BIOTIN, CHOLINE, INOSITOL, p-AMINOBENZOIC ACID, AND VITAMIN B₆ IN TRANSPLANTABLE MOUSE CARCINOMAS AND IN MOUSE BLOOD*

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The levels of biotin, choline, inositol, p-aminobenzoic acid, and of the vitamin B₆ complex in mouse epidermis treated with the potent carcinogen, methylcholanthrene, have been determined by Tatum et al. (1) as part of a study of the mechanism of the production of epidermal cancers in mice. Except for one value for choline (2), there are no reports in the literature on the levels of B vitamins in methylcholanthrene-produced skin carcinomas. The amounts of biotin, choline, inositol, p-aminobenzoic acid, and of the vitamin B₆ complex in a transplantable squamous cell carcinoma have now been determined. Since it was not possible to obtain tumor samples free of blood, it was necessary also to assay mouse blood for these vitamins. This paper summarizes the results of these determinations.

EXPERIMENTAL

Tumors—The tumor type analyzed was a squamous cell carcinoma descended from a transplantable carcinoma which Cooper, Firminger, and Reller (3) had originally produced in the epidermis of a Swiss mouse by the topical application of methylcholanthrene. There are certain definite advantages in analyzing transplanted tumors rather than carcinomas individually produced by methylcholanthrene. As has been mentioned by Suntzeff and Carruthers (4), the transplanted tumors have less necrosis and keratinization and show more uniformity in the type of malignant cells present than do tumors individually produced upon the skin. Transplanted tumor tissue is less likely to contain connective tissue mixed with the tumor tissue. Finally, the transplanted tumors grow much faster than do the tumors arising on the skin.

The tumors were grown subcutaneously in 6 week-old Swiss mice of both sexes. Suntzeff and Carruthers (4) found that the rapidly growing trans-
plants began to show some necrosis when less than 10 mm. in diameter, and therefore analyzed tumors 5 to 8 mm. in diameter. Except as indicated in Table II, we also have sacrificed the animals when the tumors measured from 5 to 8 mm. in diameter, about 12 days after implantation. Even tumors as small as this sometimes showed central necrosis. Tumors were collected from five different generations of transplants, the most recent being the thirty-eighth generation. The microscopic appearance of the different transplanted tumors was quite similar, showing solid sheets of malignant epithelial cells with relatively slight keratin formation. In preparing the tumors for analysis, the connective tissue capsule was removed, the tumor blotted with a good grade of filter paper to remove adherent blood, any necrotic tissue scraped away, and the tumor cut into small pieces. Pooled samples were dried and stored in vacuo over phosphorus pentoxide, as previously described (1). Tumors from about twenty-five mice were required to give a dried sample weighing approximately 0.5 gm.

The preparation of the tumor tissue extracts for microbiological assay and the methods of assay of biotin, choline, inositol, p-aminobenzoic acid, and the vitamin B complex with normal and mutant strains of *Neurospora* were the same as were previously used for the corresponding analyses of mouse epidermis during methylcholanthrene carcinogenesis (1). These five members of the vitamin B complex make a convenient group for investigation, since the same method of tissue hydrolysis is adequate for each vitamin, and since each substance can be satisfactorily determined with a specific strain of *Neurospora* (1). In order to facilitate the liberation of the vitamins from the tissue by the sulfuric acid hydrolysis, the dried tumor samples were finely ground in a small mullite mortar and then redried before being used.

**Blood**—Tumors, at least when still comparatively small, are usually well supplied with blood vessels. Although some blood could be removed from the mouse tumors by blotting, the minced tumor tissue had a slight but definite pink color due to the presence of blood. It was therefore necessary to determine whether any of the vitamin B factors under examination were present in mouse blood in amounts sufficiently high to affect the tumor determinations. In the previous assays of epidermis it had not been necessary to consider the vitamin levels of the blood, since epidermis is avascular.

