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NUCLEAR RIBONUCLEASES AND THE DEGRADATION OF RIBONUCLEIC ACIDS IN ISOLATED NUCLEI OF EHRLICH ASCITES CELLS

BY

ELIZABETH ANN SPEERS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Nuclear Ribonucleases and the Degradation of Ribonucleic Acids in Isolated Nuclei of Ehrlich Ascites Cells" submitted by Elizabeth Ann Speers in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

A method was developed for the isolation of nuclei from Ehrlich ascites cells in aqueous medium. The nuclei were obtained in high yield with the recovery of more than 80% of the DNA, a RNA/DNA ratio of 0.16 and a recovery of 25% to 35% of the cellular protein. In addition, the RNA degrading enzymes of the isolated nuclei were determined. Exo-RNase I and alkaline RNase II activities were found to be associated with the nuclei. However, these two RNases appeared to be soluble and were easily extracted from the nuclei during the isolation procedure. Thus, it was difficult to obtain high recoveries of the RNases in the isolated nuclei.

The relationship between the RNases associated with nuclei and metabolism of nuclear RNA was studied. The levels of RNase activities and the RNA and DNA content of the nuclei were followed during the incubation of nuclei. The degradation of ^{14}C labelled RNA was analyzed using polyacrylamide gel electrophoresis and compared to the processing of nuclear RNA in whole cells. It was found that the degradation of RNA in isolated nuclei was non-specific and probably occurred primarily by endonucleolytic attack.

The acid soluble products generated during the incubation of nuclei were analyzed by DEAE-cellulose, PEI-cellulose, and paper chromatography and high voltage paper electrophoresis. They were found to be nucleoside diphosphates and nucleoside-5'-monophosphates. Upon continued incubation these were degraded to nucleosides due to the presence of phosphatase activity in the nuclear preparation. Since the activity of endo-RNase I in Ehrlich ascites cells is very low,

the endonucleolytic degradation of RNA in isolated nuclei was probably due to alkaline RNase II. The presence of nucleoside-5'-monophosphates in acid soluble samples of incubating nuclei indicated that exo-RNase I was also involved. The occurrence of nucleoside diphosphates in the acid soluble material may indicate the presence of polynucleotide phosphorylase in the nuclei.

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LIST OF ABBREVIATIONS

RNA	- ribonucleic acid
rRNA	- ribosomal ribonucleic acid
tRNA	- transfer ribonucleic acid
mRNA	- messenger ribonucleic acid
Hn-RNA	- heterogeneous nuclear ribonucleic acid
pre-tRNA	- precursor transfer ribonucleic acid
pre-rRNA	- precursor ribosomal ribonucleic acid
DNA	- deoxyribonucleic acid
poly A	- polyadenylic acid
RNase	- ribonuclease
DNase	- deoxyribonuclease
G + C content	- guanine + cytosine content
T_m	- melting temperature
PCA	- perchloric acid
TCA	- trichloroacetic acid
Hepes	- N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
SDS	- sodium dodecyl sulfate
DEAE	- diethylaminoethyl
PEI	- polyethylenimine
EDTA	- ethylenediaminetetracetic acid
Tris	- tris(hydroxymethyl) aminomethane
NAD	- nicotinamide-adenine dinucleotide
GMP	- guanosine monophosphate
UMP	- uridine monophosphate

GDP	- guanosine diphosphate
UDP	- uridine diphosphate
GR	- guanosine
UR	- uridine
UV	- ultraviolet
S	- Svedberg unit of the sedimentation coefficient
mCi	- millicurie
µCi	- microcurie
mmol	- millimole
nm	- nanometer
POPOP	- 1,4-Bis- [2-(5-phenyloxazolyi)]-benzene
PPO	- 2,5-diphenyloxazole

INTRODUCTION

Synthesis and Processing of RNA

Mammalian cells contain several classes of RNA and these are usually designated as rRNA, tRNA, mRNA, and Hn-RNA. Eighty percent of the total RNA is made up of two species of rRNA (28S and 18S RNA) and most of the remainder is tRNA. Some of these RNAs, such as tRNA and rRNA, have been extensively characterized.

When animal cells are exposed to labelled RNA precursors, 90% of the radioactivity incorporated into the RNA of the cell within 30 minutes is found in the RNA of the nucleus. The available evidence suggests that this rapidly labelled RNA is a mixture of large molecules. These are Hn-RNA, which constitutes about 1% of the total cell RNA and is constantly being synthesized and degraded, and 45S RNA which is a precursor to rRNA (Darnell, 1968).

Messenger RNA constitutes only a very small percentage of the cellular RNA and generally has a high turnover rate. The details of the synthesis and destruction of mRNA have not been determined. The base composition of mRNA is similar to that of DNA and it is thought to carry information from the DNA of the cell to the protein synthesizing apparatus. Recently a number of mRNAs have been found to contain a large tract of poly A which is added posttranscriptionally to an end of the mRNA molecule (Darnell *et al*, 1971). The work of Philipson *et al* (1971) seems to indicate that the poly A tract is added to the 3' OH end of the molecule.

A number of workers feel that Hn-RNA is the source of mRNA in the cell and experimental evidence seems to be accumulating in support of this idea. Heterogeneous nuclear RNA ranges in size from 100S to 20S and the majority of Hn-RNA is turned over in the nucleus without entering the cytoplasm (Harris, 1959; Harris et al, 1963; Watts and Harris, 1959). Evidence presented by Soeiro et al, (1966) suggested that Hn-RNA might be the source of mRNA because it had a base composition similar to DNA. Lindberg and Darnell (1970) presented data from hybridization experiments indicating the possibility that cytoplasmic mRNA is derived from the specific cleavage of Hn-RNA. The discovery that many mRNAs contain a large tract of poly A (Darnell et al, 1971) and that a similar tract of poly A is present in Hn-RNA also suggested that Hn-RNA is the source of mRNA in the cell.

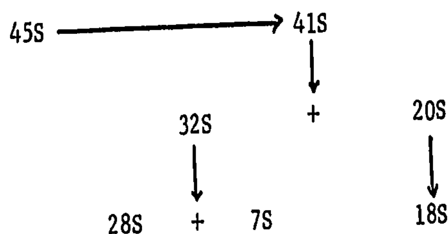
Ribosomal RNA is composed of 4 species of RNA (28S 18S, 7S and 5S) which are integral parts of the ribosomes. In various studies using actinomycin D, an antibiotic that binds to DNA preventing transcription, it has been possible to show that the rapidly labelled 45S RNA disappears on incubation of labelled cells and coincident with this disappearance is the appearance of label in 32S and 18S RNA (Penman, 1966; Scherrer et al, 1963). After longer periods of incubation the radioactivity in 32S RNA shifts to 28S RNA. These studies indicated that 45S RNA is a precursor to rRNA and other studies showed that 45S pre-rRNA synthesis occurs in the nucleolus (Perry, 1962; Perry, 1964).

Ribosomal RNA contains methyl groups attached to the nucleic

acid bases and to the 2' OH of the ribose. Studies in which ^{14}C methyl methionine was used as a precursor, showed the 45S pre-rRNA is methylated before processing occurs (Zimmerman and Holler, 1967). Approximately one-half of the 45S pre-rRNA molecule is discarded during maturation in the nucleolus. The discarded portion is unmethylated and has a base composition which is different from that of 18S and 28S rRNA (Weinberg and Penman, 1970). Ribonucleic acid molecules of various sizes less than 45S (41S, 32S, 24S and 21S) have been isolated. These are thought to be intermediates in the conversion of 45S RNA to 28S and 18S RNA.

Studies by Penman (1966) with isolated nuclei and cytoplasmic fractions have established that 18S rRNA could only be detected in the cytoplasm 30 minutes after the start of labelling of cells and coincident with the appearance of 18S RNA in the cytoplasm was the appearance of 32S RNA in the nucleus. The 32S RNA was then processed in the nucleus to 28S rRNA which did not appear in the cytoplasm until 30 minutes after the appearance of 18S rRNA. A 7S RNA molecule was found hydrogen-bonded to the 28S rRNA molecule and it seemed to arise from the 32S rRNA precursor (Pene et al, 1968).

The sequence of the processing of the 45S pre-rRNA to 28S, 18S and 7S rRNA in HeLa cells is as follows:



The 5S rRNA molecule is not derived from any of the above species of RNA (Darnell, 1968).

Transfer RNA, the carrier of amino acids for protein synthesis, was the first class of RNA to be recognized. The structure of tRNA and its role in protein synthesis have been extensively studied and are quite well understood. However, little is known about the synthesis of tRNA. In the last few years a number of reports have indicated the presence of precursors to tRNA in mammalian cells (Bernhardt and Darnell, 1969; Mowshowitz, 1970). Choe and Taylor (1972) reported kinetic evidence that there was at least one precursor to tRNA in Chinese hamster ovary cells and Ehrlich ascites tumor cells. They have characterized the precursor molecule with respect to its G + C content, methyl/uridine ratio, sedimentation coefficient and T_m value. This precursor is similar to tRNA in G + C content and T_m value. However, it is undermethylated and has a lower sedimentation coefficient.

Ribonucleic Acid Degrading Enzymes

The processing of RNA must involve the action of enzymes. A number of RNA degrading enzymes are known to occur in mammalian cells and many of these have been isolated and characterized, but their physiological functions are not known (Barnard, 1969; Razzell, 1963; Razzell, 1967; Shugar and Sierakowska, 1967). These RNA degrading enzymes appear to exist in most tissues but in varying quantities (Beard and Razzell, 1964). In order to regulate the

levels of RNA molecules in cells, the activity of RNA degrading enzymes would have to be under strict control. The action of any enzymes involved in the processing of molecules such as pre-rRNA would also have to be precise. However, nothing is known as yet about the control mechanism.

Ribonuclease I activity was originally discovered in the nuclei of guinea-pig liver (Heppel, 1966) and has since been shown to exist in the nuclei of other tissues (Razzell, 1963; Lazarus and Sporn, 1967). Lazarus and Sporn (1967) showed that RNase I activity in mouse liver cells and Ehrlich ascites cells was due to two enzymes, an endo-RNase I and an exo-RNase I. Exo-RNase I is specific for polyribonucleotides and degrades single-stranded RNA to 5' mononucleotides from the 3' OH end processively (Sporn et al, 1969). Endo-RNase I hydrolyzes single-stranded RNA to oligonucleotides bearing 5' phosphate end groups. Both endo- and exo-RNase I have a pH optimum of 7.2 to 7.6, have a requirement for Mg^{2+} and a reducing agent for activity and are labile to acid and heat. Ribonucleic acids with a high degree of helical structure are resistant to attack by both RNase I activities. Poly A is used as the substrate in *in vitro* assays because exo-RNase I is 3 times more active with poly A than with RNA (Lazarus and Sporn, 1967).

Alkaline RNase II activity was isolated from mitochondrial and soluble fractions of hog, rat and bovine liver (Beard and Razzell, 1964). This enzyme activity was found to have an endogenous inhibitor which was first described by Roth (1956). Many workers have attempted to show that the inhibitor has a control function. Roth (1956) found the highest levels of the inhibitor in brain, liver

and lung tissue and the lowest levels in kidney and muscle tissue. Kraft and Shortman (1970) examining cell samples with varying metabolic activities for levels of alkaline RNase II inhibitor concluded that, in general, the levels of inhibitor vary with the metabolic state of the tissue. High inhibitor/RNase ratios were associated with states of cytoplasmic RNA accumulation and low ratios were associated with states of RNA catabolism. They suggested that the ratio of the inhibitor/RNase activity is a factor which regulates levels of cytoplasmic RNA by controlling the rate of RNA degradation

Chakravorty and Busch (1967) reported that alkaline RNase II activity was located in the nucleus of normal rat liver and various tumor tissues. Thus, at the moment there is contradictory evidence as to the location of the enzyme activity within the cells. The mode of action of this enzyme is similar to that of RNase A. It has a pH optimum from 7.0 to 8.3 and is highly resistant to inactivation by both heat and acid. The mode of action of alkaline RNase II activity is to hydrolyze only those internucleotide bonds adjacent to the 3' phosphoryl group of pyrimidine nucleosides producing 2', 3' cyclic phosphates. A slowere hydrolysis of the cyclic phosphates to pyrimidine nucleoside 3' phosphates follows the initial hydrolytic step. Extensive hydrolysis of RNA by this enzyme leaves a resistant "core" composed of purine oligonucleotides terminated by a pyrimidine nucleoside 3' phosphate.

Acid RNase II activity has also been found in a number of mammalian tissues (Bernardi and Bernardi, 1966; Colter, Kuhn and

Ellen 1961; Roth, 1963). This enzyme has been located in the lysosomes, is active over the pH range from 4.8 to 5.8, is resistant to inactivation by acid but not by heat and cleaves polynucleotides producing oligonucleotides with free 3' phosphate ends. The mode of action of acid RNase II is similar to that of alkaline RNase II (Razzell, 1967).

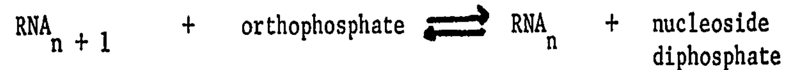
Phosphodiesterase I activity resembles that of snake venom phosphodiesterase. It has a pH optimum of 9.2, is acid labile and requires a divalent cation such as Mg^{2+} for maximal activity. The enzyme activity is located in the microsomal fraction (Razzell, 1961). Although it is non-specific with respect to nucleotide base, the enzyme shows an absolute specificity for a substrate with a free 3' OH function. The products of the hydrolysis are nucleoside 5' phosphates.

Phosphodiesterase II activity was located by Heppel and Hilmoe (1955) and Razzell (1961) in mitrochondrial fractions of the spleen. Variable portions of the activity were found in the supernatant fraction of homogenates of various tissues. The enzyme activity has no divalent cation requirement and a pH optimum of 5.9. The enzyme has not base specificity but a substrate with a free 5' OH function is required. The products of the hydrolysis are nucleoside 3' phosphates.

Both phosphodiesterase I and II are heat labile and are more active against partially degraded polynucleotides (Beard and Razzell, 1964).

Polynucleotide phosphorylase, the enzyme activity which carries

out the following reaction



$$RNA_{n+1} + \text{orthophosphate} \rightleftharpoons RNA_{n} + \text{nucleoside diphosphate}$$

has been reported by Harris (1963) to be present in HeLa cells. Siebert *et al* (1966) and See and Fitt (1970) located the activity in the nucleolus of rat liver cells and in the nuclei of guinea-pig liver cells respectively.

