
Irradiated (hr after 1000 r)		
4	1615 ± 110	875 ± 72
18	2085 ± 93	940 ± 105
24	1798 ± 132	910 ± 119
36	1208 ± 115	894 ± 76

* $\frac{\text{cpm/mg RNA}}{\text{cpm/pmole of orthophosphate in liver homogenate}}$

Rats received ^{32}P-orthophosphate (2 mCi/100 g body wt) 1 hr prior to sacrifice. Each value represents average of three independent experiments ± SEM.

Table 3

Template activity of liver chromatin and DNA after whole-body x-irradiation : effect of adrenalectomy

	Template activity of DNA (pmoles ^{14}C-AMP incorporated into RNA)*	Template activity of chromatin (pmoles ^{14}C-AMP incorporated into RNA)*
Sham-operated controls		
Unirradiated	179.2 ± 8.9	36.1 ± 7.0
18 hr after 1000 r	182.4 ± 10.2	55.9 ± 9.8
Adrenalectomised		
Unirradiated	180.6 ± 10.6	31.4 ± 8.1
18 hr after 1000 r	170.2 ± 7.9	32.2 ± 6.2

*The assay system for template activity contained 0.2 µmole ^{14}C-ATP, 20 µg of DNA either in the form of chromatin or purified as such ~~or chromatin~~, and 100 µg of rat liver RNA polymerase preparation in 0.5 ml final volume. Each value is average of three independent experiments ± SEM.

It is known that the stress mechanism, for example, that brought about in traumatic shock (8) is routed via, hypothalamus-pituitary-adrenal axes. One of the ways by which the operation of this mechanism can be tested is by protecting the head portion during irradiation so that any possible reaction arising from adreno corticotropic hormone release could be arrested. The results of such a study are presented in Table 4 and they clearly show that if only the head is protected during total-body irradiation, stimulatory effect at the transcriptory level in the liver is virtually obliterated.

DISCUSSION

The results support the contention that the whole-body radiation exposure brings about activation of transcription processes in the liver solely by abscopal mechanism. Cammarano et al. (9) found that the removal of adrenals prior to radiation did not suppress radiation-induced enrichment of liver polysomes in rat. If it is presumed that the radiation-induced enrichment of liver polysomes arises from primary activation at the transcriptional level, the findings of Cammarano et al. are in contradiction to the present findings. Yatvin (10) has however observed that in mice exposed to γ -radiation, adrenalectomy arrested increased polysome formation in the liver. He attributed failure by Cammarano et al. to observe the effect of adrenalectomy in rats to the presence of accessory adrenal glands. This suggestion indeed finds support in the studies of Goutier and co-workers (11) who found that if adrenalectomy is performed on strains of rats which do not possess accessory adrenals, whole-body radiation exposure does not lead to enrichment of polysomes.

Table 4

Liver RNA synthesis in x-irradiated rats : effect of shielding of the head during irradiation

	RNA synthetic rate (*)	
	Head Unprotected	Head Protected
Unirradiated	1420 ± 133	1415 ± 97
Irradiated (hr after 1000 r)		
8	2417 ± 96	1603 ± 85
18	2911 ± 109	1695 ± 131

* $\frac{\text{cpm/mg RNA}}{\text{cpm/pmole of orthophosphate in liver homogenate}}$

Each rat received ^{32}P-orthophosphate (2 mCi/100 g body wt) 1 hr prior to sacrifice. Each value represents average of four independent experiments ± SEM.

The present results are compelling enough to affirm that changes at transcription (and in turn translation) in the liver brought about by whole-body radiation exposure could be due to radiation-induced secretion of adrenal steroids-which may result from pituitary-adrenal overactivity (as the experiment in which the head is protected during irradiation suggests).

Thus the effect seems to be physiological in nature similar to that triggered by other kinds of stresses such as traumatic shock (8). The central nervous system has been generally considered to be radiation-resistant, because pathologists failed to find obvious lesions in mammals which received a moderate dose of ionising radiations (12). However, since early times, it was thought that the biochemical and physiological processes of irradiated mammals are altered, at least partly, by non-specific neuro-endocrine reactions which Selye (13) has termed 'adaptation syndrome'. Bacq and Alexander (8) have discussed the evidence for considering radiation as a stress condition in a manner similar to subjecting animals to cold stress or traumatic shock. That adrenal steroids, principally glucocorticosteroids, significantly activate RNA synthetic machinery in the liver is well-documented (1,14). It is therefore quite conceivable that these steroids could have been responsible for the observed changes in the chromosomal protein metabolism and in turn, on the template activity of chromatin. There are however some subtle differences between hydrocortisone (the principal glucocorticosteroid)-induced increase in RNA synthesis and enhancement in RNA

synthesis brought about after whole-body radiation exposure. Thus in the case of hydrocortisone not only is template activity of chromatin increased but there is also some increase in RNA polymerase activity (15). On the other hand, whole-body radiation exposure leads to amplification of template activity of chromatin only, while RNA polymerase activity remains unaltered (Section I). It has also been discussed earlier (Section II) that there is a difference between the selective stimulation in the turnovers of nonhistone chromosomal proteins under the condition of whole-body radiation exposure and that brought about in adrenalectomised rats after substitution of hydrocortisone (16). While experiments with hydrocortisone obviously involves only one hormone, it would seem that other hormones besides glucocorticosteroids could be involved after whole-body radiation exposure. Analysis of the hormones elaborated in response to irradiation may give further insights into the neuro-endocrine mechanisms responsible for activation of RNA and protein syntheses in the liver. Also most of the hydrocortisone effects are manifested at much higher dose levels [for example, 2.5 mg hydrocortisone/100 g body wt. (17)]. One interesting aspect of the present radiation-activated system is that the effect is amplified only in progressive manner for the period of 18 hr after radiation exposure. The question that may now be asked is whether the effects are brought about by initial triggering action of the released adrenal steroids or whether this is a manifestation of steady and progressive secretion of the adrenal steroids during post-irradiation 18 hr period.

SUMMARY

Studies were carried out to ascertain whether the biochemical events that lead to activation of transcription machinery in the liver following whole-body x-irradiation of rats originate directly from the radiation action on the liver itself or are the result of radiation-induced changes in other parts of the body. It was found that shielding of the portion of the body which includes the liver during irradiation did not obliterate the radiation-evoked stimulation in RNA synthesis in the liver. On the other hand, it was revealed that if adrenals were surgically removed prior to whole-body radiation exposure, no elevation in either liver RNA synthesis or liver chromatin template activity was apparent. Furthermore, it was seen that protection of only the head by lead shielding during total-body irradiation could also prevent the stimulatory effect on transcription in the liver. These results are strongly indicative of the involvement of neuro-endocrine reactions along the hypothalamus-pituitary-adrenal axes in the radiation-induced activation of the liver transcription machinery.

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