CHARACTERIZATION OF RNA IN RAT LIVER NUCLEI ON THE BASIS OF SENSITIVITY TO RIBONUCLEASES

by

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INTRODUCTION

For the past several years there has been considerable interest in chromosomal RNA (RNA bound to chromatin) as a possible controlling factor in the transcription process. Recent review articles reflect this interest (1, 2).

Recognition of the presence of chromosomal RNA has resulted from attempts to find ways of separating native histones from chromatin by other than the usual acid-extraction procedure. One method was to dissolve chromatin in 4 M CsCl and centrifuge until the DNA was sedimented. The high ionic strength of the environment caused rupture of the ionic bonds between the basic histones and the negatively charged DNA, permitting the chromosomal proteins to float to the surface and form a "skin" at the top of the centrifuge tube. This "skin" contained the chromosomal RNA associated with protein (2).

Chromosomal RNA has been found in all chromatins which have thus far been appropriately investigated. These include chromatins from pea bud (2), pea cotyledon (2), chick embryo (3), rat Novikoff ascites tumor (4), rat liver (5), and calf thymus (6).

Chromosomal RNA has been characterized and shown to contain about 40 to 60 nucleotides per molecule (sedimentation coefficients 3.2S to 3.8S). Base composition studies showed it to contain a relatively high content (5 to 25 moles per 100 moles) of dihydrouridylic acid (2, 4, 6).

Nascent RNA (i.e., RNA in the process of being synthesized and still attached via RNA polymerase and hybridization to its DNA template) may also be closely associated with chromatin and might be isolated by the procedure used for the preparation of chromosomal RNA. The possible

relationship between these classes of RNA and chromosomal RNA has been investigated by comparing the amount of incorporation of ^{32}P into various classes of RNA following a short exposure of whole cells to the label (4). These investigators showed that in rat ascites tumor cell, chromosomal RNA was not as rapidly labeled as was messenger RNA. From these results the authors concluded that chromosomal RNA as isolated by these procedures was not nascent RNA and suggested that chromosomal RNA was turning over at a relatively slow rate.

Chromosomal RNA has been shown to be heterogeneous with respect to base sequence and to hybridize with a substantial fraction of nuclear DNA (2). Chromosomal RNA freed of DNA by centrifugation in CsCl, freed of protein by treatment with pronase, and freed of peptides by chromatography on a diethylaminoethyl-Sephadex column with a gradient of NaCl in 7M urea has been characterized by hybridization with the DNA from which it came. At saturation it was shown to hybridize with slightly more than five percent of nuclear DNA in pea buds (7) and with about four percent of nuclear DNA in rat ascites tumor (4). Bonner (2) has concluded, both from this measure and from the slow rate of hybridization of chromosomal RNA to DNA, that chromosomal RNA was exceedingly heterogeneous in base sequences and consisted of many species of RNA, each represented but a small number of times. Neither transfer nor ribosomal RNA was found to compete with chromosomal RNA in hybridization (4, 7). Chromosomal RNA from one species did not hybridize appreciably with DNA obtained from another species, although the degree of hybridization possible between chromosomal RNA and nuclear DNA of closely related species remains to be studied (7). Furthermore, chromosomal RNAs from different tissues of the same species have been shown to be different (2).

Finally, at least 50 percent of chromosomal RNA has been shown to be confined to the nucleus (4, 7).

Two opposing views as to how this type of RNA might act to influence RNA synthesis have been clearly stated (1, 2). The first view, proposed by Bonner (2), suggested a repressor function for chromosomal RNA. According to this theory, the complex of RNA and histone binds to that portion of the chromatin in which the RNA is complementary to an appropriate DNA sequence, and thereby the histone moiety specifically represses that portion of the genome. Opposed to the Bonner view, Frenster (1) has hypothesized a derepressor function for chromosomal RNA. According to this hypothesis, polycationic histone repressors non-specifically stabilize double-stranded DNA helices against strand separation, and nuclear polyanions partially displace such histones from the DNA by forming polyanion-histone complexes within the active euchromatin. Frenster has suggested that the DNA underlying such displaced histones undergoes spontaneous strand separations. These strands are stabilized by hybrid formation of derepressor RNA with a single DNA strand. The remaining DNA strand at the gene locus is then free to serve as a template for gene-specific and strand-specific messenger RNA synthesis.

Recently Kuntzman (8) has reviewed several chemicals that are known to induce enzyme synthesis. These include compounds currently used in clinical therapy (phenothiazines, phenobarbital, meprobamate) as well as compounds with known carcinogenic activity (e.g., 3,4-benzpyrene, 3-methylcholanthrene, 1,2,5,6-Dibenzanthracene). One of

these compounds, 3-methylcholanthrene, has been shown to increase "aggregate" RNA polymerase activity (9, 10) and chromatin activity (11). Since some of these chemicals affect the transcription process, it would be of interest if the effects of these chemicals on this process were related to the amount of chromosomal RNA bound to chromatin. Such studies would help to decide between the two alternate hypotheses regarding the function of chromosomal RNA.

Unfortunately, the methods used to isolate chromosomal RNA from chromatin are neither quantitative nor reproducible (2, 4, 12). According to Dahmus (12), the yield of chromosomal RNA from either crude or purified chromatin was less than 25 percent and was variable. Therefore, it has not been possible to provide experimental data that would distinguish between the possible alternate hypotheses.

STATEMENT OF THE PROBLEM

The proposal for this thesis was to investigate other possible methods for the estimation of chromosomal RNA which would be more quantitative than the methods currently employed. If chromosomal RNA is indeed bound in the structure of chromatin in such a way as to be resistant to pancreatic ribonuclease (5, 13), this might be a useful property for the isolation of chromosomal RNA.

In addition to the fractionation and isolation procedure, a sensitive method would also be needed for the quantitation of this material. One of the most sensitive and specific methods used to quantitate RNA is the orcinol method of Volkin and Cohn (14). Thus,

it was decided to attempt the quantitation of orcinol-reacting material that was resistant to pancreatic ribonuclease in rat liver nuclei and to attempt to alter the amount of this material by experimental procedures known to cause derepression, such as partial hepatectomy.

MATERIALS AND METHODS

Animals. Unless otherwise specified, male rats of the Sprague-Dawley strain, weighing between 70 to 160 grams before sacrifice, were purchased from Simonsen Laboratories of Gilroy, California, and maintained on standard laboratory chow throughout the experiments.

Chemicals and enzymes. ^{3}H-5-orotic acid (12.1 and 16.0 c/mmole) was obtained from Schwarz BioResearch, Inc., Orangeburg, New York.

Ribonuclease A (beef pancreas 2.7.7.16, specific activity 3,000 units/mg) and Ribonuclease T_1 (Aspergillus oryzae 2.7.7.26, specific activity 30,000 units/mg) were obtained from Worthington Biochemical Corporation, Freehold, New Jersey.*

NCS solubilizer was purchased from Amersham/Searle Corporation, and Bio-Solv (BBS-3) was purchased from Beckman Instruments, Inc.

Hepatectomy. Surgical removal under ether anesthetic was carried out as described by Higgins and Anderson (15) on rats weighing between 95 to 140 grams; 65 to 75 percent of the total liver was removed, leaving within the peritoneum the right lateral lobe and the small caudate lobe. Upon recovery from the anesthetic, these animals were given one ml of 20 percent dextrose in saline, either intraperitoneally or subcutaneously depending upon the experiment, and received 20 percent

*The manufacturer gives the following criteria for the purity of their enzymes: Ribonuclease A was the purest grade available and was claimed to be monophoretic when analyzed by polyacrylamide gel electrophoresis. It was also claimed to be free of aggregates. According to the manufacturer, ribonuclease T_1 was essentially pure as determined by specific activity and disc gel electrophoresis. Purity was also verified in the laboratories of Worthington Corporation by amino acid composition determination and analytical chromatography.

dextrose in their drinking water until they were sacrificed. Other animals were sham-operated by exposing their livers under the same conditions but they were not given an injection of dextrose and were given only tap water to drink after the operation.

Preparation of Nuclear Lysate. Nuclei were prepared from rat liver cells by the method of Chauveau et al. (16) as modified by Blobel and Potter (17).* Rats were killed by decapitation. Liver tissue in the amount of 6 to 33 grams was removed quickly and chilled immediately in several volumes of ice-cold 0.25 M sucrose in TKM (0.05 M tris-HCl, pH 7.5, at 20° C; 0.025 M KCl; and 0.005 M $MgCl_2$ [See list of abbreviations, Appendix, page 50].) All subsequent operations were performed at temperatures near 0° C until preparation of the nuclear lysate was completed. Livers were blotted, weighed, and minced with scissors and then added to two volumes of ice-cold 0.25 M sucrose in TKM. They were homogenized in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle (clearance approximately 0.025 cm) with 10 to 15 strokes at 1700 rpm. The homogenate was filtered through four layers of cheesecloth.

An aliquot consisting of 7.7 ml of the resulting homogenate was mixed with 15.3 ml of 2.3 M sucrose in TKM. A 20 ml aliquot of this mixture was layered over 10 ml of 2.3 M sucrose in TKM and centrifuged

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* Nuclei prepared by this procedure were examined under phase microscopy and appeared to be free of gross cytoplasmic contamination. These nuclear preparations were also assayed for their RNA content (orcinol-reacting material, ORM) and found to contain 3.2 ± 0.1 percent (mean ± standard error) of the total cell RNA. These results are comparable with values reported by Potter and Blobel for their preparations [i.e., four to five percent of cellular RNA in the nuclear fraction (17)]. These investigators give electron micrographs to support the purity of their nuclear preparation.

for two hours at 25,000 rpm in a Spinco SW 25.1 rotor at 4° C. The supernatant was then poured off and the wall of the tube was wiped dry with tissue paper. The white nuclear pellet was taken up in 10 ml of TKM buffer* and with the aid of a vortex mixer. The nuclei were then washed by sedimentation in 15 ml centrifuge tubes at 2000 rpm in the SS 34 Sorvall rotor for 10 minutes. The nuclei were again resuspended in 10 ml of TKM buffer and sedimented as before. Finally, the nuclei were resuspended in 6.0 ml of 0.01 M tris-HCl (pH 7.5) and 0.001 M EDTA and dialyzed against five liters of this same buffer for 12 hours at 4° C. This hypotonic buffer lysed 75 to 95 percent of the nuclei as judged by inspection under the phase microscope. Complete lysis of nuclei was obtained by 30 strokes with a Dounce homogenizer using the tight-fitting "B" pestle. This suspension was then treated with RNase-free buffer, RNase T_1 or RNase A.