Isolation of Nuclei

Information about the function and specificity of an enzyme *in vivo* is often gained by the use of specific inhibitors. However, there are no effective RNase inhibitors which could be used for studies on RNA catabolism. The location of an enzyme activity within a cell should be related to its function. Therefore, exo-RNase 1, endo-RNase 1 and perhaps alkaline RNase 11 activities which are located in the nucleus of mammalian cells may be responsible for the specific cleavage of precursor RNAs to functional molecules. Thus, it was decided to isolate nuclei to determine the RNA-degrading enzymes present in nuclei and to determine their effect on the nuclear RNA.

The problems involved in the isolation of nuclei from tissues have been extensively reviewed (Blobel and Potter, 1966; Busch, Starbuck and Davis, 1959; Dounce, 1963; Mamaril, Dobrjansky and Green, 1970; Muramatsu, Smetand and Busch, 1963; Muramastu and

Busch, 1967; Siebert, 1967a; Siebert, 1967b; Zalta, Zalta and Simard, 1971). The breakage of cells and the separation of nuclei free of cytoplasm without extraction or redistribution of any nuclear components are the main problems encountered. Since the outer layer of the nucleus is continuous with the endoplasmic reticulum, a clean separation of the nucleus from cytoplasmic components is often difficult(Siebert, 1967a).

Generally, the disruption of cells is carried out in hypertonic sucrose solutions or in organic solvents. The procedure for the isolation of nuclei in hypertonic sucrose buffers involves washing the cells in a buffered sucrose solution, homogenization and then centrifugation to separate the nuclei from other cellular components. Hypertonic sucrose solutions provide the density necessary for the differential centrifugation of cellular components. However, extraction and redistribution of cellular components occurs during homogenization in sucrose solutions. The use of low concentrations of Ca^{2+} and Mg^{2+} (3 mM or less) in hypertonic sucrose buffers tends to tighten up the nuclear membrane and prevents some of this redistribution and extraction.

The isolation of nuclei in organic solvents is a more lengthy procedure. The tissue from which nuclei are to be isolated must be removed, quickly frozen in liquid nitrogen and lyophilized. The dried tissue is filtered, ground in petroleum ether for 30 to 80 hours and then centrifuged through a mixture of cyclohexane and carbon tetrachloride to remove cytoplasmic components and to obtain clean nuclei. There is much less redistribution of cellular components when organic solvents are used for the isolation of nuclei

as compared to the use of hypertonic sucrose solutions. Siebert (1967a) recommended the use of this method for the isolation of nuclei, if possible, for he believed that a more accurate representation of the *in vivo* situation was obtained. However, organic solvents may have an adverse effect on enzyme activity and the yield of purified nuclei obtained by this procedure is only of the order of 20%. Furthermore, it is not known to what extent nuclear RNA is degraded during this lengthy procedure.

To evaluate the quality or condition of isolated nuclei several criteria can be used. Microscopic examination of samples of isolated nuclei can give an estimation of cytoplasmic contamination and the number of whole cells and broken nuclei present. A fairly reliable method of evaluating the quality of isolated nuclei is to determine the RNA/DNA ratio because a close relationship between this ratio and the purity of the preparation as seen under the electron microscope was reported (Siebert *et al*, 1965). The loss of RNA from the nucleus during the isolation results in a decreased RNA/DNA ratio and contamination by cytoplasmic RNA results in an increased RNA/DNA ratio. Siebert *et al* (1965) reported values of 0.12 to 0.21 for the RNA/DNA ratio in isolated liver nuclei depending upon the method of isolation.

The recovery of nuclei during isolation is often estimated by comparing the DNA content of the cells with the DNA content of the nuclear sample. A reliable estimation of the recovery of nuclei can also be obtained by the measurement of nuclear marker enzymes. Marker enzymes have a single intracellular localization and are relatively insoluble in aqueous media (Siebert, 1963). Nicotinamide

adenine dinucleotide synthetase is a marker enzyme of the nucleus.

In summary, precursors to tRNA, rRNA and probably to mRNA are known to occur in mammalian cells. These precursors are processed to the functional units of RNA in the cell and at least two of these processing reactions occur in the nucleus. A precisely controlled mechanism, probably involving nuclear RNases, must be responsible for the specific degradation of high molecular weight RNA precursors.

It was decided to isolate nuclei from Ehrlich ascites cells and to characterize them with respect to their RNA and RNase activities and then study the effect of these RNases on the nuclear RNA during incubation. Exo- and endo-RNase I and alkaline RNase II have been reported to be present in the nucleus of Ehrlich ascites cells (Lazarus and Sporn, 1967; Chakravorty and Busch, 1967). However, the exo-RNase I is the predominant RNase I activity. It was hoped that this might simplify the analysis of RNA degradation products and that some information might be obtained with respect to the physiological role of nuclear RNases.

MATERIALS AND METHODS

Materials

Highly polymerized yeast RNA was purchased from Calbiochem and polyadenylic acid from Miles Laboratories Inc. Guanine-8-^{14}C, 31.2 mCi/mmol was obtained from Schwartz Bioresearch Inc. and uridine-2-^{14}C, 59.8 mCi/mmol from New England Nuclear. DNase was purchased from P-L Biochemical Inc. and crude snake venom (Russell's Viper) from Calbiochem. "Fisher's medium for leukemic cells of mice" was obtained from Grand Island Biological Co., Baker-Flex cellulose PEI plates for thin layer chromatography from J. T. Baker Chemical Co. and DEAE-cellulose (Cellex-D) from Bio-Rad Laboratories. Acrylamide and N,N'-methylenebisacrylamide, products from Eastman Organic Chemicals, were purified according to Loening (1967). All other substrates and chemicals were obtained commercially.

Methods

Maintenance, Collection and Washing of Tumor Cells

Ha/ICR Swiss mice bearing Ehrlich ascites tumor cells were obtained from the University of Alberta Cancer Research Unit. Cells were maintained by weekly intraperitonial injection of tumor cells into mice. Six or seven days after transplantation the mice were killed by cervical dislocation and the cells were collected by aspiration. The intraperitonial cavity was washed with 8 to 10 ml of buffered saline

(0.14 M NaCl, 0.01 M Tris-HCl, 0.004 M $NaPO_4$ and 1 mg/ml glucose, pH 7.4) containing 0.2 mg/ml heparin.

To remove the blood cells and ascites fluid, the tumor cells were washed three times with 10 to 12 ml of buffered saline in an International clinical centrifuge at 750 x g for 1 minute. The cells were then packed by centrifugation at 1600 x g for 7 minutes. All of the above procedures were carried out at room temperature. The number of cells per ml of packed cells determined by direct counting was $3.86 \times 10^8 \pm 0.2 \times 10^8$.

Enzyme Assays

Enzymatic activities were determined in extracts of Ehrlich ascites cells or in extracts of isolated nuclei of Ehrlich ascites cells. Packed cells were suspended in 8 volumes and packed nuclei in 4 volumes of 50 mM NaCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$ and 10 mM 2-mercaptoethanol, pH 7.4 (buffer 1) and disrupted at 0^oC for 20 seconds using a Branson Instruments Inc. Sonifier. After this treatment of cells and of nuclei samples, enzyme assays were carried out at 37^oC using 20 μl of cell or nuclei extract to initiate the reaction. Necessary dilutions of samples were made in buffer 1. For each assay 4 time points were taken, usually within 20 minutes.

Acid Phosphatase

Acid phosphatase activity was measured in 0.5 ml of 0.5 M ammonium acetate, 0.64 mM p-nitrophenylphosphate, pH 5.5. The reactions were stopped with 0.98 ml of 0.3 NaOH. The absorbance was

determined at 400 nm.

Ribonuclease I

Ribonuclease I activity was measured in 0.08 ml of 13.3 mM Tris acetate, 13.3 mM KPO_4, 0.66 mM Na_2EDTA, 3.3 mg/ml poly A, 2.7 mM $MgCl_2$ and 6.6 mM 2-mercaptoethanol, pH 7.4. The incubation was carried out in Beckman microfuge tubes and the reactions were stopped by the addition of 5 μl of 60% PCA. The tubes were placed in an ice bath for at least 15 minutes and then centrifuged for 30 seconds in a Beckman microfuge. Fifty μl of the supernatant were diluted into 0.25 ml of 4% PCA and the absorbance was determined at 260 nm.

In order to determine the relative amounts of exo-RNase I and endo-RNase I activities, duplicate reactions were carried out. One was stopped with 100 μl of 8% PCA and the other with 100 μl of 8% PCA plus 0.5% uranyl acetate. After centrifugation 100 μl of the supernatant solutions were diluted into 0.2 ml of 4% PCA to determine the absorbance at 260 nm.

For exo-RNase I the ratio of the enzyme activities measured with PCA and PCA plus uranyl acetate as the precipitating agent equals one since mononucleotides which are the products of the exonuclease action remain in the supernatant in both cases. The ratio in the case of endo-RNase I is greater than one since some of the products of the endonuclease action are oligonucleotides and these are precipitated with PCA plus uranyl acetate but to a lesser degree with PCA alone.

Alkaline Ribonuclease II and Acid Ribonuclease II

In order to determine the activity of these enzymes it was necessary to treat the samples with acid and with heat to inhibit interfering enzymes and the RNase inhibitor in cell or nuclei sonicates. The acid treatment involved the addition of 0.4 ml of M H_2SO_4 to 2 ml of cell or nuclei sample. The sample was incubated at 0^oC for one hour . One hundred μl of M Tris acetate, pH 7.4 was added and the final pH of the solution was adjusted to 5.7 to 5.8 by the addition of M NaOH. The sample was then centrifuged for 10 minutes at 27,000 x g. The supernatant is the "acid-treated extract". The acid treatment destroys RNase I activity, phosphodiesterase activities and other interfering enzymes but not acid and alkaline RNase II acitivities or all the alkaline RNase II inhibitor.

Two ml of "acid-treated extract" was adjusted to pH 3.5 with M H_2SO_4 and heated to 60^oC for 22 minutes. The pH was then readjusted to 5.7 to 5.8 with M NaOH and the sample centrifuged at 27,000 x g for 10 minutes. The supernatant is the "acid- and heat-treated extract". The heat treatment destroys acid RNase II activity and the alkaline RNase II inhibitor but not alkaline RNase II.

Alkaline RNase II activity was assayed in 0.08 ml of 18 mM Tris acetate, 1 mM Na_2EDTA, 16 mM KPO_4 and 1 mg/ml RNA, pH 7.8 using 20 μl of the "acid- and heat-treated extract". Acid RNase II activity was determined in the same reaction mixture except the pH was adjusted to 5.5 with acetic acid. Both the "acid-treated extracts" and the "acid- and heat-treated extracts" were assayed. The activity present in the "acid- and heat-treated extract" at pH 5.5 is that of alkaline RNase II and was subtracted from the activity obtained

with the "acid-treated extract" in order to determine acid RNase II activity in the sample.

All reactions were terminated with 5 μl of 60% PCA and prepared for absorbance measurement as outlined for RNase I.

Analytical Methods

The method of Lowry et al (1951) was used for the determination of protein in samples. Bovine serum albumin was used as the standard.

Inorganic phosphate was determined by the method of Ames (1966).

Samples for DNA determinations were precipitated with an equal volume of 10% TCA for 20 minutes at $0^{o}C$ and then centrifuged at 1400 x g for 10 minutes. The pellet was washed with 8 volumes of 5% TCA and resuspended to the original volume in water. The method of Ashwell (1957) was used for the determination of DNA in the samples. Calf thymus DNA was used as the standard.

Samples for the determination of RNA concentration were treated as for DNA determinations. The RNA content was then assayed by the method of Horecker (1957) using ribose as the standard.

The units (μmoles/hour) of enzyme activity were determined based on E = 18,000 for p-nitrophenolate, E = 11,000 for acid-soluble products of RNA and E = 14,200 for acid-soluble products of poly A (Beard and Razzell, 1964).

Isolation of Nuclei

Attempts were made to isolate nuclei from Ehrlich ascites cells using the method of Lazarus and Sporn (1967). The homogenization procedure using a Dounce homogenizer with a tight-fitting pestle was carried out only once and the pellet obtained after centrifugation was suspended in buffer 1.

The procedure for the isolation of nuclei from Ehrlich ascites cells of Mamaril et al (1970) was also attempted. After the diluted homogenate was centrifuged at 600 x g and washed with calcium free-phosphate buffered sucrose the pellet was suspended in buffer 1 for enzyme assays. High speed centrifugation through high density sucrose was not carried out.

The isolation of nuclei from Ehrlich ascites tumor cells in high yield using low ionic strength aqueous medium for washing and homogenization of cells was developed and used throughout this work. Tumor cells were obtained from mice 6 or 7 days after transplantation, washed and packed by centrifugation. These packed cells were suspended in 19 volumes of 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM NaCl and 10 mM Hepes (N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid) pH 7.0 (buffer 2). This suspension was centrifuged at 1600 x g for 3 minutes at 4^oC in an International PR-J centrifuge. This washing was repeated once. The cells were then suspended in a volume of buffer 2 equivalent to 9 times the original packed cell volume. Triton N-101 was added to a final concentration of 0.1% and the cells were homogenized with 8 to 10 strokes in a Dounce homogenizer with a tight-fitting pestle. The efficiency of this

homogenization was followed under the phase contrast microscope.

The nuclei were sedimented in an International PR-J centrifuge at 221 x g for 3 minutes at $4^{o}C$. The nuclear pellet was washed 2 times by centrifugation in buffer 2. In order to prepare extracts for enzyme assays, the final nuclear pellet was suspended in buffer 1 using an amount equivalent to 4 times the original packed cell volume.

Incubation and Labelling of Ehrlich Ascites Cells

Washed and packed Ehrlich ascites tumor cells were suspended in "Fisher's medium for leukemic cells in mice" containing 50 mM Hepes ("modified Fisher's medium") to obtain a 2% cell suspension. Incubation was carried out at $37^{o}C$ in a shaking water bath. The required label (^{14}C guanine and/or ^{14}C uridine) was added after a 5 minute preincubation period.

To determine the amount of radioactivity in acid-precipitable material, a 0.2 ml sample of the cell suspension was precipitated in 4% cold PCA and the precipitate was washed on Whatman No. 3 filter paper with 8 ml of 4% cold PCA and then with 2 ml of 5% cold TCA. These filter discs were counted in a liquid-scintillation counter using Bray's solution (Bray, 1960).

The total radioactivity in the cell suspension was measured by counting a 0.2 ml sample of the cell suspension in 5 ml of Bray's solution.

