It has been known since the work of Marshak (13) that nuclear ribonucleic acid (RNA) incorporates P\textsuperscript{32} at a much greater rate than cytoplasmic RNA. This has been confirmed by a number of investigators (2, 10). Griffin and co-workers (7) showed that nuclear RNA, as obtained by the Schmidt and Thannhauser fractionation method (17), was more active in its rate of uptake of P\textsuperscript{32} in azo dye-induced hepatomas than in a similar fraction from normal liver. Furthermore, these workers were able to show that the rate of phosphorus incorporation in the livers of precancerous animals was similar to that in tumor tissue in all fractions studied. (The term "precancerous" was used to indicate those animals which had been fed a diet containing 0.06 per cent of the dye 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) for 6-8 weeks but had not developed any liver tumors, since this usually requires 12-15 weeks.) Subsequent work by Ward et al. (21) showed that agents which delayed the carcinogenic process also depressed the rate of phosphorus uptake in precancerous animals but did not completely restore the metabolic pattern to normal levels.

In the work cited above for precancerous and cancerous animals, the Schmidt and Thannhauser method of fractionation was employed to isolate the various phosphorus-containing fractions. Since it is well known that this method of fractionation introduces some contamination of the RNA fraction by inorganic phosphate released from phosphoprotein during the alkaline degradation of RNA, it was considered desirable to extend the data with a study that provided for separation of the inorganic phosphate from the RNA fractions, to obtain data for a larger number of intervals, and, if possible, to determine whether the individual nucleotides obtained from degradation of the RNA showed any detectable differences with respect to the uptake of P\textsuperscript{32}.

METHODS

Male albino rats of the Holtzman-Sprague-Dawley strain were maintained on a purified diet (8) containing 0.06 per cent 3'-Me-DAB for a period of 6-8 weeks. These animals constituted the precancerous group. Similar rats of the same age and initial weight were also maintained on the purified diet without the azo dye for a period of 6-8 weeks. Average food intakes were calculated for the various groups and individual weight determinations made once each week during the feeding period. Food was administered ad libitum to all animals until injection of the labeled phosphate, at which time all food was withdrawn for the remainder of the experiment. Food consumption was slightly higher (10-15 per cent) in the control groups than in the dye-fed groups. All animals weighed approximately 140-150 gm. at the start of the feeding. Those receiving the control diet weighed 180-330 gm. after 6-8 weeks, while those on the azo dye diets ranged from 200 to 240 gm. Animals which showed no increase in weight on the azo dye regimen were discarded.

At the termination of the feeding period, the animals were further divided into groups of three for administration of labeled phosphate and sacrificed at various times following the injection of the P\textsuperscript{32}. Radioactive phosphorus\textsuperscript{3} as phosphate in a dilute HCl solution was diluted with 0.01 M phosphate buffer, pH 7.3, so as to give a solution with an activity of 100-150 \textmu C/ml. The solution was administered intraperitoneally, 0.1 ml/each 100 gm of rat. Experiments were staggered so that only one group of animals received the isotope

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* Portions of this work were included in a thesis submitted to the Committee on Graduate Study, Stanford University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy (20).

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each day; this allowed time for processing and fractionation within a few hours after sacrifice.

Animals were sacrificed at 1-hour intervals up to 4 hours after injection, and thereafter at 5, 8, and 18 hours. For sacrifice, the animals were anesthetized with ether and then decapitated. Livers were washed free of excess blood, then with NaOH was replaced with a hydrolysis using 14 N NH₄OH at 40° C. as suggested by Boulanger and Montreuil (3). Since this hydrolysis did not reduce the nuclear residue beyond a heavy gel stage, it was always followed by the NaOH treatment after removal of those nucleotides liberated by the NH₄OH. When present, NH₄OH

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**Chart 1.** Fractionation procedure

submitted to fractionation as described previously (7, 21), with further modifications. The modifications consisted of the removal of sodium from the nuclear RNA fraction (N-9) by ion exchange with a sulfonic acid type resin following the 18-hour hydrolysis with N NaOH at room temperature, then subjecting the resulting nucleotides to paper chromatography (3). Trichloroacetic acid was removed from the appropriate fractions by extraction with ether. In certain instances the hydrolysis was removed by concentration in vacuo. After chromatography, the spots were located by the method of Hanes and Isherwood (9). The spots were then cut from the chromatogram and affixed to aluminum planchets with a small amount of Carter's rubber cement for determination of the P³² activity. Samples were counted in a windowless, gas-flow type Geiger-Müller counter with an automatic sample changer (15) and a Tracerlab Autoscaler Model SC-1B. Counting...
efficiency was determined by means of a uranium standard in each group of planchets counted. Coincidence corrections were made for those samples that were sufficiently active for this correction to be significant. All P\textsuperscript{32} counts were corrected for decay rate. Activity of the paper chromatogram between spots was not significantly above background.

After counting, the paper spots were removed from the planchets and digested with perchloric acid for a phosphorus determination according to the method of King (11), modified for the range 20–50 \mu g phosphorus.