Ribonuclease Treatment and Fractionation of RNA, DNA and Protein.

Unless otherwise specified, 1.0 ml of nuclear lysate was treated with 1.0 ml of one of the following solutions: RNase-free buffer containing 0.01 M tris-HCl, pH 7.5, 0.001 M EDTA; ribonuclease A at 40 µg/ml in .01 M tris buffer, pH 7.5, containing 0.001 M EDTA: or ribonuclease T_1 at 10 µg/ml in 0.01 M tris buffer, pH 7.5, containing 0.001 M EDTA.

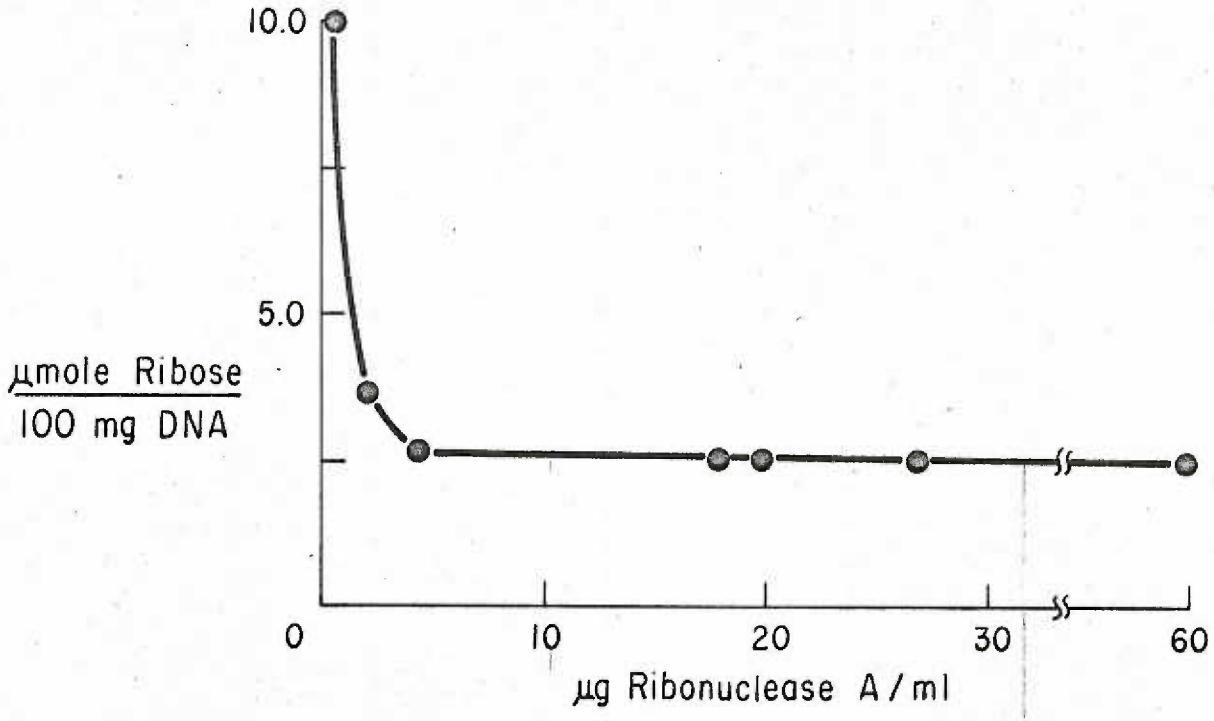
Concentration of RNase A was established from the results of an experiment in which nuclear orcinol reacting material (ORM) was hydrolyzed at various concentrations of RNase A. Fig. I shows that nuclear ORM resisted digestion by RNase A at concentrations ranging from

*For all subsequent steps all buffer solutions and glassware were freed of contaminating ribonuclease activity by the following treatment: Buffers were autoclaved at 15 lb pressure for 30 minutes at 125° C, a procedure known to destroy contaminating RNase activity (Dr. Wilbur P. McNulty, personal communication). Glassware was oven-dried for at least two hours at 160° C. All glassware had been previously acid washed.

Figure 1.

Effect of RNase A concentration on RNase A-resistant ORM in rat liver nuclei.

See text for details on methods. Each point represents the average of duplicate determinations.


10.0
5.0
µmole Ribose
100 mg DNA
0
10
20
30
60
µg Ribonuclease A / ml

5 to 60 µg RNase A/ml incubated for one hour at room temperature. In all subsequent experiments 20 µg/ml was the final concentration of RNase A used in the incubation tube.

After incubation for one hour at room temperature, material that was not rendered acid-soluble was precipitated by chilling the samples in an ice bath and adding 1.0 ml of cold 18 percent PCA. The samples were centrifuged in the SE 12 Sorvall rotor for 15 minutes at 12,000 rpm. In some experiments this pellet was washed a second time by adding 2.0 ml of six percent cold PCA and centrifuged as before.

RNA, DNA and protein were fractionated from this first pellet as shown in Fig. II. Pellet I was resuspended in 1.0 ml of 0.3 N NaOH and incubated for 18 hours (except in some experiments where incubation was for only one hour) at 37° C.

After digestion in alkali the samples were chilled to 4° C and 1.5 ml of cold 12 percent PCA was added to each sample. The samples were kept at 4° C for one hour and centrifuged as before.

Such alkaline treatment should, under these conditions, make the RNA in pellet I acid-soluble and, therefore, this RNA should appear in supernatant II (18). Pellet II should contain DNA and protein (18). Pellet II was further fractionated into DNA and protein by heating for 20 minutes at 90° C in six percent PCA (Schneider procedure) (19). The resulting mixture was chilled in an ice bath for 10 minutes and centrifuged as before. The DNA in pellet II that was made acid-soluble by this hot acid treatment appeared in supernatant III while the protein remained as acid-insoluble material in pellet III.

Table I shows that a second extraction for 18 hours of pellet I with 0.3 N NaOH converted only 16 percent as much ORM in supernatant II as did the first extraction procedure. These data indicate that the first 18 hour extraction converted the bulk of RNase A-resistant ORM into cold acid-soluble material.

Figure II.

Fractionation procedures for separation of RNA, DNA and protein from rat liver nuclear lysate.

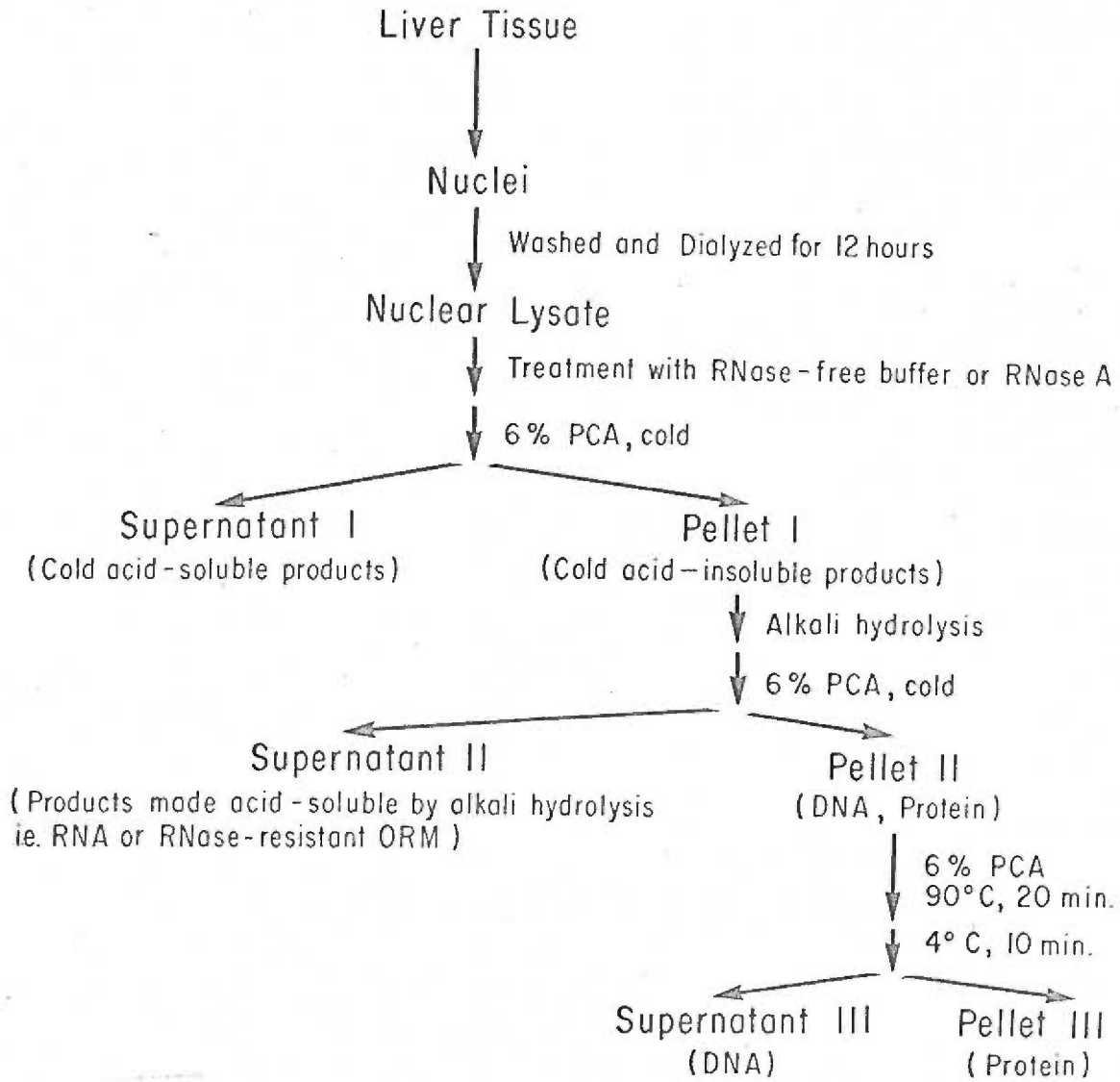

Liver Tissue
Nuclei
Washed and Dialyzed for 12 hours
Nuclear Lysate
Treatment with RNase-free buffer or RNase A
6% PCA, cold
Supernatant I
(Cold acid-soluble products)
Pellet I
(Cold acid-insoluble products)
Alkali hydrolysis
6% PCA, cold
Supernatant II
(Products made acid-soluble by alkali hydrolysis ie. RNA or RNase-resistant ORM)
Pellet II
(DNA, Protein)
6% PCA
90°C, 20 min.
4° C, 10 min.
Supernatant III
(DNA)
Pellet III
(Protein)

TABLE I.