To obtain isolated nuclei from labelled cells, the cells

were incubated as indicated above and isotope incorporation was terminated by cooling in ice. The cells were then centrifuged for 3 minutes at 1600 x g. From the resulting pellet, nuclei were isolated, using the method developed for Ehrlich ascites cells. The final nuclear pellet was suspended in "modified Fisher's medium" to 2% of the original cell volume and the incubation was continued for the times indicated.

Extraction of RNA from Ehrlich Ascites Cells and Isolated Nuclei

The method of Penman (1966) was modified and used to extract RNA from Ehrlich ascites tumor cells and isolated nuclei. Cells from 4 ml of a 2% cell suspension or isolated nuclei from 4 ml of a 2% nuclei suspension with added unlabelled carrier cells (0.4 ml of a 10% cell suspension) in either 0.15 M NaCl, 0.01 M Tris-HCl, and 0.01 M Na_2EDTA, pH 7.4 (buffer 3) or in "modified Fisher's medium" were lysed by the addition of 0.1 ml of 25% SDS. The following extractions were carried out on all samples at $55^{o}C$.

1. Four ml of phenol were added and the sample was vortexed 3 times during a 6 minute incubation. After the addition of 4 ml of 1% isoamyl alcohol in chloroform and mixing, the sample was incubated for a further 4 minutes before centrifugation. This extraction was repeated once.

2. Four ml of 90% phenol saturated with buffer 3 were added and the sample was again vortexed 3 times during a 6 minute period before centrifugation. This extraction was repeated once.

3. Four ml of 1% isoamyl alcohol in chloroform were added and the sample was incubated for 4 minutes before centrifugation.

This extraction was repeated once.

After each extraction the sample was centrifuged for 10 minutes at 1600 x g and the organic phase was removed. After the final centrifugation the aqueous phase was removed and the RNA was precipitated at -20°C for 2 hours after the addition of 0.8 ml M sodium acetate, pH 5.0, 0.04 ml of 0.1 M $MgCl_2$ and 12 ml of 95% ethyl alcohol. The precipitate was collected by centrifugation at 12,000 x g for 10 minutes and washed 2 times with 5 ml of cold 0.1 M sodium acetate in 70% ethyl alcohol. The excess alcohol was removed under reduced pressure. The pellet was then dissolved in 1 ml of 25 mM sodium acetate, 5 mM $MgCl_2$, pH 5.5 and treated with 50 μg of DNase for 30 minutes at room temperature. The RNA was reprecipitated at -20°C for at least 2 hours after the addition of 0.1 M sodium acetate, pH 5.0 and 3 ml of 95% ethyl alcohol. The final pellet was dissolved in approximately 0.5 ml of electrophoresis buffer (50mM sodium phosphate, 1 mM Na_2EDTA and 0.1% SDS, pH 7.2) containing 10% sucrose.

Polyacrylamide Gel Electrophoresis of RNA

Polyacrylamide gel electrophoresis was carried out in a 2% gel containing 0.5% agarose (Peacock and Dingman, 1968). The buffer system used was 50 mM sodium phosphate, 1 mM Na_2EDTA and 0.1% SDS, pH 7.4. To prepare 20 ml of gel the following components were combined at 40°C: 6.76 ml water, 1 ml 0.5 M sodium phosphate, pH 7.2, 2 ml acrylamide:bisacrylamide (20%:1%), 0.12 ml 10% N,N,N',N'-tetramethylethylenediamine, 10 ml 1% agarose and 0.12 ml 10% ammonium

persulfate. Gels were cast in 5 mm I.D. glass tubing and pre-run in electrophoresis buffer to equilibrate. Samples, usually 50 μl, were applied and electrophoresed for 90 minutes at room temperature using 5 mamps/gel tube. The gels (9.2 cm) were scanned at 260 nm using a Gilford Model 240 Spectrophotometer with the gel scanning attachment connected to a Photovolt recorder. In order to determine the isotope concentration in the gels approximately 2 mm slices were cut. These were incubated with 0.2 ml of concentrated NH_4OH for 18 hours at 37°C and counted in a liquid scintillation counter after addition of Bray's solution (Bray, 1960).

DEAE-Cellulose Chromatography

The DEAE-cellulose was suspended in 10 volumes of water and allowed to settle for 30 minutes. The supernatant was decanted and the procedure repeated until no fines were left in the supernatant. The DEAE-cellulose was then washed in a Buchner funnel with 10 volumes of 0.5 N HCl, followed by 10 volumes of water, then 10 volumes of 0.5 N NaOH and finally 10 volumes of water. This procedure was repeated and the DEAE-cellulose was then suspended and stored as a slurry in 2 M NaCl. Columns (1.2 cm x 35 cm) of DEAE-cellulose in 2 M NaCl were packed under pressure and washed with 1 M NH_4HCO_3, pH 8.0 until no Cl^- ion could be detected in the effluent using the $AgNO_3$ test. Before application of the sample, the column was equilibrated with 10 mM NH_4HCO_3, pH 8.0. The conductivity of the sample was adjusted until it was equal to 0.05 M NH_4HCO_3 before it was applied

to the column. The sample was eluted using a gradient consisting of 500 ml of 10 mM NH_4HCO_3, pH 8.0 and 500 ml of 0.2 M NH_4HCO_3, pH 8.0. Fractions of 7 ml were collected with a flow rate of 30 ml/hour. Four μmoles each of 5' UMP and 5' GMP were added to the sample before it was applied to the column in order to provide enough optical density for an absorbance 260 nm profile. After the completion of the gradient, elution was continued with one column volume of 0.5 M NH_4HCO_3, pH 8.0.

One-Dimensional Thin-Layer Chromatography

In order to separate purines, pyrimidines and their phosphate derivatives the method of Crabtree and Henderson (1971) was used. Lines were drawn on Baker-Flex PEI-cellulose plates with a soft pencil 2 cm from the edge (origin line), 2.5 cm from the origin and 7.0 cm from the origin. A piece of Whatman 3 MM paper 2.8 x 20.5 cm was folded to be approximately 2 to 2.5 cm long and 20.5 cm wide. This wick was attached to the top 0.5 to 0.75 cm of the chromatogram sheet with staples. The PEI-cellulose sheets plus wick were washed in glass tanks containing 4 M sodium formate buffer, pH 3.4 for 6 hours or until the yellow impurities which travel with the solvent front were well up on the wick. After drying the salt was removed by development of the plates in methanol-water (1:1) for 8 to 14 hours. The plates were then dried at room temperature.

The origin line was marked off in 1 cm segments which were 2 cm apart. Samples plus nucleotide controls (10 μmoles/ml) were spotted at the origin. Plates were developed in 0.5 M sodium formate,

pH 3.4. When the solvent reached the line 2.5 cm from the origin, the plates were transferred without drying to a tank containing 2 M sodium formate buffer, pH 3.4. The plates were finally transferred to a tank containing 4 M sodium formate buffer, pH 3.4, when the solvent front reached the line 7.0 cm from the origin. The plates were removed and dried when the final solvent front had gone onto the wick about 0.5 cm. Areas containing UV light absorbing material were marked. When labelled samples were applied, the appropriate area of the plate was cut into one cm strips and these were counted in 10 ml of toluene phosphor solution (4 gms PPO, 0.15 gms POPOP and 1000 ml toluene) in a Nuclear Chicago Liquid Scintillation Counter (Mark 1).

Paper Chromatography

Descending paper chromatography on Whatman #1 paper was used to separate the nucleosides, uridine and guanosine, from the base guanine. The solvent used was isobutryic acid plus M NH_4OH (50:30).

Base and nucleoside areas were visible under UV light and were marked. The position of labelled samples was determined by cutting the appropriate areas into 2 cm strips and these were counted in 5 ml of Bray's scintillation fluid.

Treatment of Mononucleotides from DEAE-Cellulose Columns with Crude Snake Venom

In order to determine the position of the phosphate group in

mononucleotides, samples were subjected to treatment with crude snake venom. The peaks from the DEAE-cellulose column chromatography corresponding to GMP and UMP, respectively, were freeze dried and dissolved in a minimum of water. Aliquots (0.1 ml) of each sample were adjusted to 40 mM NH_4HCO_3 and 0.4 mM $MgCl_2$ and 5 μg of crude snake venom was added to each before incubation at 37^oC. The incubation times required for complete digestion were determined by the treatment of known amounts of 5' UMP and 5' GMP with crude snake venom. The reaction was followed by the determination of inorganic phosphate. No release of inorganic phosphate could be detected when 2'3' mixed isomers of both UMP and GMP were subjected to the same treatment. After the completion of the incubation, samples of each incubation mixture, along with GMP, GR, UMP and UR as controls, were spotted on washed PEI-cellulose plates and the plates were developed as described above. Ultraviolet absorbing spots were marked and the appropriate strips in each case were cut into 1 cm segments and counted in 10 ml of toluene phosphor scintillation fluid.

High Voltage Paper Electrophoresis

Peaks from DEAE-cellulose column chromatography corresponding to GDP and UTP were freeze dried and dissolved in a minimum of water. These samples were spotted on 3 MM Whatman paper with the appropriate controls. The samples were subjected to 1500 volts, at 10^oC for 2.5 hours in a Gilson High Voltage Electrophorator, Model D, using 2.5% formic acid plus 8.7% acetic acid, pH 1.9 as the buffer system.

Varsol was used as the coolant. Ultraviolet light absorbing spots were marked and after drying, the appropriate areas were cut into 1 cm strips and counted in 5 ml of Bray's scintillation fluid.

RESULTS

The Isolation of Nuclei from Tumor Cells

In order to relate the activity of RNA degrading enzymes to RNA metabolism in the nucleus of Ehrlich ascites cells, it was necessary to develop a suitable procedure for the isolation of nuclei from these cells. This procedure should give a good recovery of nuclear material as determined by the measurement of DNA and the recovery of nuclei. The nuclei should be free of cytoplasmic contamination as determined by the activity of acid phosphatase and should not be clumpy for this might indicate damage of the nuclear membrane. In addition, the isolation procedure should be rapid in order to minimize degradation of nuclear RNA. The purity of the isolated nuclei could also be determined by assaying the nuclear marker enzyme NAD synthetase and by determining the RNA/DNA ratio for the isolated nuclei.

Preliminary Attempts to Isolate Nuclei

The isolation of nuclei from tumor cells poses a number of problems besides those described in the Introduction. The main problem is that they are more difficult to break than normal cells due to the large amount of connective tissue associated with the tumor cells (Siebert, 1967a).

A number of methods for the isolation of nuclei from tumor

cells have been published (Dounce, 1963; Lazarus and Sporn, 1967; Mamaril, Dobrjansky and Green, 1970; Zalta, Zalta and Simard, 1971). These procedures were attempted with Ehrlich ascites cells and the recoveries of enzyme activities and nucleic acids in the isolated nuclei versus those in the whole cells were compared.

The method of Lazarus and Sporn (1967) involves the homogenization of the tumor cells in a phosphate buffered 0.32 M sucrose solution. The homogenization of the cells in this system was not successful as determined by phase contrast microscopy. The tumor cells showed a high resistance to homogenization and became clumpy as the homogenization was continued in order to obtain greater breakage of the cells. The procedure was carried out only to the stage of centrifugation after homogenization and assays were performed to determine RNase I and acid phosphatase activities and the DNA in the isolated nuclei. The recoveries of RNase I activity and DNA associated with the isolated nuclei were 27% and the recovery of acid phosphatase activity (a cytoplasmic marker) was 15% as compared to the whole cell extract. The nuclei isolated by this procedure appeared to be damaged when samples were checked under the phase contrast microscope. This indication of damage is in agreement with the loss of more than 70% of the DNA from the nuclear fraction. Cytoplasmic contamination of the nuclei seemed high as indicated by the relatively high recovery of acid phosphatase activity. Lazarus and Sporn (1967) did not report on the condition of their isolated nuclei and ,therefore, a comparison of their results with those obtained here could not be made. However, it was decided that the nuclei

prepared by this procedure were not satisfactory for use in further studies.

The method employed by Mamaril et al (1970) involved allowing the cells to swell in a large volume of 2 mM EDTA, pH 7.0. After centrifugation, the cells were resuspended in one half the volume of the same buffer and homogenized with a Dounce homogenizer using a tight-fitting pestle. The homogenate was then mixed with an equal volume of phosphate buffered 0.22 M sucrose solution and the nuclei were separated from other cellular components by centrifugation. The nuclear pellet was washed by resuspension and centrifugation and purified using sucrose gradients. In the present study, this method was carried out as far as the washing of the nuclear pellet. The isolated nuclei were then assayed for RNase I and acid phosphatase activities and the DNA content was determined. The values obtained were compared to those of a cell extract. The isolated nuclei appeared very clumpy under the phase contrast microscope. Sixty-nine percent of the DNA of the cell sample was recovered in the isolated nuclei and this value was in agreement with the value of 63% obtained by Mamaril et al (1970) at this stage of isolation. The recovery of nuclei was 97% as determined by direct counts and that of the RNase I activity 73%. However, the acid phosphatase activity associated with the nuclear fraction was 30% of the cell sample, while Mamaril et al (1970) reported no detectable acid phosphatase activity in the nuclear fraction at this stage of nuclei isolation.

Due to the damaged condition of the nuclei as evidenced by clumping and the high cytoplasmic contamination of the isolated

nuclei this method was not used to isolate nuclei for further studies.

Blobel and Potter (1966) reported a method for the isolation of nuclei from rat liver cells. It is based on homogenization of tissue in buffered sucrose medium and isolation of nuclei by centrifugation. In order to compare the RNA-degrading enzyme activities associated with liver cells with those associated with Ehrlich ascites cells, nuclei of mouse liver cells were prepared via the method of Blobel and Potter (1966). Nuclei of mouse liver cells isolated by this procedure seemed to be of good quality. They were not at all clumpy and further analyses of these nuclei showed approximately 80% recovery of the DNA and less than 5% recovery of the acid phosphatase activity. However, the recovery of RNase I activity associated with the nuclei was only 3% or less.

Assays for RNA-degrading enzymes and acid phosphatase activity were carried out on samples of all fractions obtained after homogenization and after sucrose gradient centrifugation, including the 1.64 and 2.3 M sucrose solutions. The overall recovery of RNase I and acid phosphatase activities was less than 20% of the original activities in the homogenate and over 70% of the RNase I activity which was found, was in the 1.64 M sucrose solution at the top of the gradient.

