Subcellular Distribution of Some Metallic Cations in the Early Stages of Liver Carcinogenesis

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SUMMARY

In the early stages of liver carcinogenesis induced by 4-dimethylaminoazobenzene the subcellular pattern of calcium, magnesium, sodium, and potassium is sharply altered. Divalent cations decrease in the nuclear and mitochondrial fractions and increase in the residual fraction. In the case of mitochondria, however, cations decrease proportionally to the amount of proteins and nucleoproteins in the fraction, and, therefore, the specific content in calcium and magnesium of mitochondria does not change. In the nuclear fraction, on the contrary, the quantities of proteins and nucleoproteins vary only slightly, but the level of divalent cations is strongly reduced and the specific content of calcium and magnesium in nuclei is sharply altered. Monovalent cations do not undergo profound alteration in their subcellular distribution but, at a lower degree, in general follow the pattern of divalent cations.

The mineral content of primary and transplantable hepatomas of the rat shows differences from that of normal liver (2, 14, 19, 20).

Nothing is known, however, about the subcellular distribution of minerals in the hepatoma and preneoplastic liver except some rough measurements of the total content in heavy metals carried out by Arnold and Sasse (2) using a histochemical method (sulfide-silver technic).

This paper deals with investigations of the content of some divalent (calcium and magnesium) and monovalent (sodium and potassium) cations in the liver cell fractions, separated by differential centrifugation, in the early stages of carcinogenesis.

Liver carcinogenesis induced by 4-dimethylaminoazobenzene (DAB) was chosen because extensive information about the intracellular composition of rat liver after ingestion of azo dyes is already available, owing to the studies of Price, the Millers, Potter, and others (21-26).

The present study was undertaken especially on the basis of the following considerations:

1. Metals form a variety of complexes with proteins, but, essentially, three categories can be recognized (6): (a) a specific metal takes an integral part in the structure of the protein (metalloproteins); (b) the metal ion is bound reversibly but is essential to the stability of configuration of the molecule or to the association of molecular subunits; (c) the metal ion is readily reversibly bound and directly affects physical properties of the protein as net charge, aggregation, solubility, and, indirectly, configuration and biological functions. At the present it must be assumed that in the case of monovalent cations like sodium and potassium these ions play a role in the ionic atmosphere of proteins, especially as counter-ions to negatively charged macromolecules, although it seems that in part sodium and potassium are also bound in a more stable way (10, 32). A loss of cations may therefore affect structure and biological functions of proteins and nucleoproteins. On the other hand, primary damages to macromolecules could result in a release of bound cations, and therefore changes in the cellular and subcellular concentration of ions would be a sign of molecular alterations.

2. Minerals are not uniformly distributed in the subcellular fractions (10, 16, 28, 32), and selective modifications in the intracellular pattern could occur with slight changes (or none at all) in the mineral content of the cell as a whole.

MATERIALS AND METHODS
BD III (Druckrey [5]) and Wistar male rats bred in our laboratory were used. Eighty rats,
Weighing 150–180 gm each, were fed a semi-synthetic diet (4) containing 2 mg riboflavin/kg. Forty rats were fed the basal diet, and the remaining were fed the basal diet containing 0.5 gm DAB/kg. Two identical sets of experiments were carried out 8 months apart. In the first set BD III rats were used and in the second Wistar rats. On the average, every rat received a total dose of 170 mg of DAB at the rate of about 6 mg/day. Four weeks after the rats were placed on the experimental diet, they were killed. The livers were perfused with 0.25 m sucrose at 4°C. and then forced through a stainless steel tissue press with holes 1 mm. in diameter. The liver pulp (three to four livers were pooled) was rapidly weighed and a portion removed. The remaining liver pulp was homogenized in 0.25 m sucrose at 4°C. and fractionated into nuclei, mitochondria, and supernatant (microsomes + cell fluid) according to Schneider and Hogeboom (27). Occasionally the nuclei were isolated according to the method of Hogeboom et al. (7), which is recommended for the preparation of nuclei with small degree of contamination and cyto logically closely resembling those seen in the living cell. No significant difference was found in the mineral content (calcium was not measured) of nuclei prepared according to the two different methods, and therefore the results were combined. In each cell fraction and in the liver pulp proteins were determined gravimetrically (22) and DNA and RNA according to Webb and Levy (33–34).

