Intracellular Distribution of Enzymes

II. The Distribution of Succinic Dehydrogenase, Cytochrome Oxidase, Adenosinetriphosphatase, and Phosphorus Compounds in Normal Rat Liver and in Rat Hepatomas

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Interest in the particulate components of the cell has achieved considerable prominence during the last few years largely as the result of the development of techniques for the isolation of these cellular constituents in quantities sufficient for chemical and biochemical analysis. Thus, Bensley and his coworkers (4, 5, 19, 21, 22) have described the isolation of mitochondria, particulate glycogen, and a submicroscopic component of guinea pig liver. Claude has been instrumental in developing well defined methods for the isolation of large granules, microsomes, and chromatin threads from normal and neoplastic tissues (8, 9, 10, 11, 12). He has subjected these particulate components to elementary chemical analysis and more recently has described some of the enzymatic properties of the large granules and of the microsomes (11). A number of enzymes important to cellular metabolism were associated with the large granules while other enzymes were not associated with any particulate material and were apparently present in the cell in soluble form. None of the enzymes studied was found to be associated with the microsomes. The enzymatic properties of the nucleus have been studied in the case of liver by Dounce (13) but these studies have not been extended to malignant tissues because of technical difficulties encountered in the isolation of nuclei from these tissues (14, 15).

The importance to cancer research of a study of the particulate components of the cell is perhaps best emphasized by a few illustrations. In the first place, the viruses which cause several tumors have dimensions and chemical properties similar to those of particulate components isolated from normal cells (7, 8, 28). Furthermore, a preliminary report has indicated that the antigen which is specific for the Brown-Pearce tumor is associated with the microsome or small particle fraction of this tissue (24). Finally, Graffi (17, 18) has shown that the mitochondria of a large variety of tissues will preferentially absorb carcinogenic hydrocarbons in vitro and has presented some evidence that these particulate components are involved in the absorption of carcinogens painted on the skin of mice.

The present report describes the results of an investigation of some of the enzymatic and chemical properties of normal rat liver and rat hepatoma cells which have been separated by centrifugation into a nuclear fraction, a large granule fraction,1 and an unfractionated residue. Although objections have been raised to a comparison of normal liver and hepatoma on the basis that these tissues are greatly different in their degree of differentiation (11), such a comparison would nevertheless seem to be of considerable interest since the two tissues do represent the original and final stages in the carcinogenic process.

MATERIALS AND METHODS

Tissues.—The tissues were removed as rapidly as possible from animals killed by decapitation and were homogenized in 9 volumes of alkaline water (distilled water brought to pH 9.5 by the addition of 2.0 ml. of 0.1 N NaOH per liter (see 9, 10, 31)) in the apparatus of Potter and Elvehjem (26). The hepatoma samples were obtained through the courtesy of Drs. J. A. and E. C. Miller, and B. E. Kline. These hepatomas had been induced in rats by the oral ingestion of p-dimethylaminoazobenzene.

Tissue fractionation.—The homogenates were separated into a nuclear fraction, a large granule fraction,1 and an unfractionated residue by a centrifugation procedure described previously (31). The unfractionated residue consists of a supernatant and washings which remain after removal of nuclei and large granules and

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1 As Claude has pointed out (10) the large granule fraction of the liver contains secretory granules and mitochondria in unknown proportions. The large granule fraction of the rat hepatoma on the other hand may be deficient in secretory granules.
is the fraction from which microsomes and glycogen have been isolated by the use of greater centrifugal forces (9, 10, 21, 22). The time which elapsed during the centrifugation was 4 to 5 hours.

**Cytological methods.**—The whole homogenate and each of the tissue fractions were examined in the bright and in the dark field microscopes. Smears were also made and stained with aniline-acid fuchsin-methyl green (3).

**Enzyme assays.**—Succinic dehydrogenase, cytochrome oxidase, and adenosinetriphosphatase (ATPase) were measured as described previously (16, 32). The activities of the original homogenate and of each of the fractions were determined immediately after the fractionation had been completed.

**Analytical methods.**—Desoxypentose nucleic acid (DNA), pentose nucleic acid (PNA), and acid soluble, lipid, and "protein" phosphorus were determined by methods reported previously (29). Nitrogen was determined on the "phosphoprotein" fraction by the method of LePage and Umbreit (23). Dry weight was determined by drying aliquots of the tissue suspensions to constant weight in vacuo over P₂O₅.

**RESULTS**

The results of the enzyme assays and of the analyses are presented in Table I. The results were expressed in terms of 100 mgm. of fresh tissue or its equivalent so that data on the fractions could be compared with the data on the whole homogenate for recovery calculations. If such a comparison is made, it is seen that the amounts of the materials recovered in the tissue fractions agree well with the amounts found in the whole homogenate.