The poor overall recoveries of these two enzyme activities seemed to result from difficulty in assaying them in high concentrations of sucrose. The fact that most of the recovered RNase I activity was found at the top of the gradient would indicate that RNase I is a soluble nuclear enzyme and was removed from

the nuclei by increasing the solute concentration of the buffer or that it is a cytoplasmic enzyme.

It was not possible to use these isolated mouse liver nuclei for studies of enzyme activities in the nuclei due to the poor recovery of RNA-degrading enzymes.

Isolation of Nuclei from Ehrlich Ascites Tumor Cells

It was apparent that the method for the isolation of nuclei from Ehrlich ascites tumor cells would have to involve homogenization of the cells in a dilute buffer because of the difficulty of breaking these cells in sucrose solutions. The nuclei would then have to be separated from other cell components without the use of sucrose gradients, since sucrose seemed to interfere with the enzyme assays.

The recovery of DNA in the nuclei sample and the recovery of nuclei determined by counting were the only criteria used to determine the recovery of nuclei in the isolation procedure. The nuclear marker enzyme NAD synthetase could not be detected in assays of the whole cell homogenates and, thus, comparisons of cell versus nuclear activities could not be made.

A number of buffer systems for homogenization were tested at varying pH's, as well as at varying salt concentrations. Several methods of homogenization were also attempted. The pH of buffers used in published methods generally varied from pH 6 to pH 7. It was observed here that variations of pH within these limits seemed to have little effect on enzyme or nucleic acid recoveries or on

the condition of isolated nuclei under otherwise identical conditions. Therefore, a pH of 7.0 was chosen for the homogenization buffer.

The Ca^{2+} concentration in the homogenization buffer, however, was very important and a concentration of 1.0 mM seemed to be optimal. When the Ca^{2+} concentration was increased above 1.0 mM the cells were difficult to break and isolated nuclei were contaminated excessively with cytoplasmic components as evidenced by the increase in acid phosphatase activity associated with the nuclei samples. If the concentration of Ca^{2+} was lowered or if no Ca^{2+} was included in the homogenization buffer the nuclei obtained were very clumpy, indicating damage of the nuclear structure. This was also true if the Mg^{2+} was not included in the homogenization buffer. If the concentration of NaCl in the homogenization buffer was increased above 5 mM the cytoplasmic contamination of the nuclei increased as indicated by an increase in the acid phosphatase activity.

It was found necessary to use detergent during the homogenization in order to obtain satisfactory breakage of the tumor cells. Triton N-101 did not seem to interfere in the assay of enzyme activities and so it was chosen. Various levels of the detergent were tried. The use of less than 0.1% Triton N-101 resulted in higher cytoplasmic contamination and some cells could not be broken, even by increasing the number of homogenization strokes. Concentrations of 0.1% were chosen since higher concentrations might cause damage to the nuclear membrane (Siebert, 1967a).

A number of different types of homogenizers were tried in an attempt to find the one which gave efficient breakage of cells. In

the case of most homogenizers, including the Dounce homogenizer with a loose-fitting pestle and the motor driven Teflon homogenizer, 50 or more strokes of the pestle were required for breakage of 50% or more of the cells and the nuclei obtained were extremely clumpy and often appeared damaged under the phase contrast microscope. The Dounce homogenizer with a tight-fitting pestle used with a low ionic strength buffer, however, seemed to give efficient breakage with 10 strokes of the pestle or less and the nuclei obtained after the homogenization generally appeared unbroken and were not clumpy.

A procedure was eventually established which seemed to produce the best results. Ehrlich ascites tumor cells, which had been obtained from mice 6 or 7 days after transplanation, were washed and packed as described in Methods. These packed cells were then washed two times by resuspension in 19 volumes of 10 mM Hepes, pH 7.0, 1 mM $CaCl_2$, 0.05 mM $MgCl_2$ and 5 mM NaCl (Buffer 2) and centrifuged for 3 minutes at 1600 x g. The pellet was then suspended in 9 volumes of buffer 3 and Triton N-101 was added to a final concentration of 0.1%. Homogenization was carried out in a Dounce homogenizer using a tight-fitting pestle. Ten or twelve strokes were necessary and breakage of the cells was followed under the phase contrast microscope. Nuclei were then packed by low speed centrifugation (221 x g) for 3 minutes. The nuclear pellet was washed twice by resuspension and centrifugation with 9 volumes of buffer 3. The washes were found necessary in order to decrease the cytoplasmic contamination associated with the isolated nuclei. As shown in Table 1 the acid phosphatase activity associated with isolated nuclei after one wash decreases 50% with the second wash but a significant decrease in acid phosphatase activity associated

TABLE 1

THE EFFECT OF THE NUMBER OF WASHES ON ENZYME ACTIVITIES

AND DNA ASSOCIATED WITH ISOLATED NUCLEI

	Percent Recovery*			
Number of Washes	1	2	3	4
RNase I Activity	58.2%	68%	68%	65%
Alkaline RNase II Activity	56.6%	16.5%	15.6%	11.9%
Acid Phosphatase Activity	18.9%	8.9%	8.5%	5.2%
DNA	89.1%	86.2%	81.2%	82.8%
Protein	35.7%	30.1%	29.6%	25%

* The values are percentages of those obtained with cell extracts.

with the isolated nuclei was not seen with further washings. Alkaline RNase II activity associated with isolated nuclei decreased after the first wash and remained fairly constant during the remaining three washes. The DNA content and RNase I activity were constant whether the isolated nuclei were washed once or four times.

Characterization of the Isolated Nuclei of Ehrlich Ascites Tumor Cells

The nuclei isolated by the above method were characterized as to their various RNase activities and with respect to their RNA, DNA and protein content. The results are presented in Table 2 and Table 3 respectively.

The recovery of DNA, 85 to 100% of the DNA in the cell sample, was much higher than the values of 20 to 22% reported by Siebert (1967a) for rat liver nuclei isolated by various methods in aqueous buffers. The percent of nuclei recovered from cell samples corresponded to the percent of DNA recovered in the isolated nuclei. Thus, the loss of nuclei during the isolation procedure was low and the isolated nuclei seemed to have retained most of their DNA.

Siebert (1967a) reported values for RNA/DNA ratios for rat liver nuclei which ranged from 0.12 to 0.21 depending upon the method of isolation of the nuclei. The value obtained for the nuclei of Ehrlich ascites cells isolated as described was 0.16 and thus, falls into the above range.

Acid phosphatase and acid RNase II activities associated with the isolated nuclei were always less than 10% of the cell sample

TABLE 2

RIBONUCLEASE AND PHOSPHATASE ACTIVITIES IN EHRLICH ASCITES CELLS AND ISOLATED NUCLEI

Enzyme Activities	Cells μmoles/hr/ml of packed cells	Nuclei μmoles/hr/ml of packed cells	Recovery in Nuclei Percent
RNase I	138.7 ± 14.3	62.3 ± 20.9	30 to 60
Alkaline RNase II	85.3 ± 20	9.4 ± 4.5	7 to 15
Acid RNase II	37.5 ± 11	0.36 ± 0.32	0.09 to 2
Acid Phosphatase	186.8 ± 14	12.1 ± 6.7	3 to 10

The above results are the average of 5 experiments.

TABLE 3

PROTEIN, DNA AND RNA CONTENT OF EHRLICH ASCITES CELLS AND ISOLATED NUCLEI

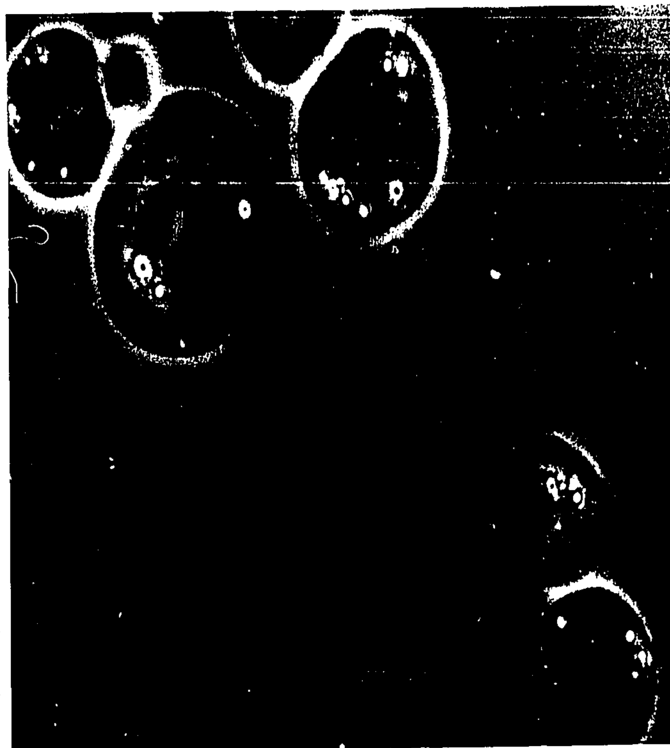
	Cells mg/ml of packed cells	Nuclei mg/ml of packed cells	Recovery in Nuclei Percent
DNA	7.8 ± 0.7	7.2 ± 0.6	85 to 100 (4)*
RNA	4.4	1.3	29.7 (1)
Protein	153 ± 20	46.5 ± 7.5	25 to 35 (4)

( )* Number of experiments averaged for results

indicating low cytoplasmic contamination. RNase I activity associated with nuclei showed considerable variation in recovery (30 to 60% of the cell sample) and the reason for this is not known. The RNase I and alkaline RNase II activity, not associated with the nuclei, was found in the supernatant of the homogenate or in the washes of the isolated nuclei. The ratio of exonuclease activity to endonuclease activity of RNase I was equal to one for isolated nuclei indicating the exo-RNase I was the main RNase I activity. The recovery of alkaline RNase II activity associated with the isolated nuclei varied with different experiments. Due to the fact that the nuclei were isolated by breakage of cells in low ionic buffer there must have been some leakage of soluble material from the nuclei. This would probably account for the variation of RNase I and alkaline RNase II activities associated with the isolated nuclei.

The appearance of nuclei isolated by the procedure outlined in Methods is shown in Figure 1. The photographs were taken with a camera mounted on a phase contrast microscope and the sample was under oil immersion. Isolated nuclei were suspended in 10 volumes of buffer 1 and this suspension was used directly. The combined data of enzyme analyses, DNA content and photomicrographs of the isolated nuclei seemed to indicate that with this isolation procedure it was possible to prepare nuclei from Ehrlich ascites cells in high yield which were intact and reasonably free from cytoplasmic material. This method of isolation was used throughout to obtain nuclei for further studies in RNA catabolism.

A



B

A

B

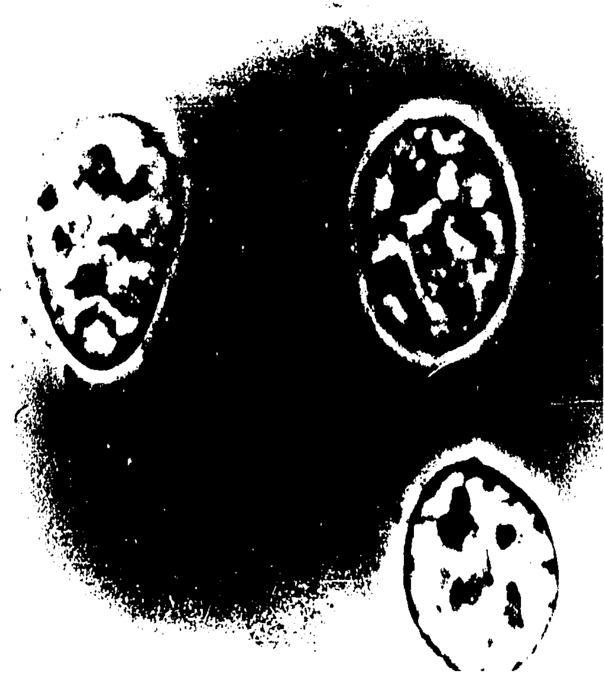


FIGURE 1A

PHASE CONTRAST PHOTOMICROGRAPHS OF EHRLICH ASCITES CELLS

Ehrlich ascites cells were collected, washed and resuspended in 16 volumes of buffer 1 as described in Methods. Samples of the cell suspension were photographed after the preparation of a wet mount. Total magnification is 4300 times.

FIGURE 1B

PHASE CONTRAST PHOTOMICROGRAPHS OF NUCLEI OF EHRLICH ASCITES CELLS

Nuclei were isolated from Ehrlich ascites cells as described in Methods and suspended in 10 volumes of buffer 1. Samples of the nuclei suspension were photographed after the preparation of a wet mount. Total magnification is 4300 times.

Incubation of Nuclei Isolated from Ehrlich Ascites Cells

Although nuclei could be isolated with a relatively high content of RNase activity, it was necessary to find out to what extent the RNases remained within the nucleus during *in vitro* incubation before the effect of these enzymes on the nuclear RNA could be determined.

Nuclei were prepared from Ehrlich ascites cells and suspended in "modified Fisher's medium" for incubation at 37°C. Samples were taken to determine the RNase I, alkaline RNase II and acid phosphatase activities and the DNA content of the nuclei during the incubation period. The values obtained for the enzyme activities and DNA associated with the freshly isolated nuclei were the control values used in the following determinations. The remainder of the suspension was incubated at 37°C. At the times indicated in Figure 2 samples were removed and centrifuged for 3 minutes at 1600 x g. The supernatant was removed and the pellet of the incubated nuclei was resuspended to its original volume in "modified Fisher's medium". 2-mercaptoethanol was added to both nuclei and supernatant samples to the final concentration of 10^{-2} M. RNase I activity was determined in both the nuclei and supernatant samples. DNA and alkaline RNase II activity was determined in the nuclei samples only.

As seen in Figure 2, when the pellet of isolated nuclei was resuspended in "modified Fisher's medium" for incubation, 50% of the RNase I activity associated with the nuclear pellet could be found in the supernatant immediately after suspension of the nuclei.