The mineral content was determined in the following way. The liver pulp and the subcellular fractions were dried at 90°C. for 6 hours and ashed at 500°C. for 12 hours. The ashes were dissolved in 10 n HCl, dried under an infrared lamp, and finally taken up in a measured quantity of 0.1 n HCl. Sodium and potassium were measured by flame photometry (Beckman DU) with comparisons of the solution of ashes with solutions of cations which had a known concentration of electrolytes similar to that of liver tissue. Calcium and magnesium were determined as follows: The solution of the sample in 0.1 n HCl was passed through a column (7 X 70 mm.) of a purified cation exchange resin (Dowex 50-X8; 200–400 mesh); and, after a washing with ion exchange water, the cations were eluted with 6 n HCl. The eluate was neutralized with NH4OH (methyl red as indicator) and calcium precipitated as calcium oxalate. Magnesium in the supernatant and calcium in the precipitate, after having been dissolved in 1 n HCl, were determined by complexometric titration according to Holasek and Flaschka (8, 9). Preliminary trials showed no intrinsic loss of calcium and magnesium from the resin under the experimental conditions used for the purification of the ash solutions. The purification of the samples by cation exchange resin was carried out because complexometric titration when applied to the nonpurified ash extracts is unreliable, according to our experience, because of uncertainty in the color-turning point of the indicator (Eriochrome T Schwarz). Extremely precise measurements are possible with the modified procedure described above.

RESULTS

Calcium and magnesium are firmly bound to the cell particulate fractions. In fact, preliminary experiments have shown that the concentration of the divalent cations in the mitochondrial and nuclear fractions did not change significantly when mitochondria and nuclei, prepared according to the method of Schneider and Hogeboom (27), were washed 2 additional times with 0.25 m sucrose at 4°C. These results are in agreement with previous work, and we refer to the paper of Thiers and Vallee (32) for comparison of data from different sources.

The levels of DNA, RNA, and protein in the liver and subcellular fractions from normal and DAB-fed rats are in agreement with those of Price et al. (23), and therefore no absolute figures are reported in this paper.

Table 1 presents the whole cell content (liver pulp) and the subcellular pattern of calcium and magnesium in livers of normal and DAB-fed rats. In normal liver the largest quantity of calcium was found in the mitochondria, and only a small portion in the residual fraction. Nuclei, however, showed the highest specific calcium content per gm. protein and nucleoprotein. Ingestion of DAB caused a decrease of the calcium content in the whole cell and altered the subcellular distribution of the cation. The decrease of calcium in the whole liver pulp after ingestion of the carcinogen was accounted for by a decrease of the nuclear and mitochondrial calcium while the cation increased in the residual fraction (Table 1).

In normal liver cells magnesium is distributed differently from calcium. The largest quantity of magnesium was found in the residual fraction. Nuclei contained only a small portion of the magnesium of the whole cell, although the concentration of this cation in the nuclear fraction differed little from that of calcium. The distribution of magnesium in the cell was closely proportional to that of proteins and RNA. In fact, magnesium content expressed per gm. protein and RNA does not vary much in the different cell fractions, whereas the specific content of calcium per gm. protein or nucleoprotein was 20–24 times higher.
in nuclei than in the residual fraction. DAB ingestion caused only a slight decrease in the magnesium content of the whole liver pulp, but the subcellular distribution of the cation was sharply altered. The changes were a decrease of the nuclear and mitochondrial magnesium while the cation increased in the residual fraction.

