Biochemical Studies of the Ehrlich Ascites Carcinoma-Bunyamwera Virus System

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The Ehrlich ascites carcinoma of mice, first described by Loewenthal and Jahn (26), has in recent years been the subject of a great many investigations. Klein et al. have studied certain of the biological properties and the nucleic acid content of this tumor (15, 17), and Christensen and co-workers (7, 8) have inquired into the capacity of the tumor to concentrate amino acids. Moldave (33) has used the cells for studies of protein turnover. Sugiura (51) has examined the effects of numerous compounds on the growth of the tumor, while LePage and others have made extensive studies of purine metabolism (9, 11, 24) and have suggested that the incorporation of glycine-2-C14 into the purines of the tumor cell be used as a convenient test system for the examination of the potential anti-cancer agents (25). Kun et al. (23), Williams-Ashman (54), McKee and his associates (30), and others (29, 40, 49) have investigated many of the metabolic properties of the Ehrlich ascites tumor.

The propagation of Mengo and Bunyamwera encephalitis viruses in cells of the Ehrlich ascites carcinoma has been reported by Koprowska and Koprowski (18, 19), and the cytological changes associated with the growth of Bunyamwera virus in these cells have been described by Love, Koprowski, and Cox (28). Flanagan and Colter (12) were successful in growing Mengo virus in Ehrlich cells in vitro and showed that the virus did not affect the respiration or glycolytic activity of the cells. The advantages of ascites tumor-virus systems for the study, not only of virus reproduction, but of the mechanism of selective cell destruction as well, have been pointed out by Koprowski and his associates (20, 21).

The present paper describes certain metabolic and biochemical properties of Ehrlich ascites carcinoma cells and the effects thereof on infection with Bunyamwera virus.

MATERIALS AND METHODS

Virus.—Bunyamwera virus (48) was obtained through the courtesy of Dr. K. Smithburn of the Division of Medicine and Public Health of the Rockefeller Foundation. The virus was stored at −25°C. in the form of a 20 per cent suspension in saline of Bunyamwera-infected mouse brains.

Tumor.—The Ehrlich ascites tumor was grown in 18–22-gm. Swiss albino mice. The tumor inoculum consisted of 0.2 ml. of pooled ascitic fluid collected on the 7th day of tumor growth and contained 10–12 million cells. The tumor was infected on the 7th day after implantation by the intraperitoneal injection of 0.5 ml. of a 1:50 dilution of Bunyamwera-infected mouse brain.

At intervals following virus infection, groups of mice were sacrificed (cervical dislocation), and ascitic fluids were aspirated from the peritoneal cavities. In the enzyme studies to be reported, the fluids were collected from a minimum of three mice in each of the uninfected and virus-infected groups. In the experiments with cell proteins, at least six mice in each group were sacrificed. Tumor cells were freed from contaminating erythrocytes by being washed in saline (30). Two washings were sufficient to remove all but traces of erythrocytes. Washed cells were suspended in physiological saline or in bicarbonate- or phosphate-buffered Krebs-Ringer solution, depending upon the system in which they were to be studied, and were enumerated in a standard bright-line Neubauer hemocytometer. Homogenates, when employed, were prepared in a motor-driven Potter-Elvehjem homogenizer.

Biochemical studies.—Succinoxidase activity was estimated by the method of Schneider and Potter (44). Malic dehydrogenase was measured by the technique described by Potter (38). Quastel and Wheatley’s method (39) was used for the estimation of choline oxidase activity, and that of Axelrod and Elvehjem (2) for the examination of xanthine oxidase.

Acid and alkaline phosphatase were studied as described by Porter and Melampy (37), with disodium phenylphosphate as substrate. The veronal-acetate buffer (pH 5.5) of Roth and Milstein (42) was used in the acid phosphatase studies, while the veronal buffer (pH 9.5) of Michaelis (32) was employed for alkaline phosphatase. Reaction mixtures were deproteinized with trichloroacetic acid, and phenol estimations were performed on the neutralized filtrates by the method of Folin and Ciocalteu (13).