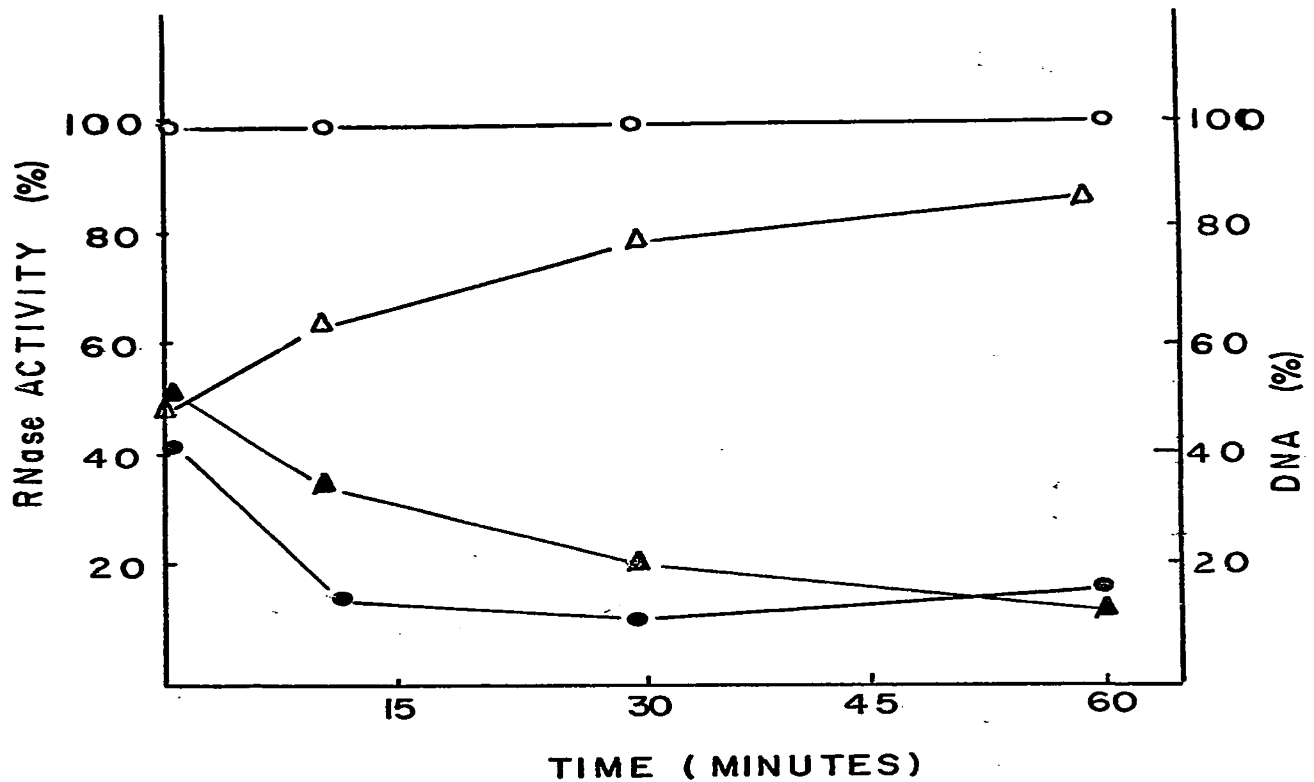

RNase ACTIVITY (%)
100
80
60
40
20
DNA (%)
100
80
60
40
20
15
30
45
60
TIME (MINUTES)

FIGURE 2

LOSS OF ENZYMATIC ACTIVITY FROM NUCLEI DURING INCUBATION

Nuclei were suspended in "modified Fisher's medium" and incubated at 37°C. At the times indicated samples were removed and the nuclei were separated from the supernatant solutions. RNase I activity was determined in each fraction and alkaline RNase II activity was determined in the nuclear fractions. All activities are expressed as a percentage of the activities present in isolated nuclei before resuspension. Values for DNA are included for comparison.

▲-▲-▲ RNase I associated with nuclei

Δ-Δ-Δ RNase I associated with supernatant solution

●-●-● alkaline RNase II associated with nuclei

O-O-O DNA associated with nuclei

After 15 minutes incubation of the nuclei the RNase I activity in the supernatant had risen to 68% and after 60 minutes incubation to 87%.

In the case of alkaline RNase II activity, only 48% of the alkaline RNase II activity remained associated with the nuclei when they were resuspended in "modified Fisher's medium". After 15 minutes of incubation, 16% of the activity was found associated with the nuclei and this value did not seem to change significantly during the incubation which was continued for one hour.

However, the amount of DNA and acid phosphatase activity associated with the nuclei during the hour of incubation remained constant. Thus, both RNases were released relatively quickly from nuclei upon the change from a hypotonic medium to an isotonic medium and during subsequent incubation. During the one hour of incubation in "modified Fisher's medium" the nuclei began to clump indicating damage.

Ribonucleic Acid Synthesis and Degradation in Ehrlich Ascites Cells

The Effect of the Incubation Conditions on RNA in Isolated Nuclei

In order to determine the effect of the incubation conditions on the RNA in the isolated nuclei, Ehrlich ascites tumor cells were prepared as described in Methods and a 2% suspension in "modified Fisher's medium" was labelled with ^{14}C guanine for 30 minutes. Nuclei were isolated from these cells and the total radioactivity in the

nuclear fraction and the amount of acid-precipitable label were determined as described in Methods. These values were used as the control values. The nuclei were then resuspended in "modified Fisher's medium" and the incubation was continued. At the times indicated in Figure 3 samples were removed and centrifuged at 1600 x g for 3 minutes. The supernatant was removed and the pellet resuspended in fresh medium. The number of acid-precipitable counts was determined in both the supernatant and nuclear fraction.

After 15 minutes incubation of the isolated nuclei, 24% of the acid-precipitable counts were no longer associated with the nuclear fraction and after 60 minutes of incubation this value was 30%. During this time period, acid-precipitable counts were detected in the incubation medium and at 60 minutes this amounted to 6% of the total acid-precipitable material in the original nuclear fractions. Thus, in one hour of incubation 24% of the acid-precipitable material which was associated with the nuclear fraction at the time of isolation had been converted to acid-soluble material. Having established the behaviour of the nuclear enzymes and the approximate extent of degradation of nuclear RNA during incubation of isolated nuclei it was possible to analyse this process more closely. To do this it was necessary to analyse the products of RNA degradation in isolated nuclei and to compare this with the results obtained with the whole cell.

Analysis of the Processing of rRNA of Ehrlich Ascites Cells

The present knowledge of the processing of rRNA in animal cells has been outlined in the Introduction. This pattern was expected to

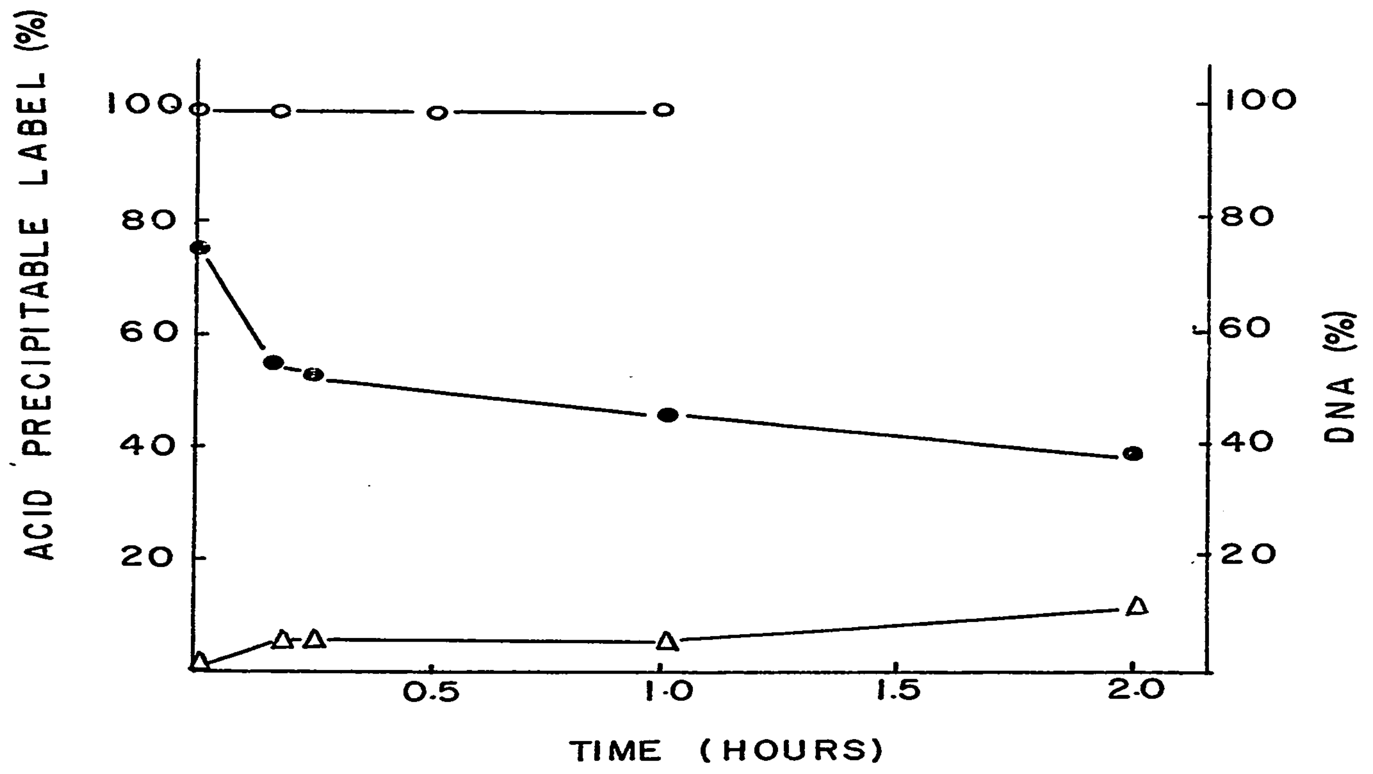

ACID PRECIPITABLE LABEL (%)
100
80
60
40
20
0.5
1.0
1.5
2.0
TIME (HOURS)
DNA (%)

FIGURE 3

DEGRADATION AND RELEASE OF RNA DURING THE INCUBATION OF NUCLEI

Nuclei were isolated from Ehrlich ascites cells after 30 minutes of labelling with ^{14}C guanine and incubated in "modified Fisher's medium" at 37°C. At the times indicated the nuclei were separated from the supernatant solution by centrifugation to determine the acid-precipitable label in each fraction. The acid-precipitable label in the samples is expressed as a percentage of that present in the cell before the isolation of the nuclei. Values for DNA are included for comparison.

●-●-● Acid-precipitable label associated with nuclei

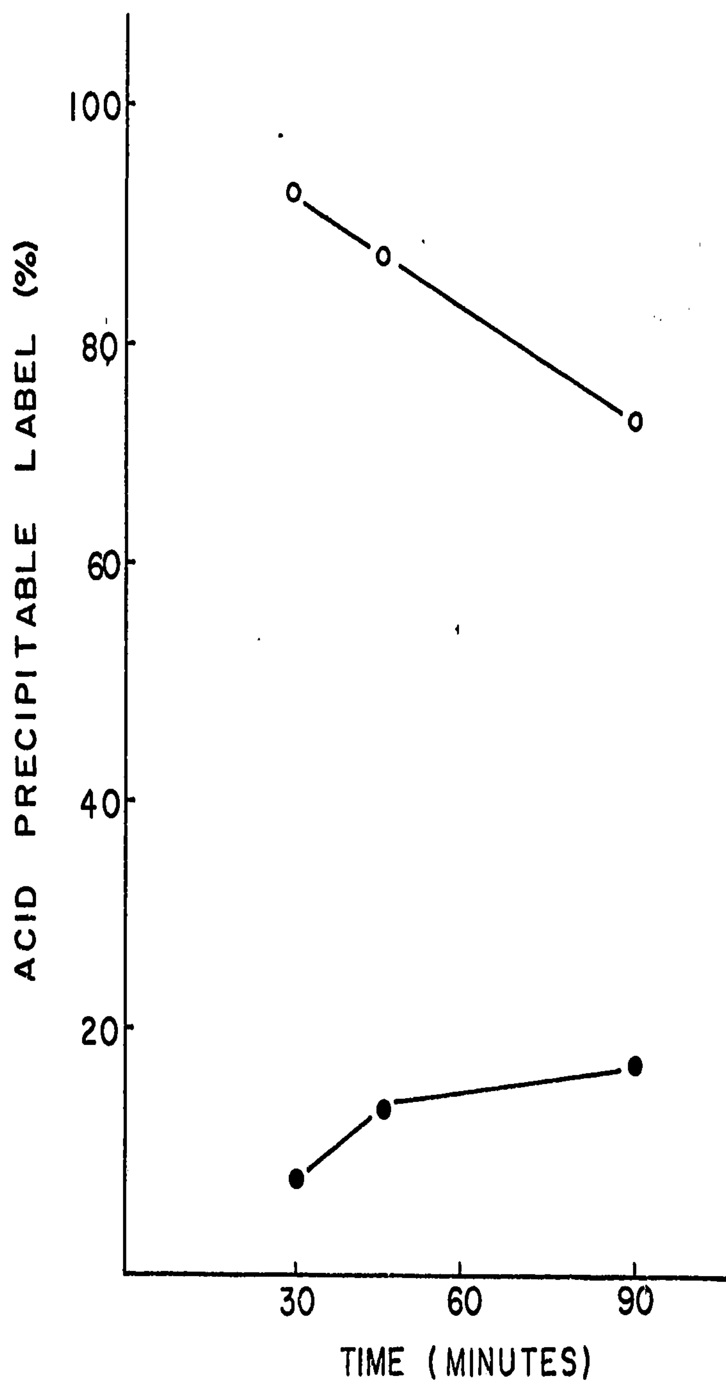
Δ–Δ–Δ Acid-precipitable label in the supernatant solutions

O-O–O DNA associated with nuclei

be the same in Ehrlich ascites tumor cells. The following experiments were carried out to show that this was the case and to test the methods used for the analysis of different classes of RNA.

A 2% suspension of Ehrlich ascites cells was incubated in the presence of 0.06 μCi/ml ^{14}C guanine and 0.03 μCi/ml ^{14}C uridine in "modified Fisher's medium". At 30, 45 and 90 minutes after the addition of label, samples were removed for the isolation of nuclei. After the homogenization of the cells, the homogenate was centrifuged at 1600 x g for 3 minutes and the supernatant was removed (cytoplasmic fraction). The nuclei were then washed as usual and resuspended in 0.15 M NaCl, 0.01 M Tris-HCl and 0.01 M Na_2EDTA (RNA extraction buffer). Unlabelled carrier was added to the nuclear suspension before the RNA was extracted to provide an absorbance profile for the gel analysis of the extracted RNA. At each sampling time the total acid-precipitable counts in the nuclei and cytoplasmic samples were determined. Figure 4 expresses the incorporation of label into nuclear and cytoplasmic RNA in terms of percent of the total label incorporated. Over 90% of the total acid-precipitable counts of the cells were found in the nucleus 30 minutes after the addition of label to the incubating cells. After 45 minutes of incubation in the presence of label this value had decreased to 87% and after 90 minutes to 73%. A corresponding rise in acid-precipitable counts in the cytoplasmic fraction accompanied the decrease that occurred in the nuclear fraction.