Healthy female Swiss mice, about 8 weeks old, which had been fed Rockland mouse pellets and water *ad libitum*, were anesthetized with ether,

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1 The tumors used for transplanting were obtained through the courtesy of Dr. V. Suntzeff.
2 The biotin used in this work was kindly supplied by Merck and Company, Inc., Rahway, New Jersey.
and venous blood was drawn from the right ventricle of the heart or from the inferior vena cava. In all cases, the blood was withdrawn at the same time of day, about 2 p.m. The blood was transferred from the syringe to a weighing bottle containing a weighed amount of sodium citrate which had been oven-dried in the bottle. On an average, 0.4 gm. of blood was obtained per mouse. The removal of this amount of blood resulted in the death of the animals. Since the blood was being studied in relationship to the tumors, the blood samples were dried, ground, extracted, and assayed in the same manner as the tumor samples.

The values obtained for the content of biotin, choline, inositol, p-aminobenzoic acid, and vitamin B₆ in mouse blood are given in Table I, calculated in micrograms per gm. of dry weight, in order to facilitate comparison with the tumor results. Both tissues had a water content of 80.5 per cent as determined by drying for 60 hours in vacuo over phosphorus pentoxide.

No figures on the levels of any of these five vitamins in mouse blood were found in the literature. Levels of inositol and vitamin B₆ have not previously been reported for the blood of any animal. As is shown in Table I, some of the blood samples, each of which consisted of the blood from four mice, differed considerably in biotin content. The average biotin value was 0.064 \( \gamma \) per gm. of dry weight, or 0.0125 \( \gamma \) per cc. Burk et al. (5) reported values of 0.015 to 0.035 \( \gamma \) per cc. in the blood of rats being fed \( p \)-dimethylaminoazobenzene. Our results indicate also considerable variations among mice in the choline content of blood. This probably reflects variations in lecithin. The average choline value was 947 \( \gamma \) per gm. of dry weight, or 185 \( \gamma \) per cc. This is about the same as Luecke and Pearson reported for the plasma of horses and cattle (6) and of dogs (7).

### Table I

**Content of Five Vitamin B Factors in Mouse Blood**

The values are given for pooled samples of blood and are expressed in micrograms per gm. of dried blood, with the averages in parentheses.

<table>
<thead>
<tr>
<th>Vitamin B factor</th>
<th>Sample I</th>
<th>Sample II</th>
<th>Sample III</th>
<th>Sample IV</th>
<th>Sample V</th>
<th>Over-all average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin</td>
<td>0.030</td>
<td>0.048</td>
<td>0.144</td>
<td>0.021</td>
<td>0.064</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.053)</td>
<td>(0.050)</td>
<td>(0.157)</td>
<td>(0.032)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline</td>
<td>1165</td>
<td>894</td>
<td>326</td>
<td>623</td>
<td>1658</td>
<td>947</td>
</tr>
<tr>
<td></td>
<td>546</td>
<td>478</td>
<td>740</td>
<td>740</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(404)</td>
<td>(614)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inositol</td>
<td>330</td>
<td>218</td>
<td>326</td>
<td>395</td>
<td>410</td>
<td>336</td>
</tr>
<tr>
<td>( p )-Aminobenzoic acid</td>
<td>1.54</td>
<td>1.54</td>
<td>1.46</td>
<td>1.40</td>
<td>1.49</td>
<td></td>
</tr>
<tr>
<td>( B_6 ) complex</td>
<td>2.10</td>
<td>2.36</td>
<td>2.01</td>
<td></td>
<td>2.16</td>
<td></td>
</tr>
</tbody>
</table>
The blood inositol level varied less than did the biotin and choline levels. The average value for inositol was 336 γ per gm. of dry weight, or 66 γ per cc. $p$-Aminobenzoic acid and vitamin $B_6$ showed almost no variations among the different groups of mice. $p$-Aminobenzoic acid averaged 1.49 γ per gm. of dry weight, or 0.29 γ per cc. Pennington found 0.03 γ per cc. of $p$-aminobenzoic acid in human blood (8). The vitamin $B_6$ assays gave an average value for mouse blood of 2.16 γ per gm. of dry weight, or 0.42 γ per cc.