### TABLE 1
#### RATIO OF LIVER WEIGHT TO BODY WEIGHT AND DEOXYRIBONUCLEIC ACID CONTENT OF THE LIVER

<table>
<thead>
<tr>
<th>TIME</th>
<th>Control Animals</th>
<th>Precancerous Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>(GROUPS)</td>
<td>Liver wt. Mg</td>
<td>Body wt. per cent</td>
</tr>
<tr>
<td>( \frac{1}{2} )</td>
<td>3.5</td>
<td>251</td>
</tr>
<tr>
<td>1</td>
<td>4.0</td>
<td>299</td>
</tr>
<tr>
<td>( \frac{1}{2} )</td>
<td>4.1</td>
<td>286</td>
</tr>
<tr>
<td>2</td>
<td>3.4</td>
<td>217</td>
</tr>
<tr>
<td>( \frac{1}{2} )</td>
<td>3.3</td>
<td>326</td>
</tr>
<tr>
<td>3</td>
<td>3.3</td>
<td>312</td>
</tr>
<tr>
<td>( \frac{1}{2} )</td>
<td>3.8</td>
<td>303</td>
</tr>
<tr>
<td>4</td>
<td>3.2</td>
<td>299</td>
</tr>
<tr>
<td>5</td>
<td>3.1</td>
<td>256</td>
</tr>
<tr>
<td>8</td>
<td>2.9</td>
<td>258</td>
</tr>
<tr>
<td>16</td>
<td>2.6</td>
<td>256</td>
</tr>
</tbody>
</table>

* Three rats per group.
† Rats fed a diet containing 0.06 per cent \( \text{3'-methyl-4-diinethylaminoazo-benzene} \). DNA = Deoxyribonucleic acid, determined by the Dische diphenylamine reaction. The reference standard used in the determinations was obtained from Schwartz Chemical Co. and contained 28.8 \mu g DNA/mg as used for the determinations and as calculated from the phosphorus content.
‡ Average of only two animals.

The fractionation procedure is outlined in Chart 1. Those fractions on the left-hand side of the chart were isolated for P and P\textsuperscript{32} determinations. In addition, an aliquot of the original homogenate from which the nuclei were prepared was also taken for analysis by the Dische diphenylamine reaction (6).

After hydrolysis of the nuclear residue (N-R) by NaOH there always remained a small residue from the nuclei of the dye-fed animals, under the conditions employed (1 ml/100 mg). This residue contained some form of azo dye, as judged by its red color in acid media. Presumably, this residue is related to the bound-dye fraction described by Miller and Miller (14). Since the residue contained no phosphorus, it was discarded.

### RESULTS AND DISCUSSION

The over-all average liver weight as per cent of body weight for the control animals was 3.4 per cent, and ranged from 2.6 to 4.1. per cent within the various time groups. For the precancerous animals the over-all average was 5.1 per cent, but the variation within the various time groups was much greater, ranging from 3.5 to 7.0 per cent (Table 1). The higher over-all average in the precancerous groups is indicative of both enlargement of the liver (16) and the lesser body growth of these animals. In fact, changes in the structure of the liver during azo dye treatment are rather profound (4, 5, 18), and comparisons of the precancerous animals with the controls must be made with caution. Although structural differences may not be so marked as in the tumor-bearing liver, conceivably the access of the labeled phosphate to the individual cell nuclei of the precancerous liver, for example, could be so altered as to obscure small differences that might exist in comparison with normal liver.

It has been shown that the deoxyribonucleic acid (DNA) content gives a reliable measure of the cellularity of the liver (4, 19). Values for the DNA content of the liver in the different groups of animals are tabulated in Table 1. This table affords some measure of the degree of cellularity together with a measure of the variation of the ratio liver weight to body weight.

In Chart 2, values are shown for the relative specific activity calculations for the various nucleic acid and phosphoprotein components of the nuclei. Values are calculated as decompositions/second/\mu g phosphorus/0.5 \mu c of P\textsuperscript{32} originally injected/gm body weight. The acid-soluble fraction (N-2, Chart 1) showed considerable variation in both phosphorus content and activity, which undoubtedly resulted from differences in the amount of soluble P\textsuperscript{32} removed from the nuclei during washing with the 2 per cent citric acid solution. Since the values for this fraction were not considered informative, they have not been included in Chart 2. Values for fraction N-3, the phospholipids, have also been omitted, since this fraction has been made the subject of a separate investigation.

The uptake of labeled phosphate into the DNA fraction (N-7) was low during the relatively short period of time included in these experiments, but is plotted in Chart 2A to afford a comparison of the relative uptake of phosphorus in this fraction as compared with the nucleotides from the nuclear RNA (Charts 2B–2E).

Examination of the specific activity values for the four constituent nucleotides of the nuclear RNA fraction revealed the following pattern: (a)
CHART 2.—Phosphorus uptake into the phosphoprotein and nucleic acids of the rat liver cell nuclei. Specific activity (decompositions/second/μg of phosphorus/0.5 μg of P32 injected/gm rat) plotted versus time after the injection of the labeled phosphate.