Effects of Alkali Treatment on the
Extraction of RNase A-Resistant ORM

	First Alkali Hydrolysis	Second Alkali Hydrolysis
μmole ribose/100 mg DNA	2.5 ± 0.1	0.4 ± 0.1

Nuclear lysates were treated with RNase A at 20 μg/ml for one hour at room temperature. After alkali hydrolysis as described in the Methods, pellet II was incubated again at 37° C for 18 hours in 0.3 NaOH. The ORM rendered acid-soluble during this second digestion was determined. These values have been corrected for diphenylamine reacting material which cross reacts with orcinol (see Appendix for details on calculating μmole ribose/100 mg DNA). Each value is given as mean ± standard error, n = 8.

When a labeled RNA was added to the nuclear lysates and treated with RNase A according to the procedure described, small amounts of radioactivity were found in pellet I. The amount of contamination was 1.2 percent (see Appendix, page 44) of the label found in supernatant I when the pellet was not washed, and this amount was further reduced so that it was no longer detectable with a single resuspension and centrifugation. The above figure was used to correct for contamination in all experiments where pellet I was not washed.

Determination of RNA and DNA. Aliquots (1.0 ml in volume) of the supernatant II fraction were used for ribose determination by the method of Volkin and Cohn (14). Aliquots amounting to 1.0 ml of supernatant II and III were used for deoxyribose determination by the method of Burton (20). Micromoles of assayed deoxyribose were converted to micrograms of DNA by assuming an average molecular weight of 327 for the deoxyribonucleotides, and that only purine-bound deoxyribose of DNA reacts with diphenylamine to give a blue color. Assuming that the DNA of rat liver is double stranded (i.e., purine = pyrimidines), this last assumption amounts to a factor of two in calculating micrograms of DNA from micromoles of deoxyribose.

Determination of total cell RNA - ribose. Total cell cold acid-insoluble ribose was determined from a 1.0 ml aliquot of rat liver homogenate after it had been filtered through cheesecloth (see above). Initially, this aliquot was precipitated with cold 6 percent PCA and washed two times with 2.0 ml of six percent cold PCA. The nucleic

acids were extracted from the final pellet with 3.0 ml of six percent PCA by heating at 90° C for 20 minutes. Ribose and deoxyribose were determined as described above.

Measurement of radioactivity. Two systems have been used for measurement of radioactivity. The first system in which acid-insoluble fractions were dissolved in 2.0 ml of NCS solubilizer for three hours at 37° C in a warm room, while the acid-soluble fraction (0.2 ml in volume) was dissolved in 2.0 ml of NCS solubilizer for three hours at room temperature. A solution of 15 milliliters of toluene scintillation counting fluid (4 grams 2,5 diphenyloxazole, 0.1 gram 1,4-bis-2-(5-phenyl-oxozylyl)-benzene per liter of toluene) was added to the resultant NCS-mixtures. Radioactivity was determined in a Packard tri-carb scintillation spectrometer. The background count was subtracted from the total count and (unless specified otherwise) correction was made for chemical quenching, using automatic external standards.

The second system was the "5 percent BBS-3" system, in which 15 milliliters of five percent volume BBS-3 toluene scintillation counting fluid was added to 0.2 ml of acid-soluble material. As with the first system all samples were corrected for background and chemical quenching.

Correction for contaminating DNA in supernatant fraction II. Since appreciable and varying amounts of diphenylamine reacting material was found in the supernatant fraction II (see Appendix, Table IX) it was necessary to establish an accurate cross reaction factor so that the

contribution of diphenylamine reacting material to the orcinol color could be eliminated. This correction factor was about 20 μmoles of apparent ribose per 100 mg of DNA (see Appendix, page 40).

RESULTS

Detection of RNase A-resistant ORM in Rat Liver Nuclei.

Cold acid insoluble orcinol-reacting material (ORM) of lysed nuclei was assayed after incubation with RNase A (20 µg/ml) or an RNase-free buffer and the results are shown in Table II. These values have been corrected for any diphenylamine reacting material (DRM) which cross-reacts with orcinol (see Appendix for details). The amount of corrected ORM resistant to RNase A (supernatant II, Figure II) ranged from 1.9 to 5.6 µmoles ribose per 100 mg DNA. The nuclear ORM which was estimated from preparations incubated with RNase-free buffer will be referred to as "total nuclear ORM".* The amount of total nuclear ORM ranged from 28 to 51 µmoles ribose per 100 mg DNA. Paired comparison analysis of the data in Table II indicate that approximately 6 to 12 percent of total nuclear ORM is resistant to RNase A.

Manipulation of RNase A-resistant ORM by Partial Hepatectomy.

The effects of partial hepatectomy on the amount of nuclear ORM resistant to RNase A are shown in Table III. Untreated animals (or

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*This total nuclear ORM may not represent the amount of ribose found in rat liver nuclei. These nuclear preparations may contain endogenous RNase activity which may result in RNA losses during dialysis or during incubation with RNase-free buffer. However, three findings suggest that these losses are not great. First, the fraction of total cell ribose found in supernatant II after incubation with RNase-free buffer compares closely with the fraction of whole cell RNA found in purified nuclei reported by Blobel and Potter (14) (see footnote, page 7). Secondly, in three experiments less than 10 percent of the nuclear ORM was rendered acid-soluble after incubation in RNase-free buffer. Third, in two experiments nuclear RNA was labeled 20 minutes *in vivo* with ^{3}H-orotate. Under these conditions 95 to 98 percent of the label was found in RNase A-sensitive ORM (i.e., RNA, since this ORM is sensitive to a ribonuclease.) In both experiments more than 85 percent of the total label before dialysis was recovered after dialysis.

TABLE II.

RNase A-Resistant ORM and Total Nuclear ORM Content in Rat Liver Nuclei

Experiment Number and Rat Weights	A RNase A-Resistant ORM $\frac{\mu\text{mole Ribose}}{100\text{ mg DNA}}$	B Total Nuclear ORM $\frac{\mu\text{mole Ribose}}{100\text{ mg DNA}}$	A/B x 100 Percent of Total Nuclear ORM Resistant to RNase A
Experiment I	4.32 ± .01 (n = 12)	37 ± 1 (n = 3)	11.7
Experiment II (100 grams)	5.6 ± 0.2 (n = 8)	51 + 1 (n = 2)	11.0
Experiment III (110-140 grams)	4.4 ± 0.4 (n = 8)	36 ± 1 (n = 2)	12.2
Experiment IV (100-110 grams)	5.3 ± 0.2 (n = 8)	49 ± 1 (n = 2)	10.8
Experiment V (130-140 grams)	2.5 ± 0.1 (n = 6)	28 ± 1 (n = 6)	8.9
Experiment VI (120 grams)	1.9 ± 0.1 (n = 4)	32 ± 1 (n = 6)	6.0
Experiment VII (95 grams)	3.4 ± 0.1 (n = 5)	39.0 ± 0.4 (n = 4)	8.7
Experiment VIII (150-160 grams)	3.2 ± 0.2 (n = 3)	43.7 ± 0.4 (n = 4)	7.3
Experiment IX (150-170 grams)	3.3 ± 0.1 (n = 4)	40.2 ± 0.4 (n = 2)	8.2
Experiment X (110-120 grams)	2.5 ± 0.1 (n = 3)	37.7 ± 0.1 (n = 3)	6.6

Nuclear lysates, treated with RNase A (20 µg/ml) or RNase-free buffer, were fractionated (Fig. II. See Methods.) Supernatant II was assayed for ORM and DRM, and the ORM component due to cross reaction with DRM substrated. Individual assays for each experiment are shown in the Appendix (Table IX), showing variability and magnitude of correction for DRM. Experiments are listed chronologically from October 1968 to January 1970. Each value is is given as mean ± standard error.

TABLE III.

Effect of Partial Hepatectomy on the Amount of RNase A-Resistant ORM in Rat Liver Nuclei

Experiment No. and Rat Weight	A Controls μmole Ribose / 100 mg DNA	B Partial Hepatectomy μmole Ribose / 100 mg DNA	P Value**	$\frac{B-A}{A} \times 100$ Percent Increase
Experiment I (100 grams)	5.6 ± 0.2 (n = 8)	7.7 ± 0.4 (n = 4)	<.01	37
Experiment II (110-140 grams)	5.3 ± 0.2 (n = 8)	7.6 ± 0.2 (n = 4)	<.001	43
Experiment III* (95 grams)	3.4 ± 0.1 (n = 5)	4.7 ± 0.1 (n = 4)	<.001	38

Standard fractionation procedure was used for each experiment (see Fig. II). For each experiment values obtained for control rats represent the mean obtained from five pooled rat livers, and mean values given for partially hepatectomized rats were obtained from 7 to 15 pooled rat livers. Values are given as mean ± standard error.

