The effect of neoplastic disease on the composition of human plasma has been studied repeatedly (11, 12—14, 18), and has been reviewed by Winzler (924). The observation of a deviation from the normal in human hosts is rendered difficult by the heterogeneity of the plasma protein patterns in normal individuals (3). The study of the effect of tumor growth on the protein composition in plasma can, therefore, best be carried out in animals of pure and inbred strains, such as in mice, where a rather high degree of uniformity in the plasma protein patterns of individual animals has been observed1 (20, 21). The plasma protein analysis in small samples obtained from individual mice became possible when reliable methods, such as micro-electrophoresis, were developed (1, 6). Even with mice of pure and inbred strains, slight variations in the electrophoretic plasma protein patterns can be observed between several animals. The influence of tumor growth on the plasma proteins can, therefore, be significantly evaluated only after experimental data from a large number of animals have been examined statistically.

EXPERIMENTAL

Animals.—All the experiments reported herein were carried out with mice of the strain C57BL/6, ranging from 4 to 12 weeks in age. Animals of each sex were used in about the same proportion. Blood was obtained after ether narcosis by sectioning the base of the heart and aspirating the blood from the thoracic cavity into a pipette, which had been wetted with a concentrated solution of sodium citrate to prevent clotting. Amounts of blood ranging between 1.0 and 1.5 ml/animal were thus obtained; the blood was centrifuged immediately.

Tumors.—Sarcoma 180, which had been grown in C57BL/6 mice for at least five tumor generations, was used. Tumors which had been allowed to grow for 10—12 days in the host animal were minced in the cytosieve (19), and the tumor cell suspension was diluted with saline to a standard concentration, as measured by the sedimentation in blood volume index tubes. At this concentration the volume of the sedimented cells after 30 minutes' spinning at 1,000 × g was 1 per cent of that of the cell suspension. Injection of 0.3 ml. of this suspension into each side of the animal yielded tumor growth in all cases of implantation. The nodules after 10—12 days were about 5—8 mm. in diameter. Unless otherwise stated, blood from tumor-bearing mice was drawn between the 10th and 12th days after implantation.

Plasma protein analysis.—Plasma protein analyses were carried out by micro-electrophoresis with the apparatus designed by Antweiler (1, 6), which operates on the principle of boundary electrophoresis according to Tiselius. Five-tenths ml. of plasma was taken from each mouse individually, and these samples were analyzed separately as follows. The plasma, containing from 10 to 20 mg. of protein, was dialyzed overnight in Visking dialysis tubing of ½-inch diameter against 50 ml. of veronal citrate buffer of pH 8.6 and ionic strength 0.1 (containing 0.06 M diethyl barbiturate and 0.008 M sodium citrate). The lower part of a fused electrophoresis cell was filled with dialyzed and undiluted plasma (about 0.4 ml. was necessary). The cell was cooled by circulating water at 9 ± 1°C., and the protein-buffer interface was shifted a short distance into the ascending channel of the cell. This was achieved by carefully adding a few hundredths of a milliliter of buffer solution to the appropriate channel of the cell, by means of a 1-ml. syringe (26-gauge needle). The beginning gradient, obtained in this way, and the protein patterns of the plasma were adjusted to a standard gradient, which was obtained by running the cell overnight at 9 ± 1°C., with a buffer reservoir at 9 ± 1°C.

Received for publication January 17, 1955.

*See preliminary report (4).
1 P. Bernfeld, unpublished results.

--The Influence of Tumor Growth on the Plasma Proteins in Mice--

PETER BERNFELD AND F. HOMBURGER

(Cancer Research and Cancer Control Unit, Departments of Biochemistry and Surgery, Tufts University School of Medicine, Boston, Mass.)
patterns obtained after 6 minutes' electrophoretic migration at 90 v difference of potential between the electrodes (corresponding to an electric field of about 9 v/cm) were photographed immediately by the use of a Schlieren cylindrical lens optical system. Each electrophoretic experiment was repeated twice, using the same plasma sample. This was achieved by refilling with new buffer solution that part of the cell (upper section) into which the protein had migrated under the influence of the electric field.

The mobilities were calculated from the distance between each protein peak and the beginning gradient; the distance of the albumin peak from the beginning gradient was used as a reference value, and the mobility of albumin was set equal to 5.9 X 10−4 cm² volt−1 sec−1 in all experiments. This value was accepted after the analysis of several samples of pooled mouse plasma (C57BL/6) in the Perkin Elmer electrophoresis apparatus showed no distinguishable difference between the mobilities of human and mouse plasma albumin.