The values obtained for the five vitamin B factors in the transplantable mouse tumor are summarized in Table II. As in the studies on epidermis (1), dry weight of tissue was used as the basis of reference. It was found

<table>
<thead>
<tr>
<th>Vitamin B factor</th>
<th>Sample I</th>
<th>Sample II</th>
<th>Sample III</th>
<th>Sample IV</th>
<th>Sample V*</th>
<th>Over-all average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin</td>
<td>0.182</td>
<td>0.140</td>
<td>0.158</td>
<td>0.133</td>
<td>0.154</td>
<td>0.149</td>
</tr>
<tr>
<td>Choline</td>
<td>5550</td>
<td>6450</td>
<td>6100</td>
<td>6602</td>
<td>6108</td>
<td>6240</td>
</tr>
<tr>
<td>(6038)</td>
<td></td>
<td></td>
<td>(6000)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inositol</td>
<td>894</td>
<td>1164</td>
<td>1255</td>
<td>1043</td>
<td>1427</td>
<td>1154</td>
</tr>
<tr>
<td>(938)</td>
<td>952</td>
<td>995</td>
<td>1310</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p$-Aminobenzoic acid</td>
<td>3.64</td>
<td>2.95</td>
<td>3.80</td>
<td>2.28</td>
<td>2.78</td>
<td>3.09</td>
</tr>
<tr>
<td>$B_6$ complex</td>
<td>3.10</td>
<td>4.64</td>
<td>2.99</td>
<td>5.54</td>
<td>4.47</td>
<td>4.10</td>
</tr>
<tr>
<td></td>
<td>3.85</td>
<td>4.29</td>
<td>4.10</td>
<td>3.56</td>
<td>(3.55)</td>
<td>(4.55)</td>
</tr>
<tr>
<td></td>
<td>(3.48)</td>
<td>(4.40)</td>
<td>(3.55)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Tumors harvested at 21 days.

unnecessary to correct the tumor values for blood for the following reasons: Suntzeff and Carruthers (4), by determining the iron content of mouse blood and of transplanted carcinomas, showed that, even if all of the iron in the tumors were present as hemoglobin, the blood content of the tumors could not exceed 5 per cent. It is probable that much of the iron in the tumors is not hemoglobin iron, since the completely avascular hyperplastic epidermis which gives rise to tumors contains about the same amount of iron (9) as the tumors. Our results show approximately 50 per cent as much $p$-aminobenzoic acid and vitamin $B_6$ in blood as in the tumors, while the other vitamins were found in the blood at lower relative levels. When the amounts of the vitamins in the tumors were corrected for blood vitamin
assumingly 5 per cent blood in the tumors, it was found that the resultant changes in the tumor values were negligible. For each of the vitamin B factors, the change in the tumor value was less than the spread between replicate assays of the same tumor sample.

For the tumor samples, the levels of the individual vitamin B factors varied less from one sample to another than was the case with the blood samples. This may have been due to the fact that, whereas four mice were represented in each blood sample, twenty-two to forty-one mice were represented in each tumor sample, except tumor Sample V (Table II). This latter sample consisted of only seven tumors, which were collected 21 days after implantation, and were therefore considerably larger, as well as somewhat more necrotic, than the 12 day carcinomas comprising the other samples. Assays for choline, inositol, and vitamin B₆ were repeated on some of the tumor hydrolysates because of a spread in the values for these vitamins (particularly B₆) among the different groups of tumors. The results of the different assay runs agreed fairly well (Table II).

The average biotin content of the transplantable mouse carcinoma was 0.149 μg per gm. of dry weight, and the p-aminobenzoic acid values averaged 3.09 μg per gm. of dry weight. No figures were found in the literature which can validly be compared with these values. The average of 6240 μg of choline per gm. of dry weight found for the mouse carcinoma is about the same as that reported by Haven and Levy (10) for transplanted rat carcinosarcoma 256. The average value found for inositol in the mouse tumor, 1154 μg per gm. of dry weight, is higher than the values reported by Pollack, Taylor, and Williams (11) for cancers of the skin and breast in mice, and the pyridoxine levels reported by these authors were somewhat lower than our findings for the whole vitamin B₆ complex. The average vitamin B₆ content of our transplantable mouse carcinoma was 4.10 μg per gm. of dry weight.