*In Experiment III, control animals were sham-operated.

**P was determined by use of the Student's t-test (two-tail).

TABLE IV.

Effect of Partial Hepatectomy on the Amount of Total Nuclear ORM

Experiment No. and Rat Weight	A Controls µmole Ribose / 100 mg DNA	B Partial Hepatectomy µmole Ribose / 100 mg DNA	P Value**	$\frac{B-A}{A} \times 100$ Percent Increase
Experiment I (100 grams)	51 ± 1 (n = 2)	75 (n = 1)	-	47
Experiment II (110-140 grams)	49.2 ± 0.2 (n = 2)	76 (n = 1)	-	54
Experiment III* (95 grams)	39.0 ± 0.4 (n = 4)	60.2 ± 0.4 (n = 2)	<.001	54

Standard fractionation procedure was used for each experiment (see Fig. II). For each experiment values obtained for control rats represent the mean obtained from five pooled rat livers, and mean values given for partially hepatectomized rats were obtained from 7 to 15 pooled rat livers. Values are given as mean ± standard error.

* In Experiment III, control animals were sham-operated.

** P was determined by use of the Student's t-test (two-tail).

sham operated animals in Experiment III) are compared with animals undergoing liver regeneration. There was a 39 percent increase in the ratioof RNase A-resistant ORM to DNA 24 to 25 hours after surgery. Table IV shows that in the same three experiments, total nuclear ORM increased 52 percent following partial hepatectomy which was statistically greater ($P < .02$, two-tail Student's t-test) than the increase found for RNase A-resistant ORM.

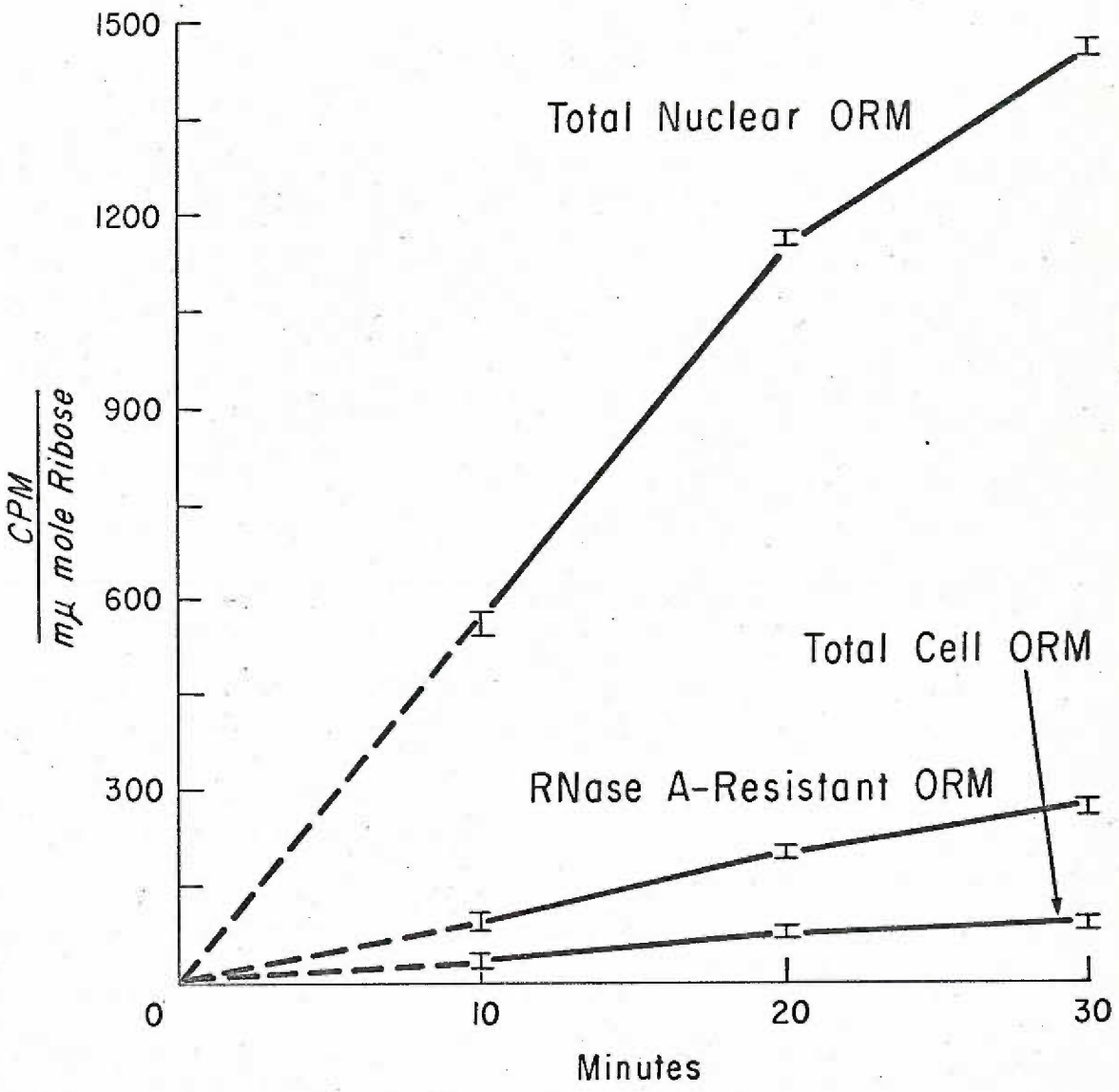
Labeling with ^{3}H-orotic Acid and Evidence for Low Rate of Synthesis and Turnover of Nuclear RNase A-resistant ORM.

The kinetics of orotate uptake into total cell ORM, total nuclear ORM and nuclear RNase A-resistant ORM after intraperitoneal injection of ^{3}H-orotate are shown in Figure III. The specific activity (cpm per µmole ribose as determined by orcinol method) of nuclear ORM after a single injection of ^{3}H-orotate increases with time and does not reach a peak before 30 minutes. The specific activity of the RNase A-resistant ORM was approximately one-sixth that of total nuclear ORM throughout the 30-minute labeling period. The specific activity of the RNase A-resistant ORM may be slightly lower than indicated because pellet I (Figure II) was not washed in this experiment and no correction was made for the small amount contamination of labeled nucleotides which might have occurred. Similar results were obtained in another experiment (Table VI, Experiment II); however, where pellet I was washed a second time. In this experiment animals were sacrificed 20 minutes after injection with ^{3}H-orotate and the specific activity of the RNase A-resistant ORM was one-eighth that of total nuclear ORM.

Figure III.

Kinetics of ^{3}H-orotate uptake into total cell ORM, total nuclear ORM and nuclear RNase A-resistant ORM after intraperitoneal injection of rats with ^{3}H-orotate.

Three rats weighing 130, 140 and 144 grams were injected intraperitoneally at time zero with 100 μc of ^{3}H-orotate (specific activity, 16 c/mmole) in 1.0 ml of saline and killed after 10, 20 and 30 minutes, respectively. The livers were quickly removed, minced and homogenized. The homogenate was then subjected to the standard fractionation procedure (see Fig. II, Methods). Whole homogenate, supernatant II after RNase-free buffer treatment and supernatant II after RNase A-treatment were assayed for their content of ORM, DRM and radioactivity. Specific activities, cpm/mμmole ribose, for the three samples are plotted above. Brackets represent the range of duplicate determinations.


1500
1200
900
600
300
0
10
20
30
Minutes
CPM / mμ mole Ribose
Total Nuclear ORM
Total Cell ORM
RNase A-Resistant ORM

The relative turnover of orotate in the RNase A-resistant fraction (supernatant II after RNase A treatment) and RNase A sensitive fraction (supernatant I after RNase A treatment) was estimated by studying the retention of label after exposure to ^{3}H-orotate. In these experiments rats which were either untreated, sham-operated or partially hepatectomized were injected with ^{3}H-orotate every 30 minutes for six hours and sacrificed 0, 6, 18 or 20 hours after the last injection. Table V shows that retention of the label expressed as percent of that found after the last injection was always greater in the RNase A-resistant fraction than that in the RNase A-sensitive fraction.

Further Fractionation of Nuclear ORM by Treatment with RNase T_1.

Table VI shows the results of two experiments in which nuclear lysates were treated with RNase-free buffer, RNase A and/or RNase T_1. The amount of ORM remaining acid-insoluble (pellet I) after each treatment is given is terms of μmoles ribose/100 mg DNA. Cold acid-insoluble radioactivity in pellet I was also determined from supernatant II in each case and the specific activity reported in terms of counts per minute per m μmole ribose. It is readily seen that about one-third of the ORM in the nuclear lysate is resistant to RNase T_1, whereas less than 10 percent is resistant to RNase A. Further, the specific activity of RNase T_1-resistant ORM is only one-half to one-third the specific activity of total nuclear ORM.

When RNase T_1 was used concurrently with RNase A there was a statistically significant decrease ($P < .01$*) in the amount of RNase

*P was determined by the use of the Student's t-test (two-tail).

TABLE V.

Retention of Label in RNase A-Resistant and RNase A-Sensitive Fractions of Liver Nuclei after Exposure of Rats to ^{3}H-Orotate

Treatment and Rat Weights	Fraction	cpm/μg DNA				P Value*	Percent Label Lost (-) or Gained (+)
		0 hour	6 hour	18 hour	20 hour		
Partially Hepatectomized Rats (120-130 gms)	RNase A-sensitive	1184 ± 7		175 ± 7		<.01	-85
	RNase A-resistant	7.6 ± 0.2		4.0 ± 0.1		<.01	-47
Partially Hepatectomized Rats (100 gms)	RNase A-sensitive	1372 ± 13		400 ± 4		<.01	-71
	RNase A-resistant	7.3 ± 0.2		10.2 ± 0.3		<.01	+40
Sham Operated Rats (70-80 gms)	RNase A-sensitive	960 ± 10		211 ± 2		<.01	-78
	RNase A-resistant	11.6 ± 0.2		4.0 ± 0.1		<.01	-57
Untreated Rats (160 gms)	RNase A-sensitive		741 ± 12		226 ± 11	<.01	-70
	RNase A-resistant		6.2 ± 0.1		3.1 ± 0.2	<.01	-50

Standard fractionation procedures (see Fig. II) were used except that the amount of radioactivity in RNase A-sensitive ORM (usually estimated in supernatant II after incubation with RNase-free buffer) was determined from supernatant I after incubation of nuclear lysates with RNase A. Radioactivity in the RNase A-resistant fraction was determined as usual in supernatant II after incubation of nuclear lysates with RNase A. Correction for radioactivity due to ^{3}H-orotate incorporation into DNA was made on the basis of the amount of DNA contamination in supernatant II and the specific activity of DNA found in supernatant III. Each corrected value is given as mean ± standard error, n = 3.