**Cytological observations.**—Microscopic examination of the fractions showed that the nuclear fraction contained some whole cells and large granules in addition to large numbers of intact nuclei which had clumped together in large masses. The large granule fraction contained large numbers of particles of a fairly uniform size which stained red with Bensley's aniline-acid fuchsin-methyl green (3). Nuclei and whole cells were apparently absent from this fraction and also from the unfracinated residue although the latter did contain some large granules.

**Enzyme assay results.**—The major portions of the succinic dehydrogenase and cytochrome oxidase present in the original homogenate were found to be associated with the large granule fractions of both tissues. In confirmation of previous results, however, the activity of these enzymes in the hepatoma was considerably lower than in the normal liver (33).

The distribution of ATP-ase in the tissues was of considerable interest because the activity of this enzyme had been shown to be essentially the same in

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**Table I: Distribution of Enzymes, Phosphorus-Containing Compounds, Protein, Nitrogen, and Dry Material in Fractions of Rat Liver and HEPATOMA HOMOGENATES**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Enzyme activities</th>
<th>DNA</th>
<th>Acid soluble phosphorus</th>
<th>&quot;Protein&quot; phosphorus</th>
<th>&quot;Organ&quot; phosphorus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Original homogenate</td>
<td>383</td>
<td>8</td>
<td>1012</td>
<td>22.6</td>
</tr>
<tr>
<td></td>
<td>Nuclear fraction</td>
<td>254</td>
<td>289</td>
<td>147</td>
<td>257</td>
</tr>
<tr>
<td></td>
<td>Large granule fraction</td>
<td>25.4</td>
<td>23</td>
<td>4.9</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>Unfractionated residue</td>
<td>1012</td>
<td>865</td>
<td>22.6</td>
<td>22.6</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>Original homogenate</td>
<td>86.7</td>
<td>322</td>
<td>802</td>
<td>82.3</td>
</tr>
<tr>
<td></td>
<td>Nuclear fraction</td>
<td>78.3</td>
<td>170.1</td>
<td>209.0</td>
<td>58.4</td>
</tr>
<tr>
<td></td>
<td>Large granule fraction</td>
<td>78.3</td>
<td>170.1</td>
<td>209.0</td>
<td>58.4</td>
</tr>
<tr>
<td></td>
<td>Unfractionated residue</td>
<td>86.7</td>
<td>322</td>
<td>802</td>
<td>82.3</td>
</tr>
</tbody>
</table>

Average values are given, with ranges of values in parentheses. The data on liver were reported previously (31). The data on hepatomas include 4 analyses with the exception of cytochrome oxidase and ATPase in which only 2 analyses were made.

* The activities are expressed as micrograms of equivalent of adrenaline per mgm. of fresh tissue in 15 minutes, by the equivalent of 100 mgm. of fresh tissue.

** The phosphorus is calculated from phosphoester measurements.
both tissues (27). This observation was confirmed in the present study (Table I). The distribution of the enzyme in the two tissues was radically different, however. In the case of the normal liver, about 48 per cent of the activity was found to be associated with the large granule fraction and 30 per cent with the unfractiioned residue while in the hepatoma 75 per cent of the ATP-ase activity was found to be associated with the unfractiioned residue and only 12 per cent with the large granule fraction. It would be of considerable interest to extend the ATP-ase studies to include an investigation of the specificity of the enzyme towards various substrates, a study of the degree of hydrolysis of ATP in the various fractions, and further fractionation of the tissues.

Analytical results.—The analyses for DNA showed that all of this nucleic acid was present in the nuclear fractions of both normal liver and hepatoma. Thus

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fraction</th>
<th>Activity per mgm. dry weight</th>
<th>Activity per mgm. &quot;protein&quot; nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Succin-oxidase*</td>
<td>Cytochrome oxidase*</td>
</tr>
<tr>
<td>Normal liver</td>
<td>Original homogenate</td>
<td>12.0</td>
<td>31.7</td>
</tr>
<tr>
<td></td>
<td>Nuclear</td>
<td>7.7</td>
<td>16.6</td>
</tr>
<tr>
<td></td>
<td>Large granule</td>
<td>50.7</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>Unfractionated residue</td>
<td>2.0</td>
<td>6.3</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>Original homogenate</td>
<td>4.1</td>
<td>15.1</td>
</tr>
<tr>
<td></td>
<td>Nuclear</td>
<td>1.9</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Large granule</td>
<td>28.5</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>Unfractionated residue</td>
<td>1.5</td>
<td>7.3</td>
</tr>
</tbody>
</table>

* Cu. mm. O₂ uptake per 10 min.
** Micrograms phosphorus liberated per 15 min.