Hexokinase activity was determined by Long’s method (97). Glucose was estimated in the incubation mixtures, after deproteinization with Ba(OH)2 and ZnSO4, according to Nelson’s modification of Somogyi’s technic (96).
The rate of aerobic and anaerobic glycolysis was followed by lactate estimations, performed as described by Barker and Summerson (8), on incubation mixtures deproteinized with trichloroacetic acid. Krebs-Ringer phosphate and bicarbonate solutions were employed in the study of aerobic and anaerobic glycolysis, respectively, and glucose was the substrate in both.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinoxidase</td>
<td>5.3 ± 0.7*</td>
</tr>
<tr>
<td>Malic dehydrogenase</td>
<td>3.0 ± 0.2*</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>124 ± 31†</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>29.0 ± 2.9†</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>10.0 ± 0.8†</td>
</tr>
<tr>
<td>Aerobic glycolysis</td>
<td>51.8 ± 2.5‡</td>
</tr>
<tr>
<td>Anaerobic glycolysis</td>
<td>41.8 ± 4.5‡</td>
</tr>
<tr>
<td>Arginase</td>
<td>18.0 ± 3.3‡</td>
</tr>
</tbody>
</table>

* Cu. mm. O₂ taken up/cell/hr (×10⁻⁶).
† μg. Glucose phosphorylated/cell/hr (×10⁻⁶).
‡ μg. Phenol liberated from disodium phenyl phosphate/cell/hr (×10⁻⁴).
§ μg. Phenolphthalein liberated from phenolphthalein glucuronide/cell/hr (×10⁻⁷).
‖ μg. Lactate produced/cell/hr.
∥ μg. Urea nitrogen produced/cell/hr.

Deoxyribonuclease was assayed by the colorimetric method of Allfrey and Mirsky (1). Highly polymerized deoxyribonucleic acid, isolated from calf thymus as described by Simmons, Chavos, and Orbach (46), was employed as substrate.

Arginase activity was measured by estimating the urea liberated from arginine upon incubation of cell homogenates with arginine at pH 9.5. The experimental conditions employed were those of Van Slyke and Archibald (53). Urea was assayed by the colorimetric method of Engel and Engel (10).

The residue remaining after fractionation of the cells was considered to represent the total cell protein. Aliquots of the residues were hydrolyzed by refluxing for 16 hours in 6 N HCl, and the hydrolysates were subjected to complete amino acid analyses by the technic of Stein and Moore (50).

RESULTS

Certain enzymes—choline oxidase, xanthine oxidase, alkaline phosphatase, and deoxyribonuclease—were found to be absent from Ehrlich ascites tumor cells. Studies of the first three were confined to uninfected cells. In the case of deoxyribonuclease, however, both virus-infected and uninfected cells were examined.

In Table 1 are listed the enzymes and enzyme systems which were found to be present in the tumor cells. Activities are listed for uninfected cells and are expressed on a per cell basis. This method of presenting data was chosen in preference to dry weight, unit of protein nitrogen or nucleic acid phosphorus, or volume of packed cells, since these properties could be altered by virus infection and so obscure any change in the amount or activity of an enzyme in each cell.

With one exception—arginase—the enzymes listed in Table 1 were unaffected by infection of the cells with Bunyamwera virus. The activity of each enzyme was measured at all stages of the infectious process, i.e., at intervals ranging from 6 to 72 hours postinfection. Under the experimental conditions employed, cells could not usually be collected later than 72 hours after administration of the virus. Very shortly thereafter, cellular degeneration became apparent and proceeded rapidly until no tumor cells remained in the ascitic fluid.

In no case did the activity of any of the enzymes—save that of arginase—deviate at any stage of the infectious process from the values observed for uninfected cells.

The investigation of the enzyme arginase arose as a natural consequence of the studies of the amino acid composition of the cell proteins. The response of arginase activity to Bunyamwera virus infection is illustrated in Chart 1. Each point on the curve represents values obtained from at least six series of experiments. In each series, the arginase activity of the infected cells was estimat
or with those at 72 hours, the ranges did not overlap.

In general, when the values for uninfected cells were compared with those at 24 and 48 hours postinfection the cells and three samples isolated at each of the post-infection times noted were subjected to complete analysis. The curve in Chart 1 was constructed from the means of the values so obtained for each time interval.

Although the extent to which arginase activity was stimulated or depressed varied from one series to another, several observations were consistent. Arginase activity in the infected cells was always stimulated during the first 30 hours of infection and was invariably depressed 72 hours after infection. Also, in any single series, maximum stimulation of activity was always seen about 24 hours following infection with the virus.