RNA was extracted from both the nuclear and cytoplasmic samples and separated on 2% polyacrylamide gels containing 0.5% agarose. The gels were scanned at 260 nm and the isotope concentration was



ACID PRECIPITABLE LABEL (%)
100
80
60
40
20
30
60
90
TIME (MINUTES)

FIGURE 4

UPTAKE OF ^{14}C GUANINE AND ^{14}C URIDINE INTO RNA OF THE NUCLEAR AND CYTOPLASMIC FRACTIONS

A 2% suspension of Ehrlich ascites cells was incubated in "modified Fisher's medium" at $37^{o}C$. After 5 minutes of preincubation ^{14}C guanine and ^{14}C uridine were added to a final concentration of 0.06 μCi/ml and 0.03 μCi/ml respectively. At the times indicated after the addition of label the nuclei were isolated and the acid-precipitable label in the nuclei and cytoplasmic fractions was determined. The acid-precipitable label in the fractions is expressed as a percentage of the acid-precipitable label in cell samples.

o—o—o Acid-precipitable label in nuclear fractions

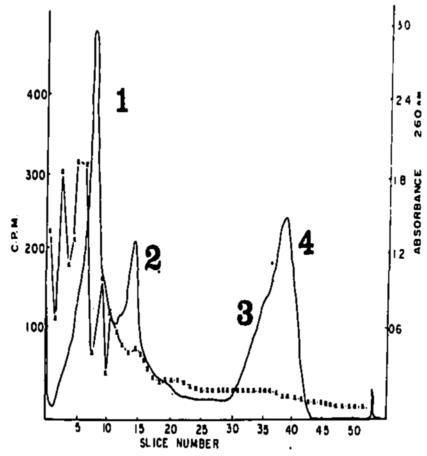
●—●-● Acid-precipitable label in cytoplasmic fractions

determined after slicing.

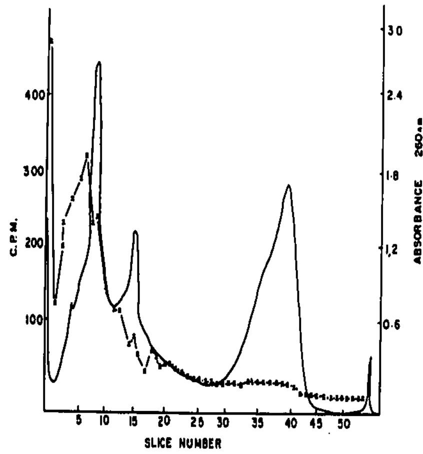
The separation of RNAs obtained by polyacrylamide gel electrophoresis is shown in Figure 5A and the absorbance profile was very similar in different experiments. Peak 1 corresponds to 28S RNA, peak 2 to 18S RNA, peak 3 to 4 and 5S RNA and peak 4 to small molecular weight UV absorbing material which had co-precipitated with the RNA after DNase treatment.

Figures 5A, 5B and 5C show the separation of the classes of RNA extracted from the nuclei isolated from cells that were incubated for 30, 45 and 90 minutes after the label was added. Figure 5A (30 minutes of incubation) shows two peaks of labelled RNA which are approximately equally labelled and seem to correspond to 45S and 32S RNA. Little or no label was present in the 18S or 4 and 5S RNA UV absorbing peaks. Using this method of separation of the classes of RNA, one always obtains an overlap of labelled 28S and 32S RNA. After 45 minutes of incubation (Figure 5B) the first peak of radioactivity (45S RNA) had decreased in size and the second peak (approximately 32S RNA) had increased and become broader, significantly overlapping the 28S RNA UV absorbing peak. Little or no label was seen in the UV absorbing peaks corresponding to 18S or 4 and 5S RNA. After 90 minutes of incubation (Figure 5C) most of the labelled RNA was in the overlapping peak corresponding to 32S and 28S RNA. The amount of label in this peak had increased during this incubation time. Little labelled RNA was seen in the area of the gel corresponding to RNA of sizes from 18S to 4S.

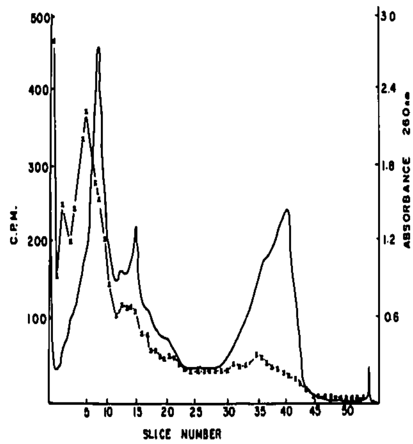
Figure 6D, 6E and 6F show the separation of RNA extracted



A



B



C

FIGURE 5

POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE NUCLEAR RNA ISOLATED FROM LABELLED CELLS

A 2% suspension of Ehrlich ascites cells was incubated in "modified Fisher's medium" in the presence of ^{14}C guanine and ^{14}C uridine at a final concentration of 0.06 μCi/ml and 0.03 μCi/ml respectively. At 30, 45 and 90 minutes after the addition of label to the incubating cells, samples were removed and the nuclei were isolated as described in Methods. Ribonucleic acid was extracted from the nuclei and separated by polyacrylamide gel electrophoresis.

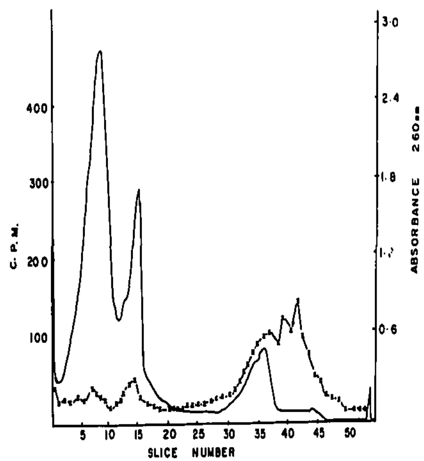
A) RNA associated with nuclei after 30 minutes

B) RNA associated with nuclei after 45 minutes

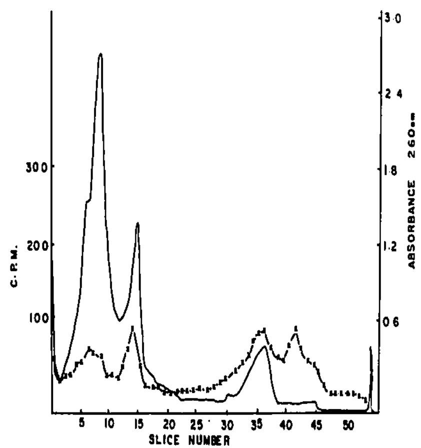
C) RNA associated with nuclei after 90 minutes

(——) Absorbance 260 nm

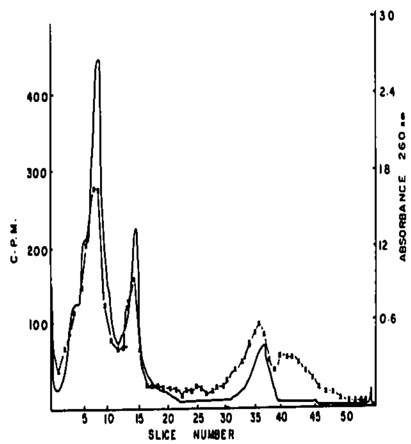
(X–X) C.P.M.



D



E



F

FIGURE 6

POLYACRYLAMIDE GEL ELECTROPHORESIS OF CYTOPLASMIC RNA ISOLATED FROM LABELLED CELLS

Cytoplasmic samples were isolated from cells labelled as indicated for Figure 5. The RNA was extracted from the cytoplasmic fractions and separated by polyacrylamide gel electrophoresis.

D) RNA in the cytoplasmic fraction at 30 minutes

E) RNA in the cytoplasmic fraction at 45 minutes

F) RNA in the cytoplasmic fraction at 90 minutes

(——)Absorbance 260 nm

(X-X) C.P.M.

from the cytoplasmic fractions of the cells incubated for 30, 45 and 90 minutes after the addition of the isotope to the incubating cells. After 30 minutes of incubation (Figure 6D) the only RNA that was significantly labelled in the cytoplasm was 4 and 5S RNA. There was also some labelled material seen in the gel profile which corresponded to the RNA that was smaller than 4 and 5S RNA in size. This small molecular weight RNA was seen in the gel profiles of all 3 cytoplasmic samples and may be due to the breakdown of RNA during isolation. By 45 minutes (Figure 6E) there was a small amount of labelled 18S RNA in the cytoplasm as well as 4 and 5S RNA. At 90 minutes there were distinctive peaks of labelled 28S and 18S RNA in the cytoplasm (Figure 6F) in addition to the label in the 4 and 5S RNA.

The results, seem to indicate that the nuclei were free of cytoplasmic RNA since little or no labelled 4 and 5S RNA was seen in any of the gel profiles of the RNA isolated from the nuclei samples. Similarly, the cytoplasmic fraction was free of nuclear RNA since, at 30 minutes after the addition of label to the incubation medium essentially no 32S RNA was found in the cytoplasmic RNA sample.

Therefore, the processing of rRNA by Ehrlich ascites tumor cells was precise under the conditions used and in general corresponded to the results obtained by Penman (1966) for HeLa cells and by Cooper and Gibson (1971) for lymphocytes.

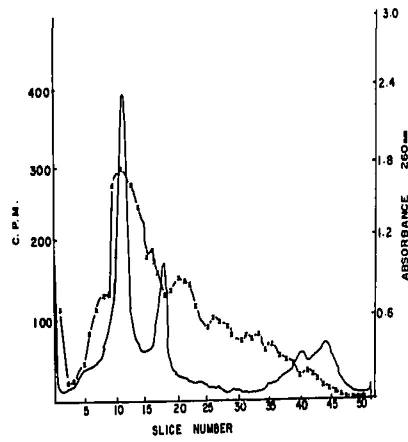
Analysis of the Acid-Precipitable Material Released from Incubating Nuclei

When nuclei were incubated in "modified Fisher's medium" for

one hour, 6% of the acid-precipitable material present in the nuclei at the time of isolation was found in the incubation medium. Experiments were carried out to determine the size and the nature of the acid-precipitable material in the incubation medium.

A 2% suspension of Ehrlich ascites tumor cells was labelled with 0.5 μCi/ml of ^{14}C guanine for 30 minutes in "modified Fisher's medium". Nuclei were isolated as described in Methods and resuspended in "modified Fisher's medium". These resuspended nuclei were incubated for 60 minutes at 37°C. A sample was then centrifuged at room temperature for one minute at 1600 x g. Ribonucleic acid was extracted from the supernatant and the nuclear pellet after addition of unlabelled carrier. The total RNA extracted in each case was separated on polyacrylamide gels. Results of these gels are shown in Figures 7A and 7B.

Figure 7A shows the classes of RNA present in the nuclei 60 minutes after the start of the incubation of labelled nuclei. The labelled RNA present in the nuclear sample was completely different from that seen in Figure 5C, although both were incubated for the same length of time. There were not just a few distinct classes of RNA for the labelled RNA ranged in size from 45S to 4S. This data suggested that the control of RNA processing in the nucleus had been lost when labelled nuclei were incubated. Gel analysis of the acid-precipitable material in the supernatant after incubation of the nuclei for 60 minutes showed it to be approximately 4 and 5S size. Therefore, only the smaller RNA was lost into the medium during incubation of the nuclei.



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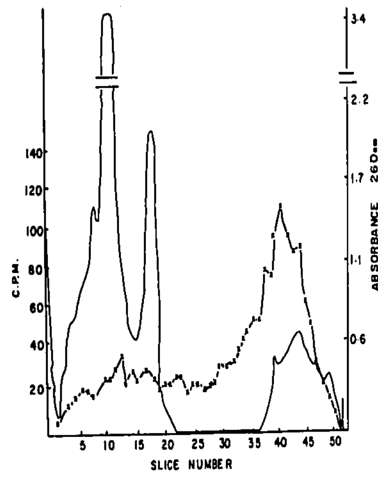


B

FIGURE 7

POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE RNA OF THE NUCLEI AND THE MEDIUM AFTER INCUBATION OF LABELLED NUCLEI

A 2% suspension of Ehrlich ascites cells was incubated in "modified Fisher's medium" in the presence of ^{14}C guanine at a final concentration of 0.5 μCi/ml for 30 minutes at 37°C. Nuclei were isolated and resuspended in "modified Fisher's medium" as described in Methods. The incubation was continued for 60 minutes and the nuclei were separated from the incubation medium by centrifugation. Ribonucleic acid was extracted from the nuclei and the incubation medium and separated by polyacrylamide gel electrophoresis.

A) RNA associated with nuclei

B) RNA in the incubation medium

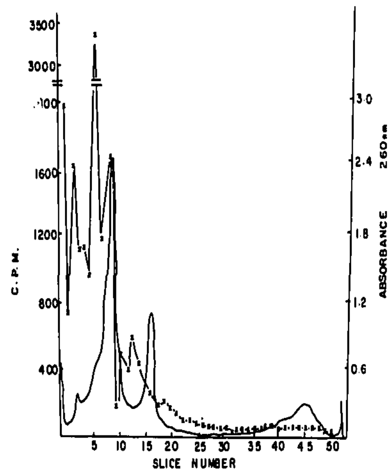
(——) Absorbance 260 nm

(X–X) C.P.M.

Analysis of the Processing of RNA in the Isolated Nuclei of Ehrlich Ascites Cells

In order to analyze the degradation of RNA in nuclei more closely, a 2% suspension of cells were incubated with 0.5 μCi/ml of ^{14}C guanine and 0.25 μCi/ml ^{14}C uridine for 30 minutes in "modified Fisher's medium". Nuclei were then isolated as before, resuspended in "modified Fisher's medium" and incubated at 37°C. At indicated times samples were removed to determine the acid-precipitable counts and for RNA extraction. Carrier cells were added to nuclei before the RNA extraction. Ribonucleic acids were again separated on polyacrylamide gels. The results are shown in Figures 8A, 8B and 8C.