Excellent chemical evidence was provided in support of the cytological observations which indicated that nuclei were present only in the nuclear fraction and also in support of the hypothesis that DNA is found only in the nucleus of the cell. The data also confirm previous observations made in this laboratory (30) which showed that DNA was present in much higher concentrations in the hepatoma than in the normal liver.

PNA, acid soluble lipid phosphorus, "protein" nitrogen, and dry material were found mainly in the unfractiioned residue. In harmony with the idea that the large granules are complexes of protein and phospholipid (10), lipid phosphorus was found to be more concentrated per mgm. of dry material in the large granule fractions. It is also of interest to note that the nuclear fraction of the hepatoma contains more dry material and "protein" nitrogen than does the nuclear fraction of normal liver while in the case of the large granule fraction the situation in the two tissues is reversed.

DISCUSSION

The observation that the succinic dehydrogenase and the cytochrome oxidase activities of normal rat liver and of rat hepatoma were associated with the large granule fractions of these tissues introduces the possibility that the decreased activity of these enzymes observed in the hepatoma as compared to the normal liver (Table I), and (33), might be rationalized on the basis of a decreased amount of large granule material in the hepatoma cell. If this explanation were correct, the activity of these enzymes per mgm. of dry material and of "protein" nitrogen should be the same in the large granule fractions of the two tissues. Table II shows the activities of these enzymes per mgm. of dry material and of "protein" nitrogen. It is evident that the enzyme activities when expressed in these terms are more similar for the large granule fractions of the normal liver and the hepatoma than are the enzyme activities of the original tissues. Thus it would seem that the decreased activities of these enzymes in the hepatoma could be explained at least in part on the basis of a diminution in the large granule fraction of the hepatoma cell. Although the data on cytochrome oxidase are in accord with such an explanation, the data on succinic dehydrogenase show a considerable discrepancy between the activity of this enzyme per unit of protein or dry weight in the large granule fraction of the hepatoma as compared with the normal liver. In addition, it must be pointed out that the chemical composition of the hepatoma granules is considerably different from the composition of the large granules of the liver; the former contain more lipid phosphorus and PNA and less "protein" nitrogen than do the latter. Thus, although the hepatoma cell does contain less large granule material than the normal liver cell, it is apparent that this difference does not provide a complete explanation for the decreased enzyme activities observed in the hepatoma. Never-
theless, in the case of enzymes which are associated with large granules or other particulate material, variations in the amount of the particulate material in the cell must be considered as a possible explanation for variations in the enzyme activities which occur under different physiological conditions. Thus it would be of considerable interest with reference to the association of enzymes with granules to re-examine the variations of the succinoxidase activity observed in vitamin deficiencies (1, 2, 20), pregnancy (25), and after adrenalectomy and thyroid feeding (34).

The data on ATP-ase (Table II) show that the activity of this enzyme was much the same in the large granule fraction of the normal liver and of the hepatoma. In the case of the hepatoma, however, only a small portion of the original ATP-ase activity was associated with the large granule fraction while in the liver most of the ATP-ase activity was associated with this fraction (Table I). The largest portion of the ATP-ase present in the hepatoma was found to be associated with the unfractionated residue. Thus the findings on ATP-ase also suggest a loss of large granule material as a characteristic of the malignant change. In the case of this enzyme, however, the activity which was associated with the large granule fraction of the liver appeared in the unfractionated residue of the hepatoma cell in contrast to the enzymes of the succinoxidase system which were lost from the cell.

An important point and a difficult one to decide is whether the DNA content of hepatoma nuclei is greater than the DNA content of liver nuclei. A comparison of the ratio of DNA to dry material in the nuclear fractions of the two tissues indicates that the DNA content of hepatoma nuclei is greater (Table I). The same conclusion was reached in a previous report from this laboratory upon different evidence (30). On the other hand Dounce found that the DNA content of transplanted hepatoma 31 nuclei was about 50 per cent as great as the DNA content of liver nuclei (15). His results were complicated by the fact that liver nuclei prepared at pH 2.4 had apparently lost histone while nuclei prepared at pH 6.0 had lost some nucleic acid. A similar situation was inferred to exist in the case of the hepatoma nuclei although no evidence was advanced in support of this assumption. Our own results would seem to be independent of these difficulties since the nuclear fraction was prepared at a pH of about 7.0 and because all of the DNA present in the original tissue was recovered in the nuclear fraction. On the other hand our results are open to the criticism that the nuclear fractions are admittedly impure. If this factor is considered, it would seem that the differences between the nuclear fraction of the hepatoma and the nuclear fraction of the normal liver would be greater since the latter would be more likely to be contaminated with large amounts of large granule material than the former.