It should be pointed out that there is a basic difference in the nature of the decrease of cations in the mitochondrial and nuclear fractions. In the case of mitochondria, in fact, the specific content in calcium and magnesium per gm. protein or nucleoprotein does not change, although the divalent cations content of the fraction per gm. fresh tissue is sharply reduced—i.e., the fraction in toto is reduced.

In the nuclear fraction, on the contrary, there is a strong decrease of the specific content of calcium and magnesium after DAB ingestion, i.e., the decrease of calcium and magnesium is due to an actual release of the divalent cations from nuclei.

During isolation in aqueous media, and despite repeated washing, a quota of the original content of sodium and potassium is retained by nuclei and mitochondria (10, 32). The figures in Table 2, therefore, should be regarded as measurements of the monovalent cations “adhering” to nuclei and mitochondria after the three washings with 0.25 M sucrose. Hence, sodium and potassium content was expressed as _meq/gm_ fresh tissue or per gm. protein, DNA, and RNA; and no attempt was made to give figures per gm. water in fraction.

Ingestion of DAB caused an identical per cent decrease (−17 per cent) of sodium and potassium.

### TABLE 1

**Distribution of Calcium and Magnesium in Liver Fractions of Rats Fed DAB**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>μeq/gm Fresh Tissue</th>
<th>μeq/gm Protein in Fraction</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
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</tr>
<tr>
<td></td>
<td>Per cent</td>
<td>Δ%</td>
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<td>Δ%</td>
</tr>
<tr>
<td>Liver pulp (Mean) (Sw)</td>
<td>3.16 ± 0.097</td>
<td>2.12 ± 0.111</td>
<td>100 ± 33</td>
<td>24.84 ± 20.00</td>
</tr>
<tr>
<td>Nuclei (Mean) (Sw)</td>
<td>1.11 ± 0.058</td>
<td>0.34 ± 0.080</td>
<td>16 ± 69</td>
<td>70.18 ± 20.44</td>
</tr>
<tr>
<td>Mitochondria (Mean) (Sw)</td>
<td>1.70 ± 0.091</td>
<td>1.29 ± 0.083</td>
<td>61 ± 24</td>
<td>45.00 ± 45.60</td>
</tr>
<tr>
<td>Residual fraction (Sw)</td>
<td>0.24 ± 0.010</td>
<td>0.37 ± 0.014</td>
<td>18 ± 54</td>
<td>3.68 ± 5.90</td>
</tr>
<tr>
<td>Recovery</td>
<td>3.05 ± 0.98</td>
<td>2.00 ± 0.95</td>
<td></td>
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Sw = standard error of mean.

* 4-Dimethylaminoaobenzene fed 6 mg. per day and per rat for 4 weeks.

† Liver after being forced through a stainless steel tissue press with holes 1 mm. in diameter, which process is effective in removing connective tissue.

‡ Microsomes and nonparticulate cell fluid.

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_Sources:_

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† Liver after being forced through a stainless steel tissue press with holes 1 mm. in diameter, which process is effective in removing connective tissue.

‡ Microsomes and nonparticulate cell fluid.
in the liver pulp. The subcellular fractions also showed reduction in their content of monovalent cations between a minimum of −12 per cent and a maximum of −31 per cent (Table 2). As for the divalent cations, sodium and potassium in mitochondria decrease proportionally to the whole fraction, whereas in nuclei their specific content is reduced. Sodium and potassium also decreased in the residual fraction.

Discussion

It is generally assumed that proteins and nucleic acids bind divalent cations to their negatively charged sites. Owing to their large number of phosphate groups, nucleic acids are particularly suitable for the binding of divalent cations. Recent evidence, however, supports the view that adenine and guanine of nucleic acids form five-membered chelates with divalent cations (35). Many authors suggest, moreover, that calcium and magnesium act as a metallic bridge between DNA and protein (12, 18). On the other hand, evidence exists that proteins are bound to nucleic acids by interference of the negatively charged phosphate groups of nucleic acids with positive groups of proteins. Free negative charges (phosphate) on nucleoproteins are, however, still available (8).