Table III compares the levels of the vitamin B factors in the transplanted carcinomas with their levels in normal and treated epidermis. By using the technique of Baumberger, Suntzeff, and Cowdry for heat separation of the two layers of the skin, epidermis uncontaminated by dermis can easily be obtained (12) and serves as an excellent control tissue for use in chemical studies of epidermal carcinogenesis (9). Similarly, epidermis rendered hyperplastic by the topical application of 0.6 per cent methylcholanthrene in benzene is obtained entirely free from dermis. Therefore, values of vitamin B factors in the transplanted tumors, which were descended from an epidermal carcinoma produced by application of methylcholanthrene and which had continued to be squamous cell carcinomas, can validly be compared with the values found for normal epidermis, for
Of the five vitamins studied, all except biotin increased in the tumors as compared both with the normal and the treated epidermis. Choline showed the greatest change, the tumor value being 2.5 times greater than in normal epidermis. This compound increased only slightly in the methylcholanthrene-treated epidermis, the rise perhaps being due to the benzene rather than to the carcinogen, since the elevation was more marked in the benzene-treated epidermis. Jacobi and Baumann (2) found less choline in a mouse skin cancer than in several normal visceral organs, but did not study skin or isolated epidermis. The level of inositol, which had been very little affected by treatment of the epidermis over a 2 month period, rose in the tumors to 219 per cent of that in normal epidermis. p-Amino-

**Table III**

Summary of Content of Five Vitamin B Factors in Mouse Tissues during Methylcholanthrene Carcinogenesis

The values given are expressed in micrograms per gm. of dried tissue, and as per cent of the values for normal epidermis.

<table>
<thead>
<tr>
<th>B factor</th>
<th>Normal epidermis</th>
<th>Benzene-treated epidermis</th>
<th>Methylcholanthrene-treated epidermis</th>
<th>Transplanted carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \gamma ) per gm.</td>
<td>( \gamma ) per gm.</td>
<td>per cent</td>
<td>( \gamma ) per gm.</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.196</td>
<td>0.194</td>
<td>99</td>
<td>0.125</td>
</tr>
<tr>
<td>Choline</td>
<td>2471</td>
<td>2802</td>
<td>113</td>
<td>2651</td>
</tr>
<tr>
<td>Inositol</td>
<td>526</td>
<td>568</td>
<td>108</td>
<td>547</td>
</tr>
<tr>
<td>( p )-Aminobenzoic acid</td>
<td>2.40</td>
<td>2.30</td>
<td>96</td>
<td>2.40</td>
</tr>
<tr>
<td>( B_6 ) complex</td>
<td>2.45</td>
<td>2.88</td>
<td>118</td>
<td>3.05</td>
</tr>
</tbody>
</table>

benzoic acid behaved somewhat similarly in that the level of this factor showed no change with methylcholanthrene or with benzene alone, but was somewhat increased in the carcinomas, reaching 129 per cent of normal. The value for vitamin \( B_6 \) increased from 118 per cent of normal in the benzene-treated epidermis, to 124 per cent in the methylcholanthrene-treated epidermis, and to 167 per cent in the tumors.

Of the five vitamin B factors, biotin alone decreased in the methylcholanthrene-painted epidermis. The biotin value was also the only one which in the tumors fell below that for normal epidermis. However, its level in the carcinomas, 76 per cent of normal, was slightly higher than in the hyperplastic epidermis, where it was 64 per cent of normal. West and Woglom (13) and Kidd, Winzler, and Burk (14) determined biotin in rabbit skin tumors, but both these groups of investigators emphasized the difficulties of obtaining good control tissue for analysis. Both groups
found biotin higher in the tumors than in the skin, but did not assay isolated epidermis.