* P was determined by the use of Student's t-test (two-tail) and indicates the level of statistical significance for the amount of label lost or gained from each fraction after the last injection.

TABLE VI.

Fractionation of Nuclear ORM by Treatment with RNase T_1 and/or RNase A

Treatments	Experiment I		Experiment II	
	µmole Ribose / 100 mg DNA	CPM / m µmole Ribose	µmole Ribose / 100 mg DNA	CPM / m µmole Ribose
RNase-Free Buffer	32 ± 1 (n = 6)	1457 ± 30 (n = 6)	37.7 ± 0.1 (n = 3)	513 ± 2 (n = 3)
RNase T_1	10.5 ± 0.2 (n = 4)	534 ± 14 (n = 4)	13.3 ± 0.1 (n = 3)	253 ± 2 (n = 3)
RNase A	1.9 ± 0.1 (n = 4)	198 ± 11 (n = 4)	2.49 ± .05 (n = 3)	65 ± 3 (n = 3)
RNase A + T_1	1.6 ± 0.1 (n = 4)	286 ± 23 (n = 4)	1.88 ± .04 (n = 3)	87 ± 4 (n = 3)

In each experiment six rats weighing 110-120 grams were sacrificed 20 minutes after the intraperitoneal injection of 100 µc of ^{3}H-orotate (specific activity 12.1 c/mmole) in 1.0 ml of saline. Standard fractionation procedures were used (see Fig. II, Methods). Radioactivity and ORM were determined as usual in supernatant II after incubation of nuclear lysate with RNase-free buffer, RNase A (20 µg/ml) and/or RNase T_1 (5 µg/ml). In Experiment I, pellet I was not washed and was corrected for contaminating labeled ribonucleotides as described in the Methods section. Each value is given as mean ± standard error.

A-resistant ORM per 100 mg DNA (compare 2.49 with 1.88 μmole ribose per 100 mg DNA). However, the decrease noted in Experiment I was not statistically significant ($P > .05$)*. In both experiments, however, there was a significant increase ($P < .05$)* in the specific activity (CPM per mμmole ribose) of RNase A-resistant ORM when RNase T_1 was used concurrently with RNase A.

Figure IV gives the relative proportions of nuclear ORM fractionated by treatment of nuclear lysates with RNase-free buffer, RNase A and/or RNase T_1. Table VII summarizes some of the characteristics found for these fractions of nuclear ORM.

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*P was determined by the use of the Student's t-test (two-tail).

Figure IV.

Relative proportions of nuclear ORM fractions in rat liver.

Relative proportions of nuclear fractions of ORM. Properties of each numbered fraction are given by corresponding numbers in Table VII. The values for Fig. IV were calculated from the data in Table VI.

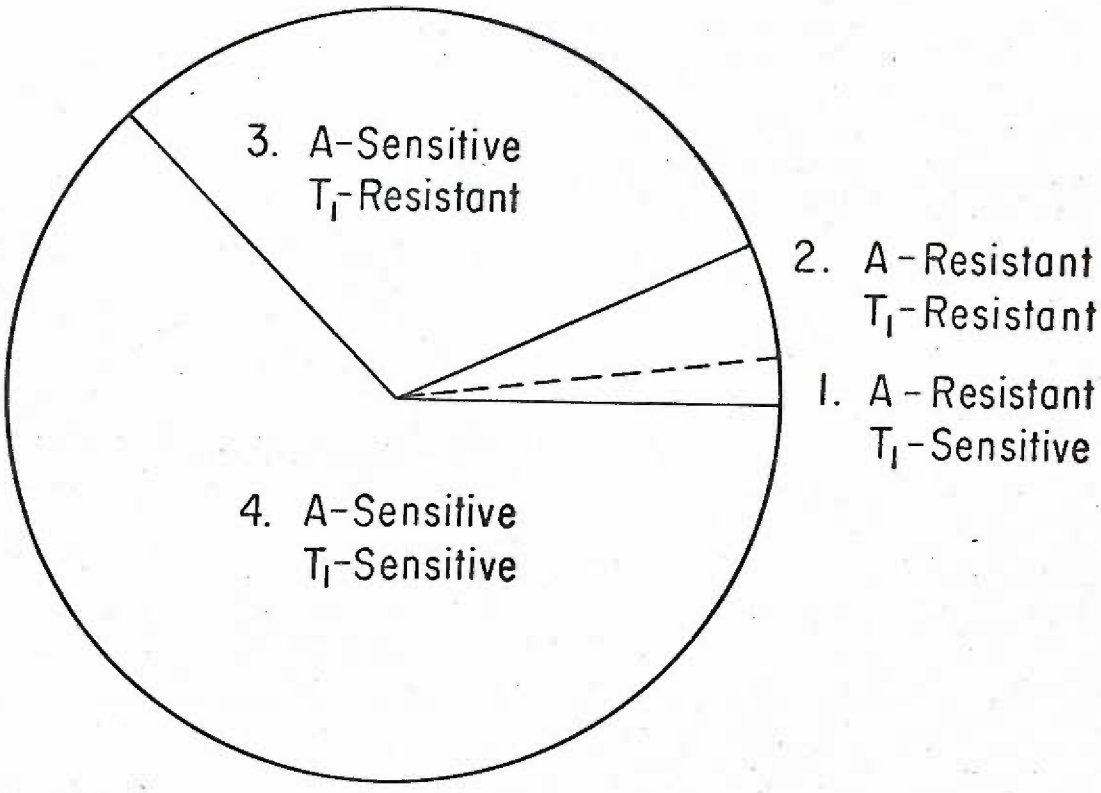
3. A-Sensitive
T_1-Resistant
2. A-Resistant
T_1-Resistant
1. A-Resistant
T_1-Sensitive
4. A-Sensitive
T_1-Sensitive

TABLE VII.

Summary of Properties for Rat Liver Nuclear ORM Fractions

ORM Fraction (see Fig. IV)	Ribonuclease Sensitivity	Chemical Nature	Percent of Total Nuclear ORM		Relative Specific Activity	
			Exp. I	Exp. II	Exp. I	Exp. II
#1	A-Resistant T_1-Sensitive	RNA	0.9	1.6	<.15	< .13
#2	A-Resistant T_1-Resistant	Unknown	5.0	5.0	.15	.13
#3	A-Sensitive T_1-Resistant	RNA	28	30	.32	.45
#4	A-Sensitive T_1-Sensitive	RNA	67	65	1.0	1.0

Properties of nuclear fractions of ORM: The relative proportion of each numbered fraction is given by corresponding numbers in Fig. IV. The values for Table VII were calculated from the data of Experiments I and II shown in Table VI.

DISCUSSION

Orcinol reacting material resistant to RNase A (which shall be referred to as RNase A-resistant ORM) was detected in rat liver nuclei. Table II shows that there was a considerable range in the ratio of RNase A-resistant ORM to DNA in ten separate experiments. Fukuda (21) has shown that in rats various dietary restrictions can cause as much as a threefold change in the amount of RNA per liver cell. Whether the variation in ORM between experiments was due to a metabolic property of RNase A-resistant ORM, the age of the rats, seasonal variation, or other factors could not be determined from the data.*

Table II shows that 6 to 12 percent of total nuclear ORM is resistant to RNase A and the remaining 88 to 94 percent is sensitive to RNase-A. This RNase A-sensitive ORM shall be referred to as RNase A-sensitive RNA since it is rendered acid-soluble by a ribonuclease.

If micromoles of ribose for RNase A-resistant ORM are converted to micrograms of RNA (see Appendix, pp. 40), the mass ratio of RNA to DNA is comparable to that reported for chromosomal RNA. The mass ratio of RNase A-resistant ORM to DNA ranges from .015 to .044 μg RNA/μg DNA; the reported mass ratio for rat Novikoff ascites cell chromosomal RNA is .04 μg RNA/μg DNA (4).

Following partial hepatectomy in rats the residual part of the liver grows rapidly and reaches the size of a normal liver in two to three weeks (15). During the first 24 hours after operation, hepatic cells increase only in size, not in number; after this latent period

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*No explanation can be provided for the obvious differences in the amounts of ORM found in Experiments I-IV as compared with Experiments V-X.

they begin to divide rapidly (22). In the rat this lag period has been characterized by several changes. The synthesis of nuclear RNA is stimulated almost immediately by partial hepatectomy, but stimulation of DNA synthesis does not occur until approximately 18 hours after surgery (23, 24, 25). The appearance of "new species" of RNA has been shown by DNA-RNA hybridization (26), and also by increase in the DNA template activity of isolated nuclei (27) and chromatin fractions (28). Because of these relatively well-characterized changes in RNA metabolism, it was felt that alterations in hepatic RNase A-resistant ORM could be most easily achieved by partial hepatectomy.

Table IV shows that at 24 to 25 hours after hepatectomy there was a 52 percent increase in the ratio of total nuclear ORM to DNA. This compares closely with the 46 percent increase in the ratio of total nuclear RNA to DNA 18 hours after partial hepatectomy in rats reported by Busch (29). Table III shows that 24 to 25 hours after hepatectomy there was a 39 percent increase in the ratio of RNase A-resistant ORM to DNA.