The possibility that ingestion of DAB could produce calcium and magnesium loss from nuclei through an indirect effect, such as loss of nucleic acids or proteins and consequently of the bound divalent cations, seems hardly acceptable. On the contrary, DNA and proteins are slightly increased in the nuclear fraction after feeding the carcinogen,

### Table 2

<table>
<thead>
<tr>
<th>Fraction</th>
<th>μEq/gm Fresh Tissue</th>
<th>% Changes</th>
<th>μEq/gm Protein in Fraction</th>
<th>% Changes</th>
<th>μEq/gm DNA in Fraction</th>
<th>% Changes</th>
<th>μEq/gm RNA in Fraction</th>
<th>% Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>Per</td>
<td>DAB</td>
<td>Per</td>
<td>None</td>
<td>DAB</td>
<td>None</td>
<td>DAB</td>
</tr>
<tr>
<td>Liver pulp† (Mean) (Sa)</td>
<td>65.30 ± 0.920</td>
<td>100</td>
<td>54.00 ± 2.340</td>
<td>100</td>
<td>510 ± 400</td>
<td>-4</td>
<td>35.50 ± 920</td>
<td>-29</td>
</tr>
<tr>
<td>Nuclei (Mean) (Sa)</td>
<td>3.95 ± 0.150</td>
<td>6</td>
<td>3.22 ± 0.180</td>
<td>6</td>
<td>247 ± 189</td>
<td>-24</td>
<td>1.995 ± 410</td>
<td>-29</td>
</tr>
<tr>
<td>Mitochondria (Mean) (Sa)</td>
<td>2.70 ± 0.090</td>
<td>4</td>
<td>2.01 ± 0.180</td>
<td>4</td>
<td>69 ± 72</td>
<td>+4</td>
<td>1.50 ± 1.650</td>
<td>+11</td>
</tr>
<tr>
<td>Residual fraction† (Mean) (Sa)</td>
<td>56.75 ± 3.410</td>
<td>87</td>
<td>46.78 ± 1.680</td>
<td>87</td>
<td>835 ± 710</td>
<td>-15</td>
<td>18.30 ± 19.100</td>
<td>+4</td>
</tr>
<tr>
<td>Recovery</td>
<td>63.40 ± 2.140</td>
<td>97</td>
<td>52.01 ± 1.680</td>
<td>97</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**K**

<table>
<thead>
<tr>
<th></th>
<th>% Changes</th>
<th>μEq/gm Fresh Tissue</th>
<th>% Changes</th>
<th>μEq/gm Protein in Fraction</th>
<th>% Changes</th>
<th>μEq/gm DNA in Fraction</th>
<th>% Changes</th>
<th>μEq/gm RNA in Fraction</th>
<th>% Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver pulp† (Mean) (Sa)</td>
<td>21.70 ± 0.840</td>
<td>100</td>
<td>18.00 ± 1.010</td>
<td>100</td>
<td>170 ± 164</td>
<td>-4</td>
<td>11.800 ± 8.460</td>
<td>-28</td>
<td></td>
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<tr>
<td>Nuclei (Mean) (Sa)</td>
<td>1.81 ± 0.050</td>
<td>6</td>
<td>1.08 ± 0.070</td>
<td>6</td>
<td>76 ± 64</td>
<td>-16</td>
<td>611 ± 474</td>
<td>-22</td>
<td></td>
</tr>
<tr>
<td>Mitochondria (Mean) (Sa)</td>
<td>1.32 ± 0.060</td>
<td>6</td>
<td>0.91 ± 0.080</td>
<td>5</td>
<td>34 ± 33</td>
<td>-4</td>
<td>734 ± 746</td>
<td>+2</td>
<td></td>
</tr>
<tr>
<td>Residual fraction† (Mean) (Sa)</td>
<td>19.16 ± 0.990</td>
<td>88</td>
<td>15.70 ± 0.840</td>
<td>88</td>
<td>262 ± 239</td>
<td>-15</td>
<td>6.180 ± 6.400</td>
<td>+4</td>
<td></td>
</tr>
<tr>
<td>Recovery</td>
<td>21.69 ± 1.784</td>
<td>100</td>
<td>17.78 ± 0.840</td>
<td>99</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Na**

Sw = standard error of mean.

