SUMMARY

The nucleic acids were determined in the lungs of rats bearing the Walker 256 tumor, and of rats bearing the Murphy-Sturm lymphosarcoma.

Significant increases in the desoxyribonucleic acid (DNA) concentration during the growth of the tumors were observed. In contrast, the ribonucleic acid, protein nitrogen, potassium, and cholesterol did not show any change.

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# THE NUCLEOTIDE COMPOSITION OF THE TOTAL RIBONUCLEIC ACID IN SUBCELLULAR FRACTIONS OF RESTING AND REGENERATING RAT LIVER*

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The majority of the activity in the field of ribonucleic acid (RNA) chemistry has been concentrated on yeast ribonucleic acid, since this material is easily accessible, and relatively simple to prepare. In recent years there has been renewed interest in the ribonucleic acid of animal cells. Various workers have analyzed such ribonucleic acid for its constituent purine and pyrimidine bases, and some have fractionated the cell into its subcellular components and then analyzed the ribonucleic acids from these[1-4]. The results obtained by different workers have shown considerable variation, depending on the method of analysis used, and primarily on the type of preparation

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to which the nucleic acid was subjected prior to analysis. The present worker believes that in much of this work the total ribonucleic acid bases were not accounted for, due to loss during preparation. The objective of this study was to fractionate rat liver cells and analyze their ribonucleic acid by methods such that no initial preparative steps would be carried out, and thus the total RNA phosphorus could be accounted for. In addition it was decided to carry out the determinations on both resting and actively regenerating rat liver to determine if rapid growth has any effect on the nucleic acid composition.

## METHODS

Female Sprague-Dawley rats, two to three months old, were partially hepatectomized by the method of BRUES *et al.*[5], and the liver tissue immediately fractionated and analyzed. Seventy-two hours later the rats were killed and the regenerating liver removed and treated similarly. The liver was immediately blotted and weighed and immersed in ice cold 0.25 *M* sucrose. Fractionation into nuclei plus cell debris, mitochrondria, submicroscopic particles (microsomes), and supernatant was then carried out at 0° C by the method of SCHNEIDER[6]. The nuclei were further purified by the method of MARSHAK[7] to give a light grey preparation which was microscopically homogeneous. The four fractions were then treated by the method of SCHNEIDER[8] to remove the acid-soluble and phospholipid phosphorus fractions. The remainder of the hydrolysis and purification was then carried out by the method of SCHMIDT AND THANNHAUSER[9] substituting sulfuric acid for hydrochloric acid. This gave an extract (T-2 fraction) containing all of the RNA constituents, presumably in the form of ribonucleotides; and the phosphorus from the phosphoproteins in the form of orthophosphate[9]. In the case of the purified nuclei the desoxyribonucleic acid is quantitatively removed by this procedure[4,9].

The T-2 extracts were then hydrolyzed by adding 1/10 volume of 10 *N* sulfuric acid and refluxing for 2 hours. This hydrolysis frees the purine bases from their ribonucleotides, but leaves the pyrimidine nucleotides intact[10]. It also destroys all the trichloroacetic acid present[11]. The resulting hydrolysate was then diluted to 25 ml and analyzed for the purine bases by precipitating them with silver sulfate[12], extracting them from the precipitate with hydrochloric acid, and analyzing this extract spectrophotometrically. The supernatant resulting from the purine precipitation was analyzed for the pyrimidine nucleotides by spectrophotometric methods. This schema is essentially that of KERR *et al.*[10].

Two milliliters of saturated hot silver sulfate solution were added to 3 ml aliquots of the RNA hydrolysates, the tubes heated in a boiling water bath for 10 min, cooled in ice overnight, and centrifuged. The precipitate was washed three times with 1 ml of ice cold 50% methyl alcohol made 0.25 *N* with respect to sulfuric acid. The combined washings and supernatant were freed of excess silver by adding solid sodium chloride, diluted to about 20 ml, adjusted to pH 2.0, and finally diluted to 25 ml. The silver purine precipitate was extracted three times by boiling gently for 5 min with 0.1 *N* hydrochloric acid, and the combined extracts diluted to 25 ml with 0.1 *N* hydrochloric acid. These solutions were then analyzed by ultraviolet spectrophotometry using a Beckman Model DU spectrophotometer with ultraviolet attachments. Each solution theoretically contained two components absorbing in the ultraviolet, adenine and guanine in the silver insoluble extract, and uridylic acid and cytidylic acid in the silver soluble fraction. In each determination the absorption curve from 240 to 300 mμ was checked against a reagent blank for general shape, and the concentrations of the two individual components calculated using the following simultaneous equations[13]:

$$c_1 = \frac{D_{\lambda_b}\,\varepsilon_{2\lambda_a} - D_{\lambda_a}\,\varepsilon_{2\lambda_b}}{\varepsilon_{1\lambda_b}\,\varepsilon_{2\lambda_a} - \varepsilon_{1\lambda_a}\,\varepsilon_{2\lambda_b}} \qquad c_2 = \frac{D_{\lambda_a}\,\varepsilon_{1\lambda_b} - D_{\lambda_b}\,\varepsilon_{1\lambda_a}}{\varepsilon_{1\lambda_b}\,\varepsilon_{2\lambda_a} - \varepsilon_{1\lambda_b}\,\varepsilon_{2\lambda_a}}$$

where $c_1$ = concentration of compound 1

$c_2$ = concentration of compound 2

$D_{\lambda_a}$ = optical density at wavelength $a$

$D_{\lambda_b}$ = optical density at wavelength $b$

$\varepsilon_1$ = molar extinction coefficient of compound 1

$\varepsilon_2$ = molar extinction coefficient of compound 2

} at the given wavelength $\lambda_a$ or $\lambda_b$

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TABLE I

MOLAR EXTINCTION COEFFICIENTS OF PURINE BASES AND PYRIMIDINE NUCLEOTIDES

| *Compound and pH* | *Molar extinction coefficients at varying wavelengths (mμ)* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *247.5* | *260* | *262* | *266* | *268* | *276* | *278* | *280* | *285* |
| Adenine, pH 1.0 | | | 13,290 | | | 7,100 | | | 1,820 |
| Guanine, pH 1.0 | | | 7,590 | | | 7,100 | | | 5,380 |
| Uridylic acid | | | | | | | | | |
| pH 2.0 | | 9,980 | | 9,390 | | | 4,160 | | |
| pH 7.0 | 7,100 | 10,090 | | | 8,600 | | 4,430 | 3,470 | |
| Cytidylic acid | | | | | | | | | |
| pH 2.0 | | 6,720 | | 9,390 | | | 12,720 | | |
| pH 7.0 | 7,100 | 7,670 | | | 8,600 | | 7,550 | 6,860 | |

The absorption curves and molar extinction coefficients of the pure compounds have been previously determined*, and the values used here agree well with published values[10,13–15]. The molar extinction coefficients necessary for this work are summarized in Table I. The optical density of the adenine-guanine solution was taken at 262 and 285 mμ; since at these points the two individual absorption curves are at maximum separation with a minimal rate of change of the molar extinction coefficient for either compound. The optical density of the uridylic acid–cytidylic acid solution was taken at 260 and 278 mμ. Additional checks on the above values were obtained by calculating the total number of moles of purine (or pyrimidine) in the solution by taking the optical density at the isobestic point (where the two individual absorption curves cross). For adenine and guanine this is 276 mμ, and for uridylic acid and cytidylic acid it is 266 mμ. Samples of the pyrimidine nucleotide solutions were also made 0.05 *M* with respect to phosphate and adjusted to pH 7.0, but these analyses gave curves showing marked end absorption and an impossible configuration. Guanine was determined independently by the method of HITCHINGS[11]. Attempts to purify the pyrimidine fraction by the *iso*propyl alcohol–silver method of KERR *et al.*[10] were not successful.

Total phosphorus was determined on each of the fractions by the method of KING[16]. Phosphoprotein phosphorus was determined on each subcellular fraction of a similar series of rats by the method of SCHNEIDER[3]. Another series of rats had to be used since the sulfuric acid used in these experiments interfered with the calcium precipitation. RNA phosphorus was then calculated by difference. The total number of moles of phosphorus was then compared with the total number of moles of purine plus pyrimidine to determine the completeness of the recovery of the bases.

Several control experiments were also carried out. Extracts were prepared containing different amounts of trichloroacetic acid and then hydrolyzed. Spectrophotometric analysis of the resulting solutions showed the results to be independent of the TCA concentration. The analytical methods were substantiated using synthetic mixtures of the pure purines and pyrimidine nucleotides, the resulting analyses agreeing within 2% with the actual mixture. Additional analyses were carried out on a twice-reprecipitated sample of a commercial yeast RNA, alone and in combination with varying amounts of casein. The results were not significantly affected by the amount or type of protein present, although the values for total pyrimidine were 5 to 10% high in all protein-containing solutions. It is true that in tissue extracts there are various substances carried over into the silver supernatant, and it is undoubtedly these compounds which interfered with the spectrophotometric analysis at pH 7.0. It is shown that these same compounds do not interfere significantly at pH 2.0 since the total number of moles of cytidylic acid plus uridylic acid agrees well with the total calculated at the isobestic point, and at wavelengths greater than 250 mμ the curve has nearly the theoretical shape. All pyrimidine values were lowered by 8% to allow for the interfering absorption due to this carry-over, since this absorption did not appear to have any significant absorption peaks and was not dependent on the amount or type of protein. In acid solution, after standing in the cold, the solubility of the silver purines is not a major error, and the precipitate is washed in acidified 50% methyl alcohol to avoid further loss.