It should be noted that the presence of hexoses, sucrose, polysaccharides, glycogen, large amounts of protein and DNA all may interfere with the orcinol reaction causing an over-estimation ribose (19). A correction was made for DNA interference. No such correction was made for possible interference by glycogen or protein. However, it is of interest to note that 24 hours after hepatectomy in rats, others have reported decreases in the ratios of glycogen to DNA (30) and total nuclear histone to DNA (31), compared to those ratios in sham-operated

rats. Such alterations in nuclear composition would tend to cause a reduction in ORM after partial hepatectomy rather than increase ORM and hence cannot explain the increases shown in Table III.

A portion of nascent RNA (i.e., RNA in the process of being synthesized and still attached via RNA polymerase and hybridization to its DNA template) may be resistant to RNase A (13). Therefore, a portion or all of RNase A-resistant ORM could merely represent nascent RNA. This possibility was evaluated by short term labeling with ^{3}H-orotate. During 10, 20 and 30 minute labeling periods with ^{3}H-orotate the specific activity of total nuclear ORM was about six times that of RNase A-resistant ORM (Fig. III). Nascent RNA by its very nature would be expected to have the highest specific activity of any RNA found in the nucleus. Since the specific activity of RNase A-resistant ORM is only one-sixth that of total nuclear ORM, nascent RNA is not a likely candidate for a major part of this fraction. Based on the specific activities shown for RNase A-resistant ORM (Fig. III), and using the specific activity of total nuclear ORM as the lowest possible estimate of the specific activity of nascent RNA, it was estimated that a maximum of 17 percent of RNase A-resistant ORM could be nascent RNA. These data also suggest that RNase A-resistant ORM is metabolically a more stable substance with respect to orotate uptake than RNase A-sensitive RNA.

The relative turnover of orotate in RNase A-resistant ORM (supernatant II after RNase A treatment, see Fig. II) and RNase A-sensitive RNA (supernatant I, after RNAse A treatment, see Fig. II) was estimated by studying the retention of label in these fractions after exposure to

^{3}H-orotate. Table V shows that in both control and partially hepatectomized rats, the retention of label expressed as percent of that found after the last injection was always greater in the RNase A-resistant ORM fraction than that in the RNase A-sensitive RNA fraction. This suggests further that RNase A-resistant ORM was metabolically a more stable substance than RNase A-sensitive RNA.

Two facts argue that RNase A-resistant ORM may be RNA. First, ^{3}H-orotate, a known precursor of RNA, is incorporated into this fraction (Tables V and VI). Second, the fraction is alkali labile (most of this material being rendered acid-soluble by digestion in 0.3 N NaOH at 37° C for 18 hours; see Table I).

Admittedly, the chemical nature of RNase A-resistant ORM is uncertain at this time and it is not possible to conclude that it represents chromosomal RNA. It can be noted, however, that RNase A-resistant ORM has three properties similar to chromosomal RNA: first, it is resistant to ribonuclease (5, 13); second, if the ORM expressed as micromoles of ribose is converted to micrograms of RNA, the calculated mass ratio of RNA to DNA is comparable to that which has been reported for chromosomal RNA (4); third, RNase A-resistant ORM, like chromosomal RNA, has a relatively slow turnover rate (4). These similarities suggest, but do not prove, that RNase A-resistant ORM is identical to chromosomal RNA.

If these substances were identical, and if chromosomal RNA were acting as a derepressor molecule according to the hypothesis of Frenster*, then an increase in the ratio of RNase A-resistant ORM

*See Introduction for a description of the models proposed by Bonner and Frenster for the function of chromosomal RNA.

to DNA would be expected in the nucleus of hepatocytes following partial hepatectomy. Conversely, a decrease ratio would be expected if chromosomal RNA was acting as a repressor molecule, according to the Bonner model. Therefore, the increase in RNase A-resistant ORM found following partial hepatectomy would be more compatible with the theory of Frenster in which chromosomal RNA is acting as a derepressor molecule.

RNase A (beef pancrease 2.7.7.16) splits the internucleotide bonds between pyrimidine nucleoside phosphoryl groups and purine or pyrimidine groups. Therefore, apyrimidinic RNA is resistant to hydrolysis by RNase A. To test for the existence of apyrimidinic RNA in RNase A-resistant ORM, RNase T_1 was added to the nuclear lysate with RNase A. RNase T_1 (aspergillum oryzae 2.7.7.26) splits the internucleotide bonds between guanylic acid groups and purine or pyrimidine groups. Apyrimidinic RNA which is resistant to RNase A is hydrolyzed by RNase T_1 (32). Table VI shows that only a small part (16 to 24 percent) of RNase A-resistant ORM is rendered acid-soluble by RNase T_1. This suggests that most of the RNase A-resistant ORM is not apyrimidinic RNA. This does not preclude that RNase A-resistant ORM may represent polyadenylic acid.

The experiment mentioned above was originally intended to test for the existence of apyrimidinic RNA. A totally unexpected finding was that a relatively large portion (one-third) of total nuclear ORM was resistant to RNase T_1. The bulk of this RNase T_1-resistant ORM could be hydrolyzed by RNase A and was labeled with ^{3}H-orotate to a lesser extent than total nuclear ORM (Table VI). On the basis of these two experiments total nuclear ORM can be fractionated into at least three, or possibly four fractions. Five IV shows the relative proportion of each fraction. Table VII lists some of the properties each fraction may have.

The existence of fraction #1 (T_1-sensitive, A-resistant) is questionable because it represents such a small fraction of total nuclear ORM and the amount of this material was statistically significant ($P < .01$) in Experiment II (Table VI) but not in Experiment I ($P > .05$). The specific activity of RNase A-resistant ORM increased ($P < .05$) in both experiments, however, when RNase T_1 was added concurrently with RNase A (Table VI). This change in specific activity of RNase A-resistant ORM suggests two kinds of RNase A-resistant ORM with different specific activities and, therefore, indirectly supports the existence of fraction #1. The ORM of fraction #1 is probably RNA since it is sensitive to RNase T_1. However, with only two experiments definite conclusions regarding fraction #1 are precluded at this time.

From Table VI it is possible to calculate that 93 to 94 percent of total nuclear ORM is sensitive to RNase A and, therefore, is probably ribonucleic acid. Of this RNA 29 to 32 percent is RNase T_1 resistant (fraction #3 of Fig. IV) and shall be called "RNase T_1-resistant RNA". The other 68 to 71 percent of this RNA that is sensitive to RNase T_1 (fraction #4 of Fig. IV) shall be called "RNase T_1-sensitive RNA".

The specific activity of RNA as determined by ^{3}H-orotate incorporation and assay for orcinol reacting material may be influenced by base composition of the particular RNA fraction such that the quantitative comparison of such data between fractions may be clouded. RNase T_1 hydrolyses specifically at guanosine nucleotide linkages and might tend to put guanidylic acid (a purine) in the acid-soluble fraction. If

true, the RNase T_1-sensitive RNA and RNase T_1-resistant RNA fractions may not contain the same proportion of purines to pyrimidines. Since orcinol reacts almost entirely with purine-bound ribose, the ribose determination may not reflect the true relative distribution of RNA in these two fractions. If the purines were enriched in the RNase T_1-sensitive fraction the estimate by the orcinol reaction of RNA will be too high and since most of the ^{3}H-orotate ends up as UMP (a pyrimidine) residues in RNA the calculated specific activity for this fraction would be artificially low. The reverse is true for RNase T_1-resistant RNA fraction. Since these considerations tend to increase the specific activity of the RNase T_1-resistant RNA and decrease the specific activity of the RNase T_1-sensitive RNA, a qualitative evaluation is still possible and it can be concluded that the specific activity of RNase T_1-resistant RNA is indeed less than that for RNase T_1-sensitive RNA.

The sensitivity of RNase T_1-resistant RNA to various concentrations of RNase T_1 has not been tested. However, two findings would suggest that RNase T_1-resistant RNA is not due to incomplete digestion of nuclear RNA. First, although RNase A and RNase T_1 were added to the nuclear lysates at final concentrations of 20 µg/ml and 5 µg/ml, respectively, the concentrations of RNase T_1 in terms of enzyme activity was at least twice that of RNase A under these experimental conditions (33). This concentration of RNase A was more than sufficient for the complete digestion of nuclear RNA (see Fig. I) and, therefore, 5 µg/ml of RNase T_1 should be sufficient for the complete digestion of nuclear RNA. Secondly, it would be difficult to explain the lower specific

activity of RNase T_1-resistant RNA compared to RNase T_1-sensitive RNA if RNase T_1-resistant RNA was merely the result of incomplete digestion of nuclear RNA. In fact, the specific activities of these RNAs should be reversed if this were the case (see above).

The structural nature and functional role of RNase T_1-resistant RNA are unknown. It is of interest to note, however, that rat liver nucleolar RNA has two properties similar to RNase T_1-resistant RNA. First, approximately 28 to 30 percent of total nuclear RNA in rat liver has been reported to be nucleolar RNA (33, 34, 35). This agrees with the 28 to 30 percent of total nuclear ORM which was found to represent RNase T_1-resistant RNA (Table VII). Secondly, during labeling periods of about 15 minutes with ^{14}C-orotate (36) or ortho-phosphate-^{32}P (29) the specific activity of nucleolar RNA has been reported to be 60 to 70 percent that of extranucleolar RNA in rat liver. The results shown in Table VII indicate that following a 20 minute labeling period with ^{3}H-orotate, the specific activity of RNase T_1-resistant RNA was 33 to 50 percent that of RNase T_1-sensitive RNA. The data of Table VII compare qualitatively with the specific activities reported for nucleolar and extranucleolar RNA, but quantitatively there is some discrepancy.

The yield of nucleoli as reported by these investigators was low (about 25 percent) and therefore may have represented a biased sample with respect to nucleolar size. It is known that the specific activity (cpm/mg RNA) of large nucleoli increase three to five times faster than the specific activity of small nucleoli when both are exposed

for short periods to labeled precursor (37). Therefore, a biased sample in favor of large nucleoli might account for the discrepancy noted above.