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† Liver after being forced through a stainless steel tissue press with holes 1 mm. in diameter, which process is effective in removing connective tissue.

‡ Microsomes and nonparticulate cell fluid.
and the observed decrease of RNA of approximately 10 per cent could account for only a small percentage of the total cation loss.

It is, therefore, necessary to conclude that the nucleic acids and the proteins of liver nuclei of rats fed the carcinogen actually bind a smaller quantity of divalent cations than under normal conditions. At the present status of our knowledge a definite mechanism for the cation release cannot be given although several reasonable hypotheses can be presented:

1. The carcinogen molecules could replace the cations on the negatively charged sites of the nuclear macromolecules. DAB is in fact a strong positively charged molecule.

2. Metabolic products of DAB could sequestrate the cations by formation of metal chelates.

3. Actual reaction of the metabolic products of DAB with nucleic acids and proteins could occur. Miller, Miller, and Hartmann (18) have found that one of the most important metabolites of 2-acetylaminofluorene is N-hydroxy-2-acetylamino-fluorene. They postulated that either this compound or the deacetylated derivative could react directly with nucleic acids or proteins. It can be foreseen that a similar mechanism would act also in the case of DAB, as well as for many other carcinogenic amines, amides, and azo dyes (18). As a consequence of the alterations in the nucleic acids and protein structure, a net loss of cations could result.

The decrease of calcium and magnesium in the mitochondrial fraction is roughly proportional to the decrease of protein and RNA of the fraction. Therefore, a quantitative reduction of the cell mitochondria accounts for the cation decrease. The decrease of liver cell mitochondria in rats fed azo dyes has already been demonstrated (1).

The lack of a specific release of cations from mitochondria in contrast with nuclei could be due to different environmental conditions (pH, redox potential, etc.) in these cell particles or because the molecules of the carcinogen are metabolized differently by mitochondria.

Monovalent cations do not undergo profound alterations in their subcellular distribution but, at a lower degree, in general follow the pattern of divalent cations. In the residual fraction, in contrast to divalent cations, monovalent cations are decreased.

Nuclei and mitochondria isolated in aqueous media retain only a part of their original content of monovalent cations (10, 29, 30). It is currently assumed that the portion of "adhering" sodium and potassium plays a part in the ionic atmosphere of negatively charged sites of macromolecules. An alteration of nuclear macromolecules which causes release of divalent cations, therefore, is expected to produce some loss of monovalent cations as well.

In the case of mitochondria a reduction of the whole fraction accounts for the cation decrease. The decrease of monovalent cations in the residual fraction, finally, seems consistent with a partial alteration of the cell capacity to retain these ions.

As stated in the Introduction, calcium and magnesium are intimately associated with protein and nucleic acids, and their release cannot be without consequences for the physical and chemical properties of the macromolecules and, hence, for their biological functions. In fact, loss of calcium and magnesium causes splitting of nucleoproteins (12, 13) and mutations (15, 17, 31); calcium and magnesium protect bacteriophage from inactivation (11), and recent evidence suggests that magnesium has a role in the mechanism of nucleoprotein reproduction (35).

ACKNOWLEDGMENTS

The authors wish to express their thanks to Mrs. Tyra Koring-Bresciani for the English translation and to Dr. Salvatore Califano, Istituto Chimico, University of Naples, for helpful discussion.

REFERENCES


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