### TABLE 2

<table>
<thead>
<tr>
<th>AMINO ACID</th>
<th>Mean % Normal</th>
<th>Range</th>
<th>24</th>
<th>Mean</th>
<th>Range</th>
<th>48</th>
<th>Mean</th>
<th>Range</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine+isoleucine</td>
<td>11.5</td>
<td>10.5-12.3</td>
<td>11.7</td>
<td>10.4-13.4</td>
<td>12.3</td>
<td>11.6-12.8</td>
<td>11.0</td>
<td>10.4-11.3</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.8</td>
<td>2.4-3.2</td>
<td>5.1</td>
<td>3.0-5.2</td>
<td>3.3</td>
<td>4.1-5.5</td>
<td>2.2</td>
<td>2.0-3.4</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>5.8</td>
<td>5.7-6.0</td>
<td>6.0</td>
<td>4.8-7.4</td>
<td>6.1</td>
<td>5.7-6.6</td>
<td>4.7</td>
<td>4.2-5.3</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6.0</td>
<td>5.6-6.2</td>
<td>5.8</td>
<td>4.5-5.8</td>
<td>4.0</td>
<td>3.7-4.5</td>
<td>3.9</td>
<td>3.4-5.0</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>4.2</td>
<td>3.9-4.6</td>
<td>4.3</td>
<td>3.7-4.8</td>
<td>5.0</td>
<td>4.4-6.0</td>
<td>3.7</td>
<td>3.5-4.1</td>
<td></td>
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<tr>
<td>Glutamic+alanine</td>
<td>17.7</td>
<td>17.4-18.2</td>
<td>18.6</td>
<td>18.9-19.5</td>
<td>17.9</td>
<td>17.7-18.0</td>
<td>17.3</td>
<td>15.1-20.0</td>
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<tr>
<td>Threonine</td>
<td>5.9</td>
<td>5.6-4.3</td>
<td>4.7</td>
<td>4.5-5.2</td>
<td>4.2</td>
<td>4.1-4.4</td>
<td>5.1</td>
<td>1.7-4.2</td>
<td></td>
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<tr>
<td>Aspartic acid</td>
<td>6.3</td>
<td>4.5-7.8</td>
<td>7.5</td>
<td>6.9-8.1</td>
<td>6.9</td>
<td>6.7-7.1</td>
<td>5.7</td>
<td>4.9-6.9</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>0.7</td>
<td>0.6-0.7</td>
<td>1.3</td>
<td>1.0-1.4</td>
<td>1.0</td>
<td>0.8-1.2</td>
<td>1.1</td>
<td>0.8-1.7</td>
<td></td>
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<tr>
<td>Glycine</td>
<td>4.7</td>
<td>4.0-5.2</td>
<td>5.4</td>
<td>4.1-6.5</td>
<td>5.2</td>
<td>4.9-6.8</td>
<td>4.3</td>
<td>2.1-5.6</td>
<td></td>
</tr>
<tr>
<td>Ammonia</td>
<td>6.7</td>
<td>6.0-7.0</td>
<td>7.7</td>
<td>5.9-8.7</td>
<td>6.8</td>
<td>6.6-9.0</td>
<td>7.0</td>
<td>6.7-7.6</td>
<td></td>
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<tr>
<td>Arginine</td>
<td>18.0</td>
<td>15.2-18.9</td>
<td>9.5</td>
<td>8.1-10.8</td>
<td>10.9</td>
<td>10.0-11.6</td>
<td>20.8</td>
<td>18.5-23.6</td>
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<tr>
<td>Lysine</td>
<td>6.0</td>
<td>5.8-6.5</td>
<td>5.5</td>
<td>4.4-6.2</td>
<td>6.5</td>
<td>6.0-6.7</td>
<td>5.3</td>
<td>4.9-5.6</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>7.5</td>
<td>6.8-8.3</td>
<td>8.5</td>
<td>8.1-8.8</td>
<td>7.9</td>
<td>7.5-8.6</td>
<td>7.8</td>
<td>7.3-8.7</td>
<td></td>
</tr>
<tr>
<td>Cystine</td>
<td>5.4</td>
<td>2.2-2.6</td>
<td>7.9</td>
<td>2.3-3.7</td>
<td>2.5</td>
<td>1.6-3.8</td>
<td>2.2</td>
<td>2.1-2.6</td>
<td></td>
</tr>
</tbody>
</table>

The results of amino acid analyses of "total" protein separated from uninfected Ehrlich cells and from Ehrlich cells at various times after infection with Bunyamwera virus are summarized in Table 2. The concentration of each amino acid is expressed as the percentage of the total amino nitrogen resolved which arose from that amino acid. Three samples of protein from uninfected cells and three samples isolated at each of the post-infection times noted were subjected to complete analysis.