Although the molecular weight of RNase T_1 (11, 127) is comparable to that of RNase A (13, 683), RNase T_1 (isoelectric point pH 2.9) differs substantially from RNase A (isoelectric point pH 7.9) in that RNase T_1 is a negatively charged protein under the incubation condition of pH 7.5 (33). Since RNase T_1 carries a net negative charge at pH 7.5, it is possible that it is repelled by the negatively charged RNA molecules which are known to be concentrated in the nucleolus (29). Such electrostatic interactions may explain the sensitivity of this RNA to RNase A which would carry a slightly positive charge at this pH.

It would be of great importance to know if RNase T_1-resistant RNA is nucleolar RNA. The isolation of nucleoli has been recently reviewed by Busch (38). A major problem in isolating nucleoli is a low yield (20 to 40 percent). This makes normalization of the amount of nucleolar RNA to total cell DNA practically impossible. Furthermore, it is possible that these methods result in the isolation of a biased sample of nucleoli. It is known that different sized nucleoli in rat liver have as much as a fivefold range in the rate of RNA synthesis (37). If RNase T_1-resistant RNA is indeed nucleolar RNA, this identity might offer a simple experimental approach to study the amounts and synthesis on nucleolar and extranucleolar RNA. Hopefully, the results would reflect the changes in the total cell population of nucleoli.

The possibility that RNase T_1-resistant RNA might be nucleolar RNA should be further investigated. One experimental approach might be to isolate nuclei and determine, by the use of either chemical staining or audioradiography, whether RNase T_1-treatment removes RNA from nucleoli.

SUMMARY

Orcinol reacting material (ORM) in rat liver nuclei has been fractionated and characterized on the basis of its sensitivity to RNase A and/or RNase T_1. Four fractions have been defined. Fraction #1 was defined as being RNase A-resistant and RNase T_1-sensitive; fraction #2 was RNase A-resistant and RNase T_1-resistant; fraction #3 was RNase A-sensitive and RNase T_1-resistant; fraction #4 was RNase A-sensitive and RNase T_1- sensitive.

The amount of ORM in fraction #1 bordered on the limits of the assay system used.

Fractions #1 and #2 were studied together as a single substance (RNase A-resistant ORM); combined, they represent about six percent of total nuclear ORM. RNase A-resistant ORM was shown to increase about 40 percent 24 hours after partial hepatectomy and to be considerably more stable than nuclear RNA which was sensitive to RNase A. Its chemical nature is not yet certain, but it has some characteristics expected of RNA (hydrolyzed by alkali and labeled after exposure to ^{3}H-orotate). Although it would be premature to conclude that this substance was chromosomal RNA, the data so far available are consistent with this suggestion.

Fractions #3 and #4 (approximately 28 and 66 percent of total nuclear ORM, respectively) were probably RNA since they were labeled after exposure to ^{3}H-orotate and hydrolyzed by alkali and RNase A. Following a 20 minute labeling period with ^{3}H-orotate, the specific activity of fraction #3 was one-half to one-third that of fraction #4. Based on these properties fractions #3 and #4 appear to represent

two relatively large pools of nuclear RNA with different labeling characteristics. The possible significance of fraction #3 is discussed because it was found to have two properties similar to nucleolar RNA.

APPENDIX

Correction for Contaminating DNA in Supernatant Fraction II.

Orcinol reaction is subject to interference by DNA (39). Several authors have confirmed Schneider's original observation (39) that DNA gives about 12 percent of the color given by RNA on a mass basis (40, 41); other authors (42, 43, 44) have reported values varying from 0.3 to 19 percent.

RNA interferes with the diphenylamine reaction to a lesser degree than DNA interferes with the orcinol reaction. In this laboratory yeast RNA (Worthington Biochemicals) reacts with diphenylamine only one percent as well as DNA on a mass basis.

Since diphenylamine reacting material (DRM) was detected in supernatant II in varying amounts (see Table IX), it was important to establish an accurate correction factor for this contamination. Once a correction factor was determined it was found that the amount of color produced by DRM (presumably DNA) in supernatant II could amount to as much as 25 percent of the total color produced by orcinol. This amount varied greatly among replicate samples (i.e., 0 to 25 percent) within the same experiment.

To determine the extent of cross-reaction in this laboratory, rat liver DNA was isolated as outlined in Figure II (see Methods). The duration of alkali-treatment was varied (8, 18 and 24 hours at 37° C). Table VIII shows that when pellet I was treated with alkali for 18 hours the DRM appearing in supernatant III cross-reacts with orcinol to the extent that 100 mg of DNA is equivalent to 20 μmoles of ribose. Assuming orcinol reacts almost entirely with purine-bound ribose (45,

TABLE VIII.

Effects of Alkali Digestion for Varying Periods

on the Determination of DNA Cross Reaction with Orcinol

	Period of Digestion in Alkali		
	8 hour	18 hour	24 hour
Apparent $\frac{\mu\text{mole ribose}}{100\text{ mg DNA}}$	23 ± 1	20 ± 1	20 ± 1

See text for details on Methods. Each value is given as mean ± standard error, n = 4.

46, 47), 50 percent of rat liver nuclear ribonucleotides are purines (48) and an average molecular weight of 343 for ribonucleotides, micromoles of assayed ribose can be converted to micrograms of RNA. On this basis DNA was calculated to give 14 percent as much color with orcinol as RNA on a mass basis.

It is still possible that the correction factor for cross reaction of DNA with orcinol as determined for purified DNA (supernatant III) may not be valid when applied to supernatant II because of other contaminating factors present in supernatant II which were not present in supernatant III. A statistical analysis of the relationship between ORM and DRM in supernatant II indicates that application of this correction factor is valid. The basic principle underlying this analysis is as follows: the concentration of ribose, $(R)_c$ (which can be considered non-diphenylamine reacting), is calculated by the following equation:

$$(R)_c = (ORM_{II}) - k(DRM_{II})$$

where (ORM_{II}) is the assayed concentration of ribose; (DRM_{II}) is the concentration of DRM in supernatant II, and k is the correction factor (20 µmole ribose/100 mg DNA) determined from supernatant III. If we assume that orcinol reacting material $(R)_c$ and diphenylamine reacting material (DRM_{II}) appear in supernatant II independent of one another, then there should be minimal variability in estimating the "true" concentration of orcinol reacting material $(R)_c$ when the correction for diphenylamine reacting material k is "true". That is, the correction factor will be "accurate" when the ratio of the standard deviation over the mean for $(R)_c$ is a minimum for a large number of samples.

Figure V.

Statistical analysis of the relationship between ORM and DRM in supernatant II.

See text for details on computations and methods.

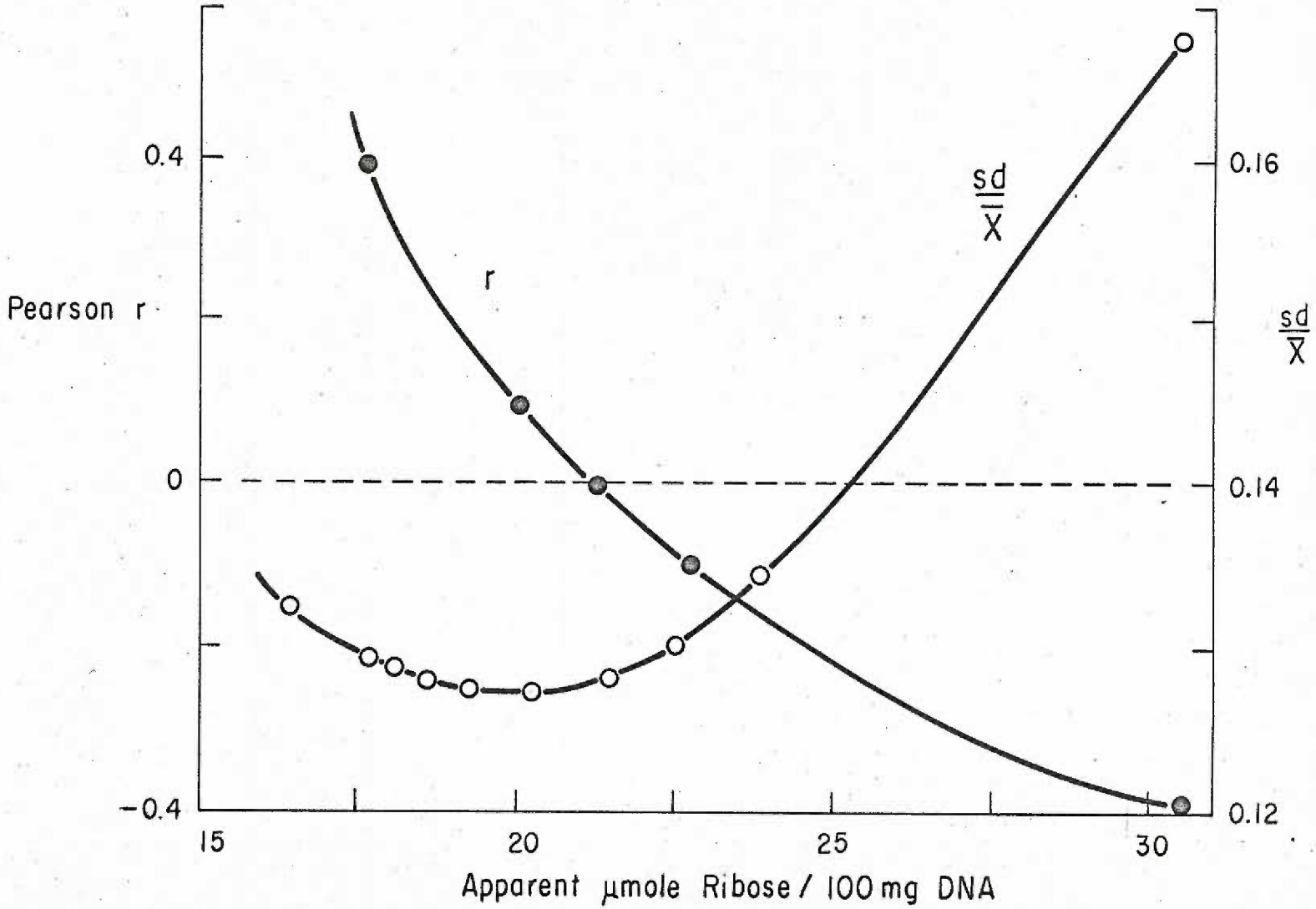
0.4
0
−0.4
Pearson r
r
sd/X
0.16
0.14
0.12
sd/X
15
20
25
30
Apparent µmole Ribose / 100 mg DNA

Figure V shows a minimal amount of variability among 16 samples when the correction factor is approximately 20 µmole ribose per 100 mg DNA. A corollary to this first proposition is that there should be minimal correlation between the amount of diphenylamine reacting material (DRM_{II}) and corrected orcinol reacting material, $(R)_c$, when the coefficient is accurate (i.e., the Pearson r should equal zero). Figure V shows that there is minimal correlation when the correction factor is about 20 µmoles ribose per 100 mg DNA.