## RESULTS

The values for the analyses of the RNA of the four subcellular fractions are summarized in Table II. As noted above, the pyrimidine nucleotide values are lowered

* J. MCT. PLOESER, H. W. BORTNER AND H. S. LORING, unpublished data cited in [22].

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TABLE II

NUCLEOTIDE ANALYSES OF THE RIBONUCLEIC ACID OF RAT LIVER SUBCELLULAR FRACTIONS

(Values given are arithmatical averages followed by the number of rats used in parentheses, and the total range of values is given in the second line)

| Subcellular fraction | *Resting liver* — Molar ratios | | | | *Regenerating liver* — Molar ratios | | | |
|---|---|---|---|---|---|---|---|---|
| | *Guanine / Adenine* | *Uridylic / Cytidylic* | *Pyrimidine / Purine* | *Analytical P / Calculated P** | *Guanine / Adenine* | *Uridylic / Cytidylic* | *Pyrimidine / Purine* | *Analytical P / Calculated P** |
| Mitochondria | 3.6 (6)<br>3.0–4.4 | 0.89 (6)<br>0.82–0.99 | 2.2 (6)<br>1.9–2.7 | 0.96 (4)<br>0.94–0.97 | 4.0 (6)<br>3.5–4.9 | 0.91 (6)<br>0.85–0.96 | 2.2 (6)<br>1.8–2.6 | 0.93 (6)<br>0.90–0.97 |
| Submicroscopic particles | 3.9 (9)<br>3.5–4.2 | 0.92 (9)<br>0.81–1.00 | 1.5 (9)<br>1.3–2.1 | 0.91 (9)<br>0.84–0.99 | 3.7 (7)<br>3.1–4.1 | 0.94 (7)<br>0.87–1.0 | 1.4 (7)<br>1.2–1.6 | 0.88 (7)<br>0.84–0.91 |
| Supernatant | 3.3 (6)<br>3.0–4.2 | 0.84 (6)<br>0.80–0.86 | 2.0 (6)<br>1.9–2.2 | 0.84 (6)<br>0.77–0.92 | 3.0 (6)<br>2.6–3.8 | 0.82 (6)<br>0.74–0.85 | 1.6 (6)<br>1.5–1.7 | 0.82 (6)<br>0.77–0.87 |
| Purified nuclei | 3.0 (3)<br>2.9–3.1 | 1.1 (3)<br>0.98–1.2 | 1.8 (3)<br>1.7–1.9 | 0.90 (2)<br>0.90–0.91** | 2.7 (2)<br>2.7–2.7 | 0.98 (2)<br>0.98–0.98 | 1.6 (2)<br>1.5–1.6 | Unreliable |

* This ratio is the total phosphorus found analytically in the SCHMIDT-THANNHAUSER T-2 fraction corrected for phosphoprotein phosphorus, divided by the total number of moles of purine and pyrimidine found spectrophotometrically.

** Not corrected for phosphoprotein phosphorus.

by 8% to correct for silver soluble substances which interfere. All values are expressed as ratios so they may be compared. As already noted, the ultraviolet absorption curves for the purine mixtures, and the pyrimidine nucleotide mixtures at pH 2.0, give curves of roughly the theoretical shape above 250 mμ, and show excellent agreement between the concentrations of the two substances calculated by the simultaneous equations, and the total number of moles calculated at the isobestic point. The guanine values determined colorimetrically checked within 5% those calculated from the spectrophotometric data. In the case of the nuclei fractions the solutions were so dilute that the guanine to adenine ratio determined spectrophotometrically was not considered reliable; so it was calculated from the colorimetric guanine value and the total number of moles of purine calculated at the isobestic point.

A series of phosphoprotein phosphorus determinations on 10 different rat livers gave the following average values for the percentage of the phosphoprotein plus RNA phosphorus (total T-2 phosphorus) which was RNA phosphorus: mitochondria 79.6%, submicroscopic particles 88.7%, and supernatant 89.1%. It will be seen from Table II that the total RNA phosphorus determined by analysis was consistently slightly less than that calculated from the sum of purines plus pyrimidines.