Figure 8A shows the RNA present in the nuclei isolated from cells that had been incubated in the presence of label for 30 minutes. The gel analysis of this RNA showed the label to be present in 45S RNA and the overlapping peak of 32S and 28S RNA of the nucleus. The main peak of label was in the UV absorbing peak corresponding, approximately, to 32S RNA. There was little or no labelled 5 and 4S RNA in the sample of nuclear RNA. After 15 minutes incubation of the labelled nuclei the label in the 45S RNA had decreased and shifted to RNA of approximately 32S. The peak of radioactivity was very broad and overlapped with the UV absorbing peak of 28S RNA. There was also labelled RNA in the sample which ranged in size from 28S to 4S (Figure 8B). Sixty minutes after the start of the incubation of the nuclei (Figure 8C) most of the labelled RNA was approximately 28S RNA and the labelled RNA varied from 28S to 4S. This dispersion



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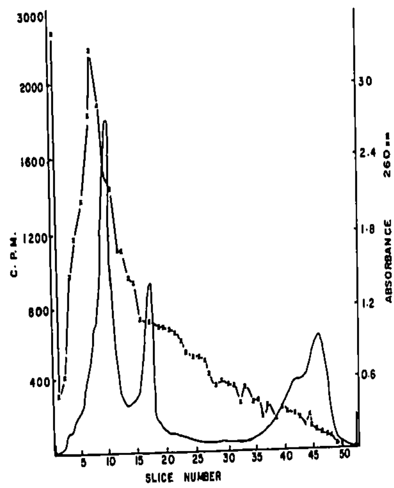

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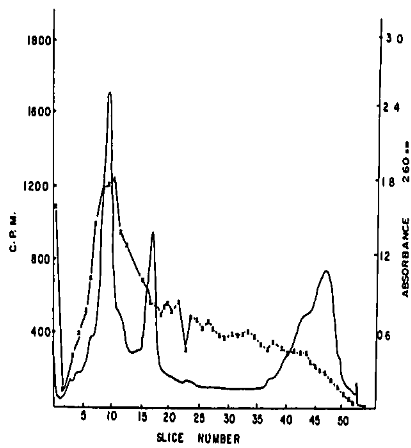


C

FIGURE 8

POLYACRYLAMIDE GEL ELECTROPHORESIS OF NUCLEAR RNA AFTER THE INCUBATION OF ISOLATED NUCLEI

A 2% suspension of Ehrlich ascites cells was incubated in "modified Fisher's medium" in the presence of ^{14}C guanine and ^{14}C uridine at a final concentration of 0.5 μCi/ml and 0.25 μCi/ml respectively for 30 minutes at $37^{o}C$. Nuclei were isolated and resuspended for continued incubation as described in Methods. At the indicated times, samples were removed and RNA was extracted and separated by polyacrylamide gel electrophoresis.

A) RNA associated with nuclei after 0 minutes of incubation of the nuclei

B) RNA associated with nuclei after 15 minutes of incubation of the nuclei

C) RNA associated with nuclei after 60 minutes of incubation of the nuclei

(——) Absorbance 260 nm

(X—X) C.P.M.

of the sizes of RNA was even more dramatic than at 15 minutes after the start of the nuclei incubation.

The precise processing of the nuclear RNA seen on the incubation of cells did not occur when the isolated nuclei were incubated under the same conditions.

Analysis of the Acid-Soluble Products Produced when Nuclei were Incubated

During the incubation of labelled nuclei for one hour, as indicated above, 24% of the acid-precipitable counts were converted to acid-soluble products. The acid-soluble products which appeared during the incubation of isolated nuclei were analyzed to determine if either of the RNase activities of the nuclei were involved in the conversion of acid-precipitable material to acid-soluble material.

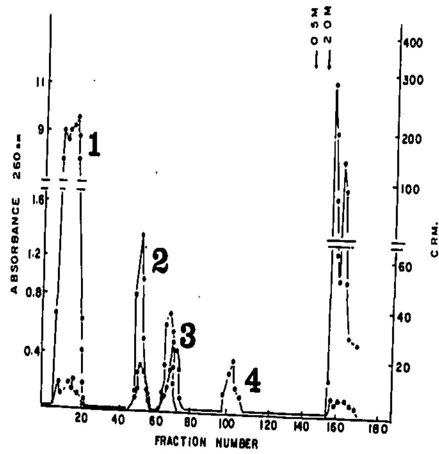
A 2% suspension of Ehrlich ascites tumor cells was incubated with labelled uridine and guanine for 30 minutes as for the RNA analysis described above. Nuclei were isolated as described in Methods and resuspended in "modified Fisher's medium". At 0, 15 and 60 minutes after the resuspension of nuclei, samples were taken to be carried through the RNA extraction procedure only as far as the end of the second chloroform extraction. The aqueous phase in each case was then diluted into sterile water until the salt concentration was equivalent to 0.05 M NH_4HCO_3 and applied to a 40 cc. DEAE-cellulose column. Four μmoles each of 5' GMP and 5' UMP were added to the samples as markers before they were applied to a DEAE-

cellulose column. Samples were eluted using a linear gradient consisting of 500 ml of 10 mM NH_4HCO_3, pH 8.0 and 500 ml of 0.2 M NH_4HCO_3, pH 8.0. After the gradient was completed, the elution was continued with one column volume of 0.5 M NH_4HCO_3, pH 8.0 and four column volumes of 2 M NH_4HCO_3, pH 8.0.

The elution profile is shown in Figure 9A. Peaks 2 and 3 of the absorbance profile are a result of the nucleotide markers. The main radioactive peaks followed the absorbance profile closely. However, in the cases of the samples taken at 0 and 15 minutes there was a radioactive peak which was eluted from the DEAE-cellulose column at approximately 0.14 M NH_4HCO_3, (peak 4) which showed no UV absorbance (Figures 9A and 9B). This peak of radioactivity did not appear in the elution profile of the 60 minute nuclei sample (Figure 9C). The salt concentration at which this material was eluted corresponded to the salt concentration at which both GDP and UTP had been eluted from a DEAE-cellulose column under similar conditions.

The 3 peaks of UV absorbing material were each pooled and the absorption spectra were determined using a Unicam spectrophotometer SP8000 with the appropriate controls. Peak 1 had a spectrum that corresponded exactly to that of phenol. Thus, it seemed that not all of the phenol was removed using 2 chloroform extractions. Absorbance peak 2 and peak 3 corresponded to UMP and GMP respectively.

In the chromatography of all three samples no label could be detected in the fractions collected when the column was eluted with 0.5 M NH_4HCO_3, pH 8.0. However, radioactivity was seen in the fractions of all three samples after one column volume of 2 M



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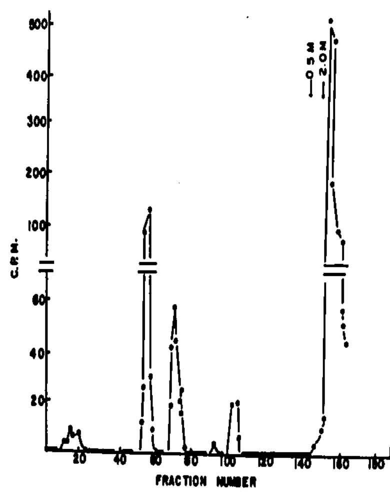

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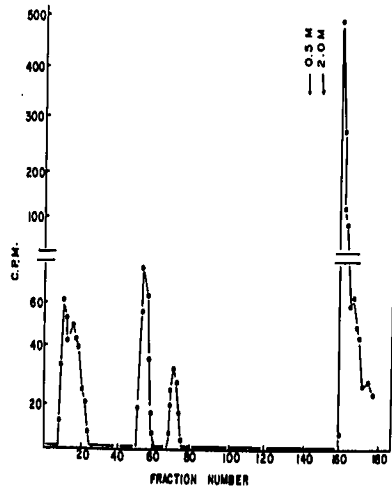

C

FIGURE 9

DEAE-CELLULOSE CHROMATOGRAPHY OF NUCLEAR RNA

AND RNA DEGRADATION PRODUCTS

A 2% suspension of Ehrlich ascites cells was incubated in "modified Fisher's medium" in the presence of ^{14}C guanine and ^{14}C uridine to a final concentration of 0.5 μCi/ml and 0.25 μCi/ml respectively for 30 minutes at 37°C. Nuclei were isolated and resuspended for continued incubation as described in Methods. At 0, 15 and 60 minutes after the start of the incubation of the nuclei, samples were removed and carried through the RNA extraction procedure until the end of the second chloroform extraction. In each case the aqueous phase was applied to a DEAE-cellulose column and eluted as described in Methods.

A) Acid-soluble products after 0 minutes of incubation

B) Acid-soluble products after 15 minutes of incubation

C) Acid-soluble products after 60 minutes of incubation

(○-○) Absorbance 260 nm

(●-●) C.P.M. / 100 μl

NH_4HCO_3, pH 8.0 was applied.

The amount of label in peaks 1, 2 and 3 for all three incubation samples is plotted in Figure 10. The label corresponding to GMP and UMP increased 2.5 times and 8 times respectively during the first 15 minutes of incubation. During the following 45 minutes of incubation the label corresponding to GMP and UMP decreased 50%. The label in peak 1 increases only slightly in the first 15 minutes of incubation but in the following 45 minutes there was a drastic increase in the label in this peak. The decrease in counts in GMP and UMP after the first 15 minutes of incubation of the isolated nuclei was equal in value to the increase that occurred in the counts in peak 1 during this incubation time. This seemed to indicate a precursor-product relationship which was due probably to the conversion of nucleoside monophosphates to nucleosides by contaminating phosphatases.

Analysis of the Labelled Material in Peak 1 from the DEAE-Cellulose Columns

The material from peak 1 was lyophilized and dissolved in the minimum volume of sterile water. Aliquots from the 60 minute sample were chromatographed on Whatman 3 MM paper and on PEI-cellulose plates as described in Methods. The separation obtained is shown in Table 4. The labelled compounds were determined to be 34.5% guanosine and 65.5% uridine. No labelled guanine was detected Since ^{14}C guanine and ^{14}C uridine were originally used to label the RNA, ^{14}C guanosine could only have arisen

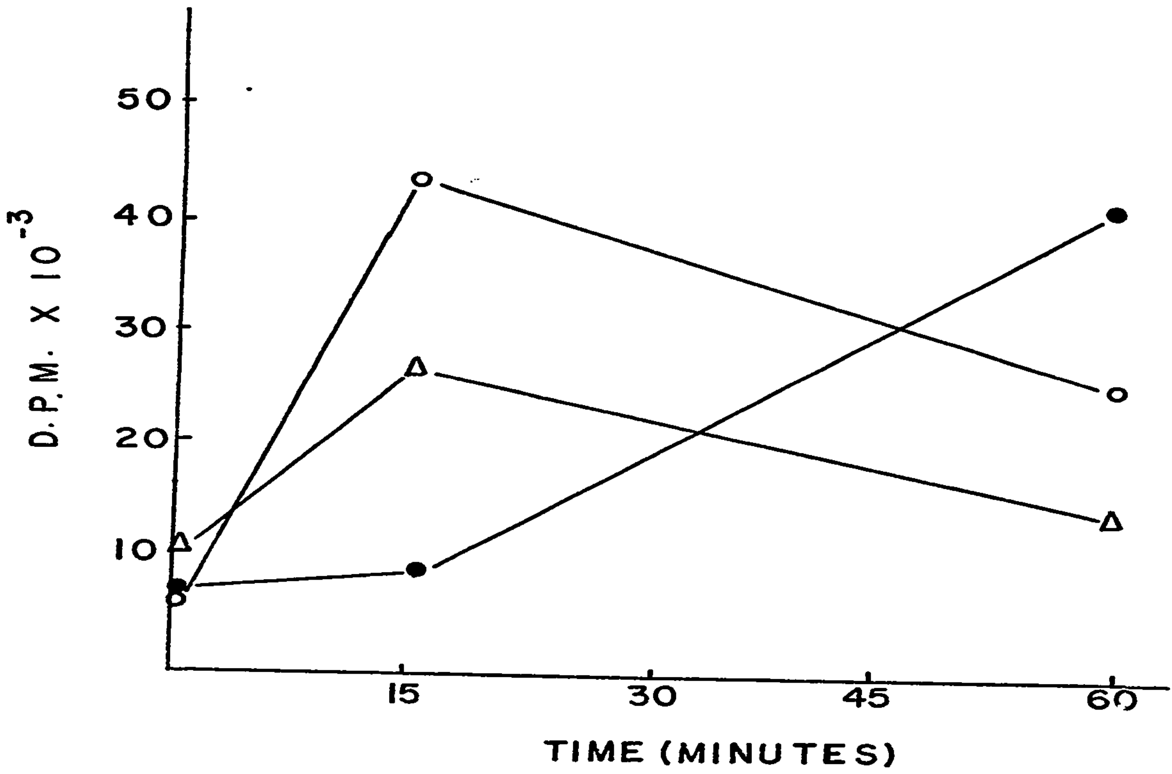
D.P.M. X 10^-3
50
40
30
20
10
15
30
45
60
TIME (MINUTES)

FIGURE 10

NUCLEOTIDE AND NUCLEOSIDE DEGRADATION PRODUCTS OF RNA

OBTAINED DURING THE INCUBATION OF ISOLATED NUCLEI

The absorbance peaks of the 3 columns described in Figure 9 were pooled separately and samples of each were counted in 5 ml of Bray's scintillation fluid using a Nuclear Chicago Liquid Scintillation Counter.

(●-●-●) D.P.M. in peak 1 at indicated times

(○-○-○) D.P.M. in the UMP peak at indicated times

(Δ-Δ-Δ) D.P.M. in the GMP peak at indicated times

TABLE 4

CHROMATOGRAPHY OF LABELLED MATERIAL IN PEAK 1 FROM THE DEAE-CELLULOSE CHROMATOGRAPHY OF THE 60 MINUTE NUCLEI SAMPLE

A. Paper Chromatography Results

Sample	R_f Values
Guanine	0
Guanosine and Uridine	0.24
Peak 1	0.26

B. PEI-Cellulose Chromatography Results

Sample	Distance Travelled (cm)
Guanosine	12
Uridine	10
Peak 1	10 and 12

from the degradation of ^{14}C GMP. The presence of ^{14}C uridine could have been due in part to some of the original ^{14}C uridine used to label the RNA and in part to the degradation of ^{14}C UMP.

Samples of peak 1 from the DEAE-cellulose column chromatography of the 15 minute nuclei sample were chromatographed on Whatman 3 MM paper as described in Methods. This analysis indicated that 31% of the labelled material was ^{14}C guanine and that the remainder was ^{14}C guanosine and ^{14}C gridine. There was not enough label in peak 1 from the DEAE-cellulose column chromatography of the 0 minute sample to do any chromatographic anlysis.