- Control animals.
- Precancerous animals.

CHART 2A.—Uptake in the deoxyribonucleic acid fraction. Since no purification other than that indicated in the text was applied, the values indicate the order of magnitude only for this fraction.

CHART 2B.—Uptake in the uridylic acid fraction of the nuclear ribonucleic acid.

CHART 2C.—Uptake in the guanylic acid fraction of the nuclear ribonucleic acid.

CHART 2D.—Uptake in the cytidylic acid fraction of the nuclear ribonucleic acid.

CHART 2E.—Uptake in the adenylic acid fraction of the nuclear ribonucleic acid.

CHART 2F.—Uptake in the phosphoprotein fraction of the nucleus as determined on the inorganic phosphate liberated after the alkaline hydrolysis indicated.
a steady rise during the first 2-hour period, (b) a high specific activity during the next 3-hour period, and (c) a slight decrease after 8 hours, with no appreciable decline after 18 hours. This same pattern was followed by both the precancerous animals and the controls. No significant difference was indicated for the precancerous animals as compared with that for the control animals in the rate of incorporation of labeled phosphate into the nuclear RNA fraction, since the specific activity in both groups was approximately equal at all times. The two seemingly low points (precancerous animals at 3½ hours and control animals at 4 hours) may have resulted from failure to absorb all the P³² owing to faulty injection, for example. Unfortunately, the experiments did not include a means of evaluating this possibility.

The specific activities of the four constituent nucleotides from the nuclear RNA fraction of any given group of animals did not differ significantly from one another, with the possible exception of adenylic acid. After the first 2 hours, adenylic acid values were 25-80 per cent higher than the values for the other nucleotides in some of the groups, while in the 18-hour control group the value was about 30 per cent lower than that in the other nucleotides. The significance of the observed difference between the adenylic acid and the other nucleotides is questionable on the grounds that it was not uniformly observed in all groups. However, MacIndoe and Davidson (19) and also Anderson and Åqvist (1) have observed similar differences for adenylic acid from normal rat liver nuclear RNA and also, in the latter work, for regenerating liver. Both groups of workers questioned the significance of this difference.

In those experiments where ammonium hydroxide was used for the hydrolysis (10 ml/100 mg of lipid extracted residue—N-R), the inorganic phosphate liberated from phosphoprotein, presumably, had a lower specific activity than that subsequently split off by N NaOH from the same residue. The ammonium hydroxide-labile phosphate had a specific activity approximately equal to that of the nucleotides from nuclear RNA at all the time intervals, while that liberated by the sodium hydroxide treatment was about 2-4 times greater during the period 1-8 hours after injection and decreased to about the same specific activity as the ammonium hydroxide-labile fraction after 18 hours. The amount of phosphate liberated by either hydrolysis, as estimated from the chromatograms and the aliquots used, was approximately equal. The ammonium hydroxide hydrolysis was carried out for only four of the precancerous groups, but no differences were observed in the behavior of this fraction as compared with that of the controls. Since our attention was not directed toward this difference in specific activity according to the method of hydrolysis until the experiments had progressed considerably, the significance of this heterogeneity of incorporation into what was presumably the phosphoprotein fraction was not adequately investigated. We merely note the difference at this time.

These results indicate that there is no significant difference in the incorporation of P³²-labeled phosphate into the nuclear RNA of liver cells from normal and azo dye-fed animals. Previous results suggesting a difference in the rate of incorporation into this fraction from similar experimental animals (7, 21) perhaps resulted from failure to remove all the inorganic phosphate liberated from hydrolysis of phosphoprotein, since the present work also demonstrates that the latter fraction under certain conditions of hydrolysis may have a specific activity considerably greater than that of the nuclear RNA.

**SUMMARY**

The uptake of radioactive phosphate into the nuclear ribonucleic acid fraction from the liver of normal and precancerous animals has been studied. Measurements were made at 1-hour intervals following injection of the isotope for periods up to 18 hours, with further measurements at 5, 8, and 18 hours after administration. Liver nuclei were isolated, and the residue after acid and lipid extraction was used to isolate the nuclear ribonucleic acid as the constituent nucleotides following alkaline hydrolysis and paper chromatography.

The uptake of phosphorus into nuclear ribonucleic acid did not differ significantly in either the precancerous or control animals when compared with each other.

The constituent nucleotides did not differ significantly from one another in their uptake, except for adenylic acid, which was observed to be as much as 30 per cent higher than the other nucleotides in some instances.

The inorganic phosphate from phosphoprotein (presumably) liberated by hydrolysis with 14 N NaOH had a specific activity one-half to one-fourth as great as that liberated by a subsequent hydrolysis with 1 N NaOH.

**REFERENCES**


2. Bannum, C. P., and Hunter, R. A. The Intracellular Heterogeneity of Pentose Nucleic Acid as Evidenced by
Phosphorus Incorporation into Nucleic Acids and Proteins of Liver Nuclei of Normal and Azo Dye-fed Rats

Darrell N. Ward and A. Clark Griffin