The data presented in Table I support the suggestion advanced previously on entirely different evidence that the hepatoma contains approximately twice as many cells per volume of tissue as does the normal liver (30). This follows from the data on the DNA and the dry material contents of the nuclear fractions of the two tissues if it is assumed that the DNA content per nucleus is approximately the same for the two tissues. If it is true that the hepatoma contains twice as many cells per volume of tissue as does normal liver, it is of interest to note that the amount of dry material found in the hepatoma cytoplasm (large granules plus unfractionated residue) is only about 50 per cent as great as the amount of dry material found in the liver cytoplasm. This is considerably less than would be predicted on the basis that the nucleus is the same size in both tissues (6) and occupies about 6 per cent of the volume of the liver cell (13).

From the data presented in this paper the following picture of the difference between the normal liver cell and the hepatoma cell can be obtained. The hepatoma tissue contains cells which are about one-half as large as the normal liver cells. The volume of the nucleus in both tissues is approximately the same, however, and therefore the volume of the cytoplasm of each hepatoma cell must be about one-half as large as the cytoplasm of the normal liver cell. The concentration of large granule material and of other cytoplasmic material is much less in the hepatoma cell than in the liver cell. The enzymatic properties of the cytoplasm have also been altered; a large portion of the activity of the succinoxidase system present in the normal liver cell is absent from the hepatoma cell while the distribution of ATP-ase in the hepatoma cell is different from that of the liver cell although the ATP-ase activities of the two tissues are essentially the same. The concentration and distribution of chemical compounds are also different in the cytoplasm of the hepatoma cell than in the normal liver cell. Thus it is evident that the differences between the normal and the malignant cell are exceedingly complex. A further understanding of these differences will undoubtedly be obtained when the studies are extended to include a larger number of enzymes, a more complete fractionation of the tissues, a study of embryonic and regenerating liver as well as livers obtained from animals at various stages of the carcinogenic process, and a wider variety of normal and cancerous tissues.
SUMMARY

1. Homogenates of normal rat liver and rat hepatomas were separated by centrifugation into a nuclear fraction, a large granule fraction, and an unfraccionated residue.

2. Succinic dehydrogenase, cytochrome oxidase, adenosine triphosphatase, pentose and deoxypentose nucleic acids, acid soluble and lipid phosphorus, “protein” phosphorus and nitrogen, and dry material were determined on the original tissue homogenate and on each of the tissue fractions.

3. The activities of succinic dehydrogenase and cytochrome oxidase were much lower in the hepatoma than in the normal liver. However, the major part of the enzyme activities associated with the original tissues was found to be associated with the large granule fractions of these tissues, and the enzyme activities per unit of dry material or of “protein” nitrogen were similar in the large granule fraction of the two tissues. Thus the decreased enzyme activity observed in the hepatoma seemed to be due at least in part to a decrease in the amount of large granule material.

4. The activity of adenosinetriphosphatase was essentially the same in the hepatoma as in the normal liver although the distribution of the enzyme was profoundly different in the two tissues. In the latter about 50 per cent of the activity was associated with the large granules and 30 per cent with the unfraccionated residue, while in the hepatoma 75 per cent of the enzyme activity was associated with the unfraccionated residue and only 12 per cent with the large granules. The enzyme activity per unit of dry weight or of “protein” nitrogen was similar for both tissues in the large granule fraction.

5. The deoxypentose nucleic acid content of the hepatoma was found to be more than twice as great as that of normal liver. All of the deoxypentose nucleic acid present in the whole homogenates was recovered in the nuclear fractions. The increased content of this nucleic acid in the hepatoma was considered to be due to an increase in the number of cells in this tissue.

6. The pentose nucleic acid content of the two tissues was found to be essentially the same and the major part of this nucleic acid was found in the unfraccionated residue. The nucleic acid content per mgm. of dry material was higher in the large granule fraction and in the unfraccionated residue of the hepatoma than in normal liver.

7. The major portions of the other components measured were found in the unfraccionated residue. The lipid phosphorus was more concentrated in the large granule fractions of the two tissues and more concentrated in the cytoplasm fractions of the hepatoma than in the corresponding fractions of normal liver.

REFERENCES


Intracellular Distribution of Enzymes. II. The Distribution of Succinic Dehydrogenase, Cytochrome Oxidase, Adenosinetriphosphatase, and Phosphorus Compounds in Normal Rat Liver and in Rat Hepatomas

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