Correction for Radioactive Contamination of Pellet I (Fig. II, Methods)

When ^{14}C-ribosomal RNA* was added to nuclear lysate and treated with RNase A (20 µg/ml) for one hour at 37° C and pellet I isolated by one step centrifugation (no further washing of pellet) a small amount of radioactivity was found in the pellet. Of the label in supernatant I, 1.2 ± 0.1 percent (mean ± standard error, n = 3) appeared in pellet I as "RNase A-resistant ^{14}C-RNA counts". Furthermore, when pellet I was washed in 2.0 ml of six percent cold PCA, virtually no radioactivity was detected in the final pellet. Unless specified otherwise, experiments were pellet I was not washed a correction of 1.2 percent of the labeled RNA in supernatant I was applied to pellet I.

*Label ^{14}C-ribosomal RNA was obtained through the courtesy of Dr. Wilbur P. McNulty at approximately 750 dpm/µg RNA. Quenching in this experiment was corrected by using ^{14}C-toluene as an internal standard.

Legend to Table IX.

Nuclear lysates were treated with RNase A or RNase-free buffer and fractionated as shown in Figure II (see Methods). Supernatant II was always assayed for both ORM (ORM_{II}) and diphenylamine reaction material (DRM_{II}) in order that an internal correction be made for cross reacting material. Each value is the result of a single assay on one ml of nuclear lysate. The corrected ORM per 100 mg DNA was calculated by the following equation:

$$\text{corrected ORM} = \frac{(ORM_{II}) - k\,(DRM_{II})}{(DRM_{II}) + (DRM_{III})} \times 10^5$$

where $k = 2 \times 10^{-4}$ apparent µmole ribose per µg DNA (see pages 40-44, Appendix for details concerning the determination of k).

TABLE IX.

RNase A-Resistant ORM and Total Nuclear ORM Content in Rat Liver Nuclei

Sample #		Supernatant II (ORM_{II}) μmole ribose / ml x 10^3	Supernatant II (DRM_{II}) μg DNA / ml	Supernatant III (DRM_{III}) μg DNA / ml	Corrected ORM μmole ribose / 100 mg DNA
Exp. I	1	4.32	2.9	78.6	4.58
	2	3.69	2.5	75	4.12
	3	3.81	2.5	77	4.16
	4	4.19	2.2	72.3	5.03
	5	4.70	2.9	70.6	5.61
	6	5.21	5.0	77.9	5.08
	7	3.56	.5	77.6	4.43
	8	3.81	0	78.3	4.87
	9	3.81	.2	83.7	4.49
	10	2.92	0	78.2	3.73
	11	3.56	0	82.7	4.30
	12	3.68	3.1	83.4	3.54
				x ± s.e. = 4.32 ± .01	
Exp. II	1	5.84	1.4	81.9	6.67
	2	7.87	11.7	75.7	6.33
	3	8.00	11.8	77.4	6.32
	4	6.22	5.4	82.4	5.85
	5	6.60	7.34	75.7	6.18
	6	6.73	10.8	74.6	5.35
	7	6.60	7.5	75.7	6.13
	8	6.48	13.4	74.6	4.32
				x ± s.e. = 5.6 ± 0.2	
Exp.III	1	4.95	.4	115	4.22
	2	5.21	.1	114	4.55
	3	6.86	.9	114	5.81
	4	4.32	.1	118	3.64
	5	5.84	6.3	101	4.27
	6	5.08	6.2	104	3.48
	7	4.32	.1	109	3.94
	8	6.73	0	104	6.47
				x ± s.e. = 4.4 ± 0.4	

(continued next page)

TABLE IX: Continuation
RNase A Treated

Sample #		Supernatant II (ORM_{II}) μmole ribose / ml x 10^3	Supernatant II (DRM_{II}) μg DNA / ml	Supernatant III (DRM_{III}) μg DNA / ml	Corrected ORM μmole ribose / 100 mg DNA
Exp. IV	1	19	2.5	329	5.6
	2	16	2.1	338	4.6
	3	17	.4	348	4.9
	4	16	2.9	339	4.5
	5	25	2.1	397	6.2
	6	22	4.3	431	4.9
	7	28	15.7	390	6.1
	8	28	20	406	5.6
				x ± s.e. = 5.3 ±	0.2
Exp. V	1	14.0	15	420	2.5
	2	14.0	14	413	2.6
	3	15.2	9	499	2.6
	4	15.2	15	493	2.4
	5	15.2	13	392	3.1
	6	14.0	18	403	2.5
				x ± s.e. = 2.5 ±	0.1
Exp. VI	1	10.2	18	293	2.1
	2	11.4	18	303	2.3
	3	11.4	24	335	1.7
	4	11.4	22	3.2	2.0
				x ± s.e. = 1.9 ±	0.1
Exp. VII	1	17.8	11	414	3.7
	2	18.3	16	425	3.4
	3	19.3	29	407	3.1
	4	20.1	24	425	3.4
	5	22.7	22	442	3.9
				x ± s.e. = 3.4 ±	0.1
Exp.VIII	1	18.4	6.2	446	3.8
	2	13.8	7.2	447	2.7
	3	16.4	6.2	462	3.2
				x ± s.e. = 3.2 ±	0.2
Exp. IX	1	22	36	383	3.5
	2	22	42	383	3.2
	3	25	48	369	3.7
	4	25	47	379	3.7
				x ± s.e. = 3.3 ±	0.1
Exp. X	1	17.6	23	502	2.5
	2	17.2	18.5	494	2.6
	3	16.9	18.5	481	2.6
				x ± s.e. = 2.6 ±	0.1

TABLE IX: Continuation

Buffer-Treated

Sample #		Supernatant II (ORM_{II}) µmole ribose / ml x 10^3	Supernatant II (DRM_{II}) µg DNA / ml	Supernatant III (DRM_{III}) µg DNA / ml	Corrected ORM µmole ribose / 100 mg DNA
Exp. I	1	29.8	3.4	79.9	35
	2	32.5	4.5	76.9	39
	3	31.4	1.1	81.2	38
				x ± s.e. = 37 ± 1	
Exp. II	1	44.8	3.5	80.3	52
	2	44.4	9.8	76	50
				x ± s.e. = 51 ± 1	
Exp.III	1	41.0	4.4	113	34
	2	43.8	8.3	105	37
				x ± s.e. = 36 ± 1	
Exp. IV	1	167	4.7	331	49
	2	206	6.0	404	50
				x ± s.e. = 49 ± 1	
Exp. V	1	126	15	430	28
	2	121	15	438	26
	3	147	15	477	29
	4	145	15	502	27
	5	131	16	420	29
	6	135	15	393	32
				x ± s.e. = 28 ± 1	
Exp. VI	1	118	18	308	35
	2	123	24	288	38
	3	74.9	12	249	28
	4	80.0	17	243	29
	5	90.2	16	293	28
	6	94.0	13	293	30
				x ± s.e. = 32 ± 1	
Exp.VII	1	169	4	418	39.9
	2	173	11	417	39.9
	3	187	43	415	39.0
	4	176	34	411	38.0
				x ± s.e. = 39.0 ± 0.4	

(continued next page)

TABLE IX: Continuation

Buffer-Treated

Sample #		Supernatant II (ORM_{II}) µmole ribose / ml x 10^3	Supernatant II (DRM_{II}) µg DNA / ml	Supernatant III (DRM_{III}) µg DNA / ml	Corrected ORM µmole ribose / 100 mg DNA
Exp.VIII	1	190	7.7	419	44.2
	2	199	8.7	441	43.9
	3	193	10	420	44.4
	4	193	11	445	41.8
				x ± s.e. =	43.7 ± 0.4
Exp. IX	1	165	36	361	39.7
	2	176	36	376	41.0
				x ± s.e. =	40.2 ± 0.4
Exp. X	1	195	27	476	37.7
	2	193	23	476	37.8
	3	195	24	477	38.0
				x ± s.e. =	37.7 ± 0.1

LIST OF ABBREVIATIONS

1. TKM solution = .05 M tris-HCl, pH 7.5, at 20° C; .025 M KCl; and .005 M $MgCl_2$.
2. EDTA = Ethylenediamine-tetraacetic acid.
3. RNase = ribonuclease.
4. ORM = orcinol reacting material.
5. PCA = perchloric acid.
6. rpm = revolutions per minute.
7. RNA = ribonucleic acid.
8. DNA = deoxyribonucleic acid.
9. DRM = diphenylamine reacting material
10. ORM_{II} = orcinol reacting material in supernatant II (see Fig. II)
11. DRM_{II} and DRM_{III} = diphenylamine reacting material in supernatants II and III, respectively (see Fig. II).
12. UMP = uridine monophosphate.
13. s.e. = standard error of the mean.

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