The relative amounts of the protein components were determined from enlarged drawings of the photographic records by the method of Tiselius and Kabat (22). Total plasma protein was determined on 0.1-ml samples, drawn before dialysis, by the biuret method of Robinson and Hogden (16), with crystalline ovalbumin (Worthington Biochemical Laboratory) as the reference protein.

RESULTS

Electrophoretic mobilities.—To identify the components in mouse plasma which are evident from electrophoretic analysis, the mobilities pertaining to each boundary have been calculated and compared with the values obtained for human plasma proteins, as described in the literature (8) (see Table 1).

Although the mobilities of the globulin components in mouse plasma are different from those of the corresponding proteins of human plasma, we shall call them α-, β-, and γ-globulins, respectively, for the purpose of identification. No separate peak corresponding to fibrinogen could be detected in mouse plasma.

Plasma protein concentrations.—Figure 1 shows typical electrophoretic diagrams, chosen at random among 85 normal mice and 126 tumor-bearing mice. It is evident from these patterns that there was a considerable difference in the plasma protein concentration between normal and tumor-bearing mice.

The full data calculated from all these experiments are presented in Chart 1, and the average values with standard deviations are given in the last two columns of Table 2. The amounts of protein are indicated as per cent of total protein. The statistical evaluation of the differences between normal and tumor-bearing mice yields the following z values: 10.3 for albumin, 8.7 for α-globulin, 3.5 for β-globulin, and 1.1 for γ-globulin. It is evident from these data that the relative amount of albumin decreased during tumor growth, that the relative amount of α-globulin increased considerably during this period, whereas the relative amount of β-globulin increased slightly and that of γ-globulin remained unchanged. The changes appear to be statistically significant.

The bars at 0 per cent protein in the chart of

<table>
<thead>
<tr>
<th>Proteins in C57BL/6 Mice and in Humans</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mice</strong></td>
</tr>
<tr>
<td>U</td>
</tr>
<tr>
<td><strong>Albin</strong></td>
</tr>
<tr>
<td>α-globulin</td>
</tr>
<tr>
<td>β-globulin</td>
</tr>
<tr>
<td>γ-globulin</td>
</tr>
</tbody>
</table>

* Average values from 178 experiments.
† According to Edsall (8).
‡ U = Mobilities X 10⁴ cm² volt−1 sec−1, in veronal buffer, pH 8.6, μ = 0.1.
§ S = Standard deviation.

α-globulin and γ-globulin mean that, in a certain number of animals, these protein components could not be detected. The absence of α-globulin in some mice is probably explained by an artifact, i.e., an incomplete electrophoretic separation. It is interesting to note, however, that this phenomenon, whenever it occurred, did appear consistently in the duplicate and triplicate experiments performed with the same plasma sample. The apparent absence of γ-globulin may be due to a partial overlapping of its electrophoretic boundary with the well known δ-salt anomaly, thus yielding inaccurate results for γ-globulin.

Total plasma protein.—Table 3 shows that the total plasma protein decreased markedly during the growth of Sarcoma 180. When the plasma protein components are expressed on a gm/100 ml basis, referred to in the following as absolute protein amounts, there was a significant increase of about 50 per cent in the α-globulin during tumor growth, a significant decrease of 40 per cent in albumin, whereas the changes in β-globulin and γ-globulin were not significant.
CHART 1.—Plasma protein components in C57BL/6 mice (per cent of total plasma protein). Black bars: 85 normal mice; cross-hatched bars: 126 mice bearing Sarcoma 180.

TABLE 2

AVERAGE VALUES OF PLASMA PROTEINS IN C57BL/6 MICE
AS PER CENT OF TOTAL PLASMA PROTEIN*

<table>
<thead>
<tr>
<th></th>
<th>Liver tissue</th>
<th>Soluble tumor cell constituents</th>
<th>Embryonic tissue</th>
<th>Sarcoma 180</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>53.9 (4.9)</td>
<td>61.1 (7.7)</td>
<td>60.6 (9.3)</td>
<td>47.3 (7.0)</td>
<td>59.3 (9.0)</td>
</tr>
<tr>
<td>α-Globulin</td>
<td>10.4 (5.4)</td>
<td>8.9 (6.7)</td>
<td>8.9 (6.5)</td>
<td>17.6 (6.8)</td>
<td>9.5 (6.9)</td>
</tr>
<tr>
<td>β-Globulin</td>
<td>28.1 (5.3)</td>
<td>23.4 (5.2)</td>
<td>24.2 (4.5)</td>
<td>29.1 (5.0)</td>
<td>25.2 (5.2)</td>
</tr>
<tr>
<td>γ-Globulin</td>
<td>10.2 (5.5)</td>
<td>6.5 (4.0)</td>
<td>6.2 (5.4)</td>
<td>7.9 (5.8)</td>
<td>7.1 (5.6)</td>
</tr>
<tr>
<td>No. animals</td>
<td>20</td>
<td>19</td>
<td>20</td>
<td>126</td>
<td>85</td>
</tr>
</tbody>
</table>

* Standard deviations in parentheses.
Effect of time on protein changes.—Five groups of about twenty mice each were given implants of Sarcoma 180 in the usual way, and every 2–3 days the blood of two mice of each series was collected. The mortality rate in animals more than 20 days after implantation was high, and only in two cases was it possible to obtain blood from mice 24 days after implantation.