Among the amino acids, the level of arginine alone was significantly affected by the virus infection, being markedly depressed at 24 and 48 hours and markedly elevated 72 hours after infection of the cells. Particularly gratifying is the fact that, when the values for uninfected cells were compared with those at 24 and 48 hours postinfection or with those at 72 hours, the ranges did not overlap.

The other amino acids appeared to be unaffected by the virus infection. In general, when the arginine content of the protein was depressed (24 and 48 hours after infection) the contribution of each of the other amino acids to the total amino nitrogen resolved was slightly elevated. When the arginine content was elevated, the contribution of each of the other amino acids to the total amino nitrogen was slightly decreased.

### DISCUSSION

The mechanism of viral reproduction is a problem which has attracted the attention of many investigators. Much of the work done in this area has, like that reported here, been directed toward the search for specific biochemical and metabolic lesions which could be related to the process of viral multiplication. Bauer (4-6) described increased xanthine oxidase activity of mouse brain following infection with neurotropic viruses, and Sellers and Jann (45) found an increase in the activity of this enzyme in influenza virus-infected mouse lung. Smith and Kun (22, 47) reported that a number of viruses produced an increased rate of anaerobic glycolysis in chick embryo chorionallantoic membrane without altering the respiration of the tissue, while Franklin and co-workers (14) showed that poliomyelitis virus-infected cultures of human embryo brain and cord utilized less glucose than did uninfected control cultures. McLimans et al. (31) reported that the O2 consumption of Newcastle disease virus-infected embryonated eggs remained normal until the terminal stage of the infection. Moldave and others (34, 35) studied the in vitro uptake of C14 from labeled glucose into the amino acids of mouse brain. They found that infection of the tissue with Theiler's GD VII virus stimulated the incorporation of label into most of the amino acids but inhibited incorpora-
tion into lysine and histidine. Rafelson et al. (41) had similar results using the same system. Johnson, Kempf, and Bergeim (16) described the decrease of free lysine and histidine in chick chorioallantoic membranes as a result of infection with influenza virus.

It is difficult to assess the importance of these many observations in terms of the process of viral multiplication. Similarly, it would be fruitless to speculate on the significance of the changes in arginase activity and in the level of protein-bound arginine in Ehrlich ascites cells insofar as they relate to the reproduction of Bunyamwera virus. However, these changes were evident during a period when the activities of a sizeable number of enzyme systems were unaffected. Thus, it is tempting to suggest that, whatever their significance, the changes represent a specific response of the cell to infection with the virus. The apparent relationship between arginine content of the cell proteins and the arginase activity of the cells is striking. The fact that the protein-bound arginine is at its lowest level when arginase is maximally stimulated, and is at its highest level when arginase activity is lowest, suggests that the incorporation of this amino acid into cell proteins is directly regulated by the arginase activity of the cell.

SUMMARY

1. Ehrlich ascites carcinoma cells were shown to be deficient in the enzymes choline oxidase, xanthine oxidase, alkaline phosphatase, and deoxyribonuclease.
2. Infection of Ehrlich ascites cells with Bunyamwera virus was without effect at any stage of the infectious process on the succinoxidase, malic dehydrogenase, hexokinase, acid phosphatase, xanthine oxidase, alkaline phosphatase, and deoxyribonuclease activities of the cells.
3. The arginase activity of Ehrlich cells was stimulated during the first 30 hours of infection with Bunyamwera virus. Thereafter, the activity diminished until, terminally, it was significantly lower than normal.
4. At intervals of 24 and 48 hours after infection of Ehrlich cells with Bunyamwera virus, the arginase content of the "total" cell protein was lower than that of uninfected cells but was higher than that of uninfected cells 72 hours post-infection.

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