The results from the above analyses indicated that the acid soluble products from the incubation of nuclei were mononucleotides which with further incubation were degraded to nucleosides due to the presence of phosphatase activity in this nuclei preparation (Table 2).

Analysis of the Mononucleotides obtained from the DEAE-Cellulose Chromatography of Nuclei Incubation Samples

Exo-RNase I activity hydrolyzes RNA to 5' mononucleotides. This enzyme activity was shown to be associated with the isolated nuclei (Table 2). It was the only RNase activity found associated with the isolated nuclei which produced mononucleotides. Thus, both UMP and GMP found in the incubation samples would be expected to be 5' mononucleotides. To determine if the GMP and UMP produced during the incubation of labelled nuclei were 5' or 3' mononucleotides the pooled GMP and UMP fractions from the DEAE-cellulose column

chromatography of the nuclei sample were freeze dried. They were resuspended in a minimum of water and samples of each were treated with crude snake venom and chromatographed on PEI-cellulose as described in Methods. Crude snake venom contains a 5' nucleotidase which removes only 5' phosphates from mononucleotides producing nucleosides. After this treatment of the GMP and the UMP samples with crude snake venom, 100% of the label was recovered as guanosine and uridine respectively. Thus both GMP and UMP produced during the incubation of nuclei were 5' mononucleotides and probably products of exo-RNase I activity.

Analysis of Labelled Material in Peak 4 from DEAE-Cellulose Chromatography

The fractions in peak 4 from the DEAE-cellulose chromatography of nuclei samples that were incubated for 0 and 15 minutes were pooled and lyophilized separately. They were then dissolved in a minimum of sterile water and samples of each were chromatographed on Whatman 3 MM paper using isobutryic acid: M NH_4OH (50:30) as described in Methods. Unlabelled UTP, GDP, UDP and UMP were chromatographed with the samples. The separation of the nucleotides by this method was poor but UTP was separated from the other nucleotides and shown not to be one of the labelled compounds in peak 4.

Aliquots of peak 4 material in each case were applied to Whatman 3 MM paper for separation by High Voltage Paper Electrophoresis. The results are shown in Figure 11 They indicate that the only

GDP	UDP	SAMPLE 1	SAMPLE 2

FIGURE 11

HIGH VOLTAGE ELECTROPHORESIS

Fractions of peak 4 of the DEAE-cellulose chromatography of nuclei samples that had been incubated as described in Methods for 0 and 15 minutes were pooled and freeze dried. After suspension of the samples in sterile water, samples of each plus nucleotide controls (UDP, GDP and UMP) were electrophoresed on Whatman 3 MM paper under 1500 volts for 2.5 hours. The positions of the nucleotide controls were determined under UV light and are indicated by the marked circles. The positions of the labelled samples were determined by cutting the appropriate areas of the paper into 2 cm strips and counting these in 5 ml of Bray's scintillation fluid. The location of the radioactivity is indicated by the shaded areas.

Sample 1: 0 minute incubation sample

Sample 2: 15 minute incubation sample

labelled compound in peak 4 was GDP.

In summary, the acid-soluble products produced during the incubation of labelled nuclei include nucleosides nucleotides and nucleoside diphosphate(s).

DISCUSSION

The purpose of this study was to try to determine the physiological function of nuclear RNases and their relationship to the processing of nuclear RNA. Ehrlich ascites cells were chosen for the study because they are a homogeneous cell population and they take up RNA precursors rapidly. The RNases of these cells have also been studied and exo-RNase I activity was found to be the predominant RNase activity in the nuclei (exo/endo ratio 50:1) (Lazarus and Sporn, 1967) which might simplify the analysis of the system. It is clear, however, that an endonucleolytic attack must occur in order to explain the processing of pre-rRNA to rRNA.

The isolation from Ehrlich ascites cells of nuclei suitable for such a study was a difficult problem. High yields of intact nuclei, obtained by a rapid isolation technique, were required. As indicated in the Results, nuclei isolated in aqueous buffers, using several different published methods were not suitable for this study and the recovery of nuclei was generally low. Therefore, a new method of isolation of nuclei in an aqueous buffer was developed. The nuclei isolated from Ehrlich ascites cells using this procedure retained at least 85% of their DNA, were intact and free of cytoplasmic material including cytoplasmic RNA and the destruction of nuclear RNA during the isolation procedure was minimal.

The enzymes, exo-RNase I and alkaline RNase II, were found to be associated with the nuclear fraction from Ehrlich ascites cells. To what extend these activities were located inside the nucleus or

just associated with the nucleus could not be determined directly.

To obtain more information about the functions of RNases it was hoped that the enzymes could be located more specifically within the nucleus. However, after a number of attempts to isolate the nuclei in aqueous buffers with high recoveries of RNase I, and during attempts to isolate nucleoli, it was apparent that this enzyme was easily extracted during the isolation procedure. The same was true for alkaline RNase II. Although alkaline RNase II and exo-RNase I activities were always found to be associated with nuclei isolated in low ionic strength buffers, the recoveries of these enzymes varied and were generally of the order of 10% and 45% respectively (Table 2). Upon resuspension in isotonic medium there was a loss of 50% of exo-RNase I activity and 60% of alkaline RNase II activity associated with the nuclei. Thus the isolation of nucleoli to attempt a more specific localization of these enzymes did not seem practical.

During the incubation of Ehrlich ascites cells the processing of pre-rRNA to rRNA in the nucleus and transport of 18S and 28S rRNA to the cytoplasm was found to be similar to that observed by Penman (1966) in HeLa cells and by Cooper (1971) in lymphocytes. Thus, the conditions used for the incubation of the cells, which were also used for nuclei, did not seem to interfere with the processing of pre-rRNA to rRNA. In addition, nuclear RNA was not degraded during the procedure used to isolate the nuclei, since the profiles of the nuclear RNA for incubating cells showed only high molecular weight RNA. When isolated nuclei were incubated, however, a rapid degradation of nuclear RNA could be observed.

When Ehrlich ascites cells were labelled for 45 minutes with RNA precursors before the isolation of the nuclei, it was found that the labelled RNA of the nuclei corresponded to 32S and 28S RNA. After 90 minutes of incubation of the cells the labelled RNA of the nuclei still corresponded to 32S and 28S RNA. However, if the cells were labelled for 30 minutes and then the nuclei were isolated and resuspended it was found that after 15 minutes of incubation, the labelled RNA in the nuclei varied in size from 32S to 4S. As the incubation of the nuclei was continued for another 45 minutes the amount of degradation of the high molecular weight RNA in the nuclei increased (Figure 8C). Only acid-soluble material and small molecular weight RNA, approximately 5 and 4S, but not high molecular weight RNA, were lost into the incubation medium during one hour of incubation, indicating that the nuclei probably remained intact. There was also a loss of the RNase activities from the nuclei and this was most noticable during the first 15 minutes of incubation (Figure 3). Most of the RNA degradation also occurred during these initial 15 minutes of incubation which suggests that the RNases had actually been inside the nucleus initially.

The products of the degradation of nuclear RNA during the incubation of nuclei were RNAs ranging in size from 28S to 4S. Therefore, the cleavage of the nuclear RNA during the incubation of the nuclei was non-specific. There seemed to be a loss of control of the processing of pre-rRNA in the nucleus when the nuclei were isolated. This could be due to the loss of or the redistribution of the constituent or constituents necessary for processing from the nuclei

or the loss of some control factor which was cytoplasmic. The presence of RNA ranging from 32S to 4S in the incubating nuclei probably indicates an endonucleolytic attack on the high molecular weight RNA.

During the incubation of the isolated nuclei some of the RNA was converted to acid-soluble material. After 0 minutes of incubation 3% of the labelled nuclear RNA was converted to acid-soluble products. After 15 minutes of incubation this value was 18% and after 60 minutes of incubation 24% (Figure 3). Analysis of these products was carried out to determine the enzyme or enzymes involved in the degradation. The acid-soluble material from nuclei samples after incubation for 0, 15 and 60 minutes was analyzed by DEAE-cellulose and PEI-cellulose chromatography and by high voltage paper electrophoresis.

The cells were incubated in the presence of 0.25 μCi/ml of ^{14}C uridine and 0.5 μCi/ml of ^{14}C guanine and 15% of this label was incorporated into the acid-precipitable material of the cell in 30 minutes. The nuclei were isolated from these cells and resuspended for continued incubation. At 0 and 15 minutes after the start of the incubation of the labelled nuclei the majority of the acid-soluble products in the samples were mononucleotides (^{14}C GMP and ^{14}C UMP). During the first 15 minutes of incubation the amount of labelled GMP increased 2.5 times and the amount of labelled UMP increased 8 times. In the following 45 minutes of incubation of the nuclei the amount of both labelled GMP and UMP decreased 50%. There was 2 times as much lablled UMP as labelled GMP in the 15 and 60 minute samples.

The decrease in labelled mononucleotides during the incubation was accompanied by a corresponding increase in nucleosides indicating a precursor-product relationship due, probably, to the presence of phosphatase activity associated with the isolated nuclei(Table 2). Analysis of the nucleosides present after 60 minutes of incubation of the nuclei showed there was twice as much uridine as guanosine. Although ^{14}C uridine was originally used to label the RNA of the cells, the ^{14}C uridine present in the 60 minute samples was due to the degradation of ^{14}C UMP for the decrease in ^{14}C UMP from the 15 minute to the 60 minute sample corresponded to the increase in ^{14}C uridine. The presence of ^{14}C guanosine in the 60 minute incubation sample was due to the degradation of ^{14}C GMP only, for ^{14}C guanine was used to label the RNA originally and guanine is converted directly to GMP in the synthesis of RNA (Brockman, 1960; Henderson and Khoo, 1965). Again there was twice as much labelled uridine compounds as guanine compounds in the acid-soluble samples.

The mononucleotides produced during the incubation were 5' mononucleotides. Exo-RNase I activity, the predomainant RNase I in Ehrlich ascites nuclei, was associated with the isolated nuclei and it degrades RNA to 5' mononucleotides. Phosphodiesterase I activity also degrades polynucleotides to 5' mononucleotides but it is present in very low levels in Ehrlich ascites cells (von Tigerstrom, 1972) and could not be detected in the nuclei of Ehrlich ascites cells. Thus, it would seem that exo-RNase I activity was responsible for the conversion of some of the acid-precipitable material to acid-soluble material on the incubation of the isolated nuclei.

A small quantity of guanosine diphosphate was detected in

the 0 and 15 minute samples but none was detected at 60 minutes. At 15 minutes there was approximately 2 times as much labelled GDP as in the 0 minute sample. Polynucleotide phosphorylase degrades RNA to nucleoside diphosphates. This enzyme has not yet been detected in Ehrlich ascites cells but Siebert et al (1966) reported the existence of polynucleotide phosphorylase in the nucleoli of rat liver cells. A search for polynucleotide phosphorylase in Ehrlich ascites cells should be attempted. The major difficulty in detecting this enzyme activity in cell and nuclei extracts is that there is no accumulation of nucleoside diphosphates (GDP) due to their conversion to nucleoside monophosphates (GMP) by nucleoside diphosphatase and acid and/or alkaline phosphatases. No uridine diphosphate was detected in any of the samples, although its presence was expected in view of the recovery of labelled GDP. The absence of labelled UDP in the samples might, however, indicate that labelled GDP in the acid-soluble material was not due to the action of polynucleotide phosphorylase. It is conceivable that labelled GMP may be converted to labelled GDP in the nuclei under these conditions.

In all 3 incubation samples analyzed by DEAE-cellulose chromatography there was labelled material which was eluted from the column with 2 M NH_4HCO_3, pH 8.0. This material is probably heterogeneous and several nucleotides in length and was not further analyzed.

In summary, when labelled nuclei of Ehrlich ascites cells were isolated and incubated, the control of the processing of pre-rRNA to rRNA broke down and the high molecular weight RNA of the nucleus was probably degraded by endonucleolytic and exonucleolytic atttack.

A relatively large proportion of the acid-soluble products were 5' mononucleotides which would seem to have arisen from the action of exo-RNase I.

Giannitsis et al (1967) isolated nuclei from rat liver in high density sucrose solutions and in organic solvents and compared the RNase activity associated with the nuclei in both samples. The conditions under which the RNase activity was assayed were similar to those used for the alkaline RNase II assays as outlined in the Introduction. These workers found that the solvent nuclei had 8 times more RNase activity than the sucrose nuclei. The sucrose nuclei contained a non-extractable RNase activity and when solvent nuclei were extracted using 0.14 M NaCl they were found to have the same level of non-extractable RNase activity as the sucrose nuclei. A soluble enzyme was defined by Giannitsis et al (1967) as one that is extractable in 0.14 M NaCl and a non-soluble enzyme as one that is extractable in 1 M NaCl. Using the above definition Giannitsis et al (1967) found that 80% of the RNase activity in rat liver nuclei was extractable. This data would help to account for the facts that 100% recovery of RNase activity in nuclei isolated from Ehrlich ascites cells in aqueous solution was not possible and that 50% of the RNase I activity and 30% of the alkaline RNase II activity were lost from isolated nuclei of Ehrlich ascites cells when they were suspended in "modified Fisher's medium" which is isotonic. This loss of RNase activity from nuclei during the initial incubation period is a serious limitation in analyzing RNA degradation by this method. Attempts should be made to overcome this by using incubation media

of different composition.

In view of the results presented in the paper by Giannitsis et al (1967) the isolation of nuclei from Ehrlich ascites cells using organic solvents instead of aqueous medium should be carried out. The RNase activities in the nuclei prepared by this procedure should be studied in detail. The time required to prepare nuclei by the organic solvent method would make a study of RNA metabolism difficult but it might help to localize RNases in the nuclei. It might be possible to isolate nucleoli from organic solvent nuclei and thus give a more specific location for particular RNases. The specific location of an RNase activity might be useful in defining its function when one considers that the processing of pre-rRNA to rRNA occurs in the nucleolus.

Another approach to obtain information about the role of nuclear RNases might be to compare the degradation of RNA in the nuclei from different tissues. Lazarus and Sporn (1967) found that exo-RNase I activity was the predominant RNase activity in the nuclei of Ehrlich ascites cells (ratio exo/endo 50:1) and that in liver cell nuclei endo-RNase I was the main activity (ratio exo/endo 1:3). It would be interesting to compare the products of RNA degradation in Ehrlich ascites cells with those in liver cells.

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