## DISCUSSION

By the use of the methods discussed, the purine and pyrimidine analyses were carried out on tissue preparations which were treated in such a manner that the nucleic acid components were never isolated, and they were purified by only the mildest procedures. There was thus little chance for losing any of the constituent portions of the

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nucleic acid macromolecule. The results obtained showed marked variation between different rats, but a similar phenomenon has been observed by MARRIAN* in studying the incorporation of labeled purines into the nucleic acid of rat liver. In the present work it is felt that the agreement between the independent methods of analysis for the purine components substantiates the results obtained. There is a slight loss of purines due to the solubility of the silver purines, but the controls show it to be insignificant. At pH 2.0 the pyrimidine nucleotide curve has approximately the theoretical shape above 250 m$\mu$, and the total calculated pyrimidine agrees well with that found at the isobestic point. It should be pointed out that some error is introduced by the different absorption of the two optical isomers of the pyrimidine nucleotides, some breakdown to nucleosides, and cytidine to uridine, but this is slight[13,15]. Excellent agreement was obtained in each case between duplicate samples, and between two different preparations of the same liver when this was done.

An analysis of the results in Table II shows that the corresponding fractions of resting and actively regenerating liver have no essential differences in composition. The various fractions also have essentially the same composition, except the uridylic acid to cytidylic acid ratio in the nuclei is significantly higher than in the other fractions. It should also be stressed that the analytical results between the resting and regenerating fractions of the same rat always gave excellent agreement, and the wide spread of values was due to individual differences between different rats. As has been mentioned the ratio of analytical RNA phosphorus to calculated RNA phosphorus was consistently less than 1.0, an indication that there are nitrogenous bases in the nucleic acid molecule which are not associated with their own phosphorus atom. FLETCHER *et al.*[17] had previously reported such a phosphorus deficiency in what they considered to be a nearly native preparation of yeast RNA.

When these data are calculated in terms of milligrams of RNA per unit weight of tissue the previous observation of NOVIKOFF AND POTTER[18] that regenerating liver contains a higher concentration of nucleic acid than its resting counterpart is confirmed. These data also confirm the previous work of THOMSON *et al.*[19] that this increase is due to an increased concentration of RNA in all the cytoplasmic subcellular fractions, and especially in the more difficultly sedimentable fractions, as shown by BRACHET AND CHANTRENNE[20]. Although these changes in concentration are quite marked it should be emphasized that no significant differences in the composition of the ribonucleic acids of resting and regenerating liver could be shown.

When these results are compared with those of other investigators[1-4] it is noted that the guanine to adenine ratios are significantly higher, as are the pyrimidine to purine ratios. Most previous workers have used some type of purification or preparation of the nucleic acid, and have not demonstrated recovery of all the RNA phosphorus, although DAVIDSON *et al.*[20] have analyzed some samples without prior preparation and obtained significantly different results than on their prepared samples. CROSBIE *et al.*[2] have previously shown an increased uridylic acid to cytidylic acid ratio in purified nuclei, although some other workers[3] did not find this. All of the results in the literature show considerable variation, and at this time it is not possible to establish all the variables which produce this. The present worker believes one of the most important is the loss of fragments of the macromolecule during preparative procedures, and has therefore used a method which determines the constituents

* D. H. MARRIAN, personal communication.

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without prior isolation. The inherent inaccuracies of the methods are pointed out, but the methods appear to be valid within 10%, and it is felt to be very significant that all of the RNA phosphorus is accounted for by the resulting purine and pyrimidine values. The essentially constant ratios obtained between various fractions and between resting and regenerating liver neither confirm nor deny the possible existence of qualitatively different ribonucleic acids within the cell, except within the nucleus.

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SUMMARY

The ribonucleic acid in the mitochondria, submicroscopic particles, supernatant, and purified nuclei of both resting and regenerating rat liver has been analyzed for its constituent purine and pyrimidine components without prior isolation of the nucleic acid. All of the fractions show essentially the same ratio of guanine to adenine, uridylic acid to cytidylic acid, and pyrimidine to purine, except for a significantly higher uridylic acid to cytidylic acid ratio in purified nuclei. Phosphorus analyses show that all the RNA phosphorus has been accounted for in the form of purine or pyrimidine bases, with the ratios of analytical RNA phosphorus to calculated pyrimidine plus purine phosphorus averaging about 0.90. This suggests that the intact ribonucleic acid macromolecule has more than one mole of base per mole of phosphorus. The significance of these results is discussed.

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