**DISCUSSION**

It is perhaps significant that the two factors which showed the greatest rise in concentration in the carcinoma tissue compared with their concentrations in normal epidermis are known to be concerned in lipide metabolism and to occur as constituents of animal lipides. Choline rose to a level of 252 per cent of that in normal epidermis, and inositol increased to 219 per cent. The increase in choline, as a constituent of lecithin, may well be related to the increased phospholipide content of mouse epidermal tumors, both induced and transplanted, found in other experiments by one of us.3 The increase in inositol may also be correlated with phospholipide if inositol is present in tumors in a combined form such as the inositol-containing phosphatides found by Folch (15) in brain "cephalin." These considerations might suggest the possibility of modifying tumor growth by controlling the availability of choline and inositol. The available experimental results bearing on this question are difficult to interpret. Jacobi and Baumann (2) failed to detect any effect of the dietary choline level on the survival time of mice bearing epithelial tumors produced by methylcholanthrene. Laszlo and Leuchtenberger (16) claimed that inositol given intravenously markedly decreased the size of tumors in mice in a 48 hour period, but found that oral or subcutaneous administration of inositol had no effect.

The changes in the levels of the other three vitamins studied may be interpreted as accompanying alterations in nitrogen metabolism in the tumor, since each vitamin has been implicated more or less directly with some phase of metabolism of nitrogen compounds. The rôle of vitamin $B_9$ in amino acid metabolism and synthesis has been well established (17–19). The suggestion from the work of Winzler, Burk, and du Vigneaud (20) that biotin is concerned with nitrogen assimilation has been strengthened by the finding of Stokes, Larsen, and Gunness (21) that this vitamin plays a rôle in aspartic acid synthesis in lactic acid bacteria. In addition to the presence of $p$-aminobenzoic acid in the folic acid molecule (22), this substance has been linked with the synthesis of methionine and certain purines in bacteria (23–25).

One is perhaps justified in viewing the change from normal epidermis to carcinoma as a temporal sequence of changes in the biochemistry and metabolism of the cells involved. From this aspect, a change which is evident in early carcinogenesis with methylcholanthrene may be more directly related to the process of carcinogenesis than a change detectable

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3 Wicks, L. F., unpublished findings.
only at a later stage in the developed tumor. The data summarized in this paper indicate, in line with this interpretation, that biotin is of primary significance in methylcholanthrene carcinogenesis. This may also be true for vitamin B₆, since the validity of the increase previously found for hyperplastic epidermis (1) is strengthened by the still higher value found for the tumor. Perhaps the other three vitamin B factors, choline, inositol, and p-aminobenzoic acid, are less directly concerned with carcinogenesis than with growth and metabolism of the tumor itself, since none of these factors changed significantly in epidermis during methylcholanthrene-induced hyperplasia.

Information is now available on the changes in lipides (26), in ascorbic acid and some of the metals (27), and in five members of the vitamin B complex during epidermal carcinogenesis. However, more information on the biochemistry of carcinogenesis, as well as on the metabolic functions of all of these tissue constituents, will be required for a critical evaluation of the significance of the changes so far observed.

SUMMARY

The levels of biotin, choline, inositol, p-aminobenzoic acid, and vitamin B₆ have been determined in a transplanted epidermal carcinoma in mice and in mouse blood. Neurospora mutant strains were used for the bioassays.

The levels of these vitamin B factors in mouse blood are lower than in the epidermal carcinoma.

Choline and inositol, which had shown little change in methylcholanthrene-treated epidermis, increased most in the tumors, choline reaching 252 per cent of the value for normal epidermis and inositol 219 per cent of the normal.

The concentration of biotin in the tumors was somewhat below the level for normal epidermis, but was not as low as in the hyperplastic epidermis.

The vitamin B₆ complex, which had risen to 124 per cent in the hyperplastic epidermis, was elevated to 167 per cent in the carcinoma.

p-Aminobenzoic acid, which had remained at the normal level in the treated epidermis, increased to 129 per cent in the tumors.

The possible significance of these results is discussed in relation to methylcholanthrene-induced epidermal carcinogenesis:

BIBLIOGRAPHY

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