The changes in α-globulin as a function of time after implantation are shown in Chart 2. It is evident that the α-globulin started to increase as early as 4 days after transplantation, reached a maximum at 8–14 days, and decreased to almost normal levels at about 21 days. At this time, all tumors had grown to a very large size, and no spontaneous regression had been observed.

### TABLE 3

AVERAGE CONCENTRATIONS OF PLASMA PROTEIN COMPONENTS IN C57BL/6 MICE

<table>
<thead>
<tr>
<th>Component</th>
<th>Normal mice</th>
<th>Tumor-bearing mice</th>
<th>α Values of Statistical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein</td>
<td>3.4 ± (1.15)</td>
<td>2.65 ± (0.6)</td>
<td>4.0</td>
</tr>
<tr>
<td>Albumin</td>
<td>1.10 (0.54)</td>
<td>1.16 (0.37)</td>
<td>7.6</td>
</tr>
<tr>
<td>α-Globulin</td>
<td>0.51 (0.18)</td>
<td>0.47 (0.20)</td>
<td>4.9</td>
</tr>
<tr>
<td>β-Globulin</td>
<td>0.80 (0.35)</td>
<td>0.60 (0.32)</td>
<td>1.5</td>
</tr>
<tr>
<td>γ-Globulin</td>
<td>0.22 (0.16)</td>
<td>0.23 (0.16)</td>
<td>0.26</td>
</tr>
</tbody>
</table>

No. animals: 51

*Standard deviations in parentheses.

A corresponding analysis of the albumin (Chart 3) shows that the concentration of this protein remained constant until about the 10th day after transplantation, after which time it started to decrease slowly. No significant changes in the β-globulin concentration could be detected during the whole period of 24 days under investigation (Chart 3).

Influence of other factors on plasma protein concentration.—To establish whether the changes in plasma protein concentration described above were actually due to neoplastic growth and not to other factors, several series of control experiments were carried out.

1. Liver cell suspension, obtained from the livers of normal C57BL/6 mice, was injected into twenty mice. The concentration and technic were the same as in the tumor transplantations. Plasma protein analyses on these animals were made 12 days after the injection.

2. Sarcoma 180 cells were disintegrated by repeated slow-freezing at −15° C. and thawing of a cell suspension. The mixture was then freed from cell debris and surviving cells by spinning for 15 minutes at 40,000 r.p.m. in a Spinco Model L preparative ultracentrifuge (100,000 × g). The clear supernatant, containing all soluble cell con-
studies, had a protein content of 2.3 mg/ml. It was injected into a group of nineteen mice in the usual manner, and the plasma protein of these animals was analyzed after 12 days.2

3. Embryonic tissue, obtained from animals of the same strain, was passed through the cytosieve and implanted into a group of twenty mice. The resulting nodules after 12 days are comparable in size with those observed after 12 days' growth of Sarcoma 180 (Figs. 2–4).

The results obtained in these control experiments are summarized in Table 2. It is evident that, under our experimental conditions, only the growth of Sarcoma 180 was capable of changing the plasma protein composition. Neither injection of a liver cell suspension or of the soluble constituents of Sarcoma 180 cells nor the growth of embryonic tissue affected the plasma proteins.

Influence of food intake.—The changes in total body weight during the 12-day period following transplantation of tumor were determined in 36 animals. The average increase was 1.95 per cent, with individual changes of ±15 per cent. The weight increase of a 20-gm. mouse due to the tumor growth calculated from the average tumor size of 5 × 8 mm. would be 0.5–2.5 per cent of the total body weight, which is in satisfactory agreement with the experimental data.

A comparison of the food intake of 24 normal mice with that of 26 animals observed during the 12 days following implantation of Sarcoma 180 revealed no significant difference.

DISCUSSION

Various observations on the effect of tumor growth on the plasma proteins in animals have been reported in recent years. In rats, tumors induced by methylcholanganthrene caused an elevation in the β-globulin fraction and a decrease in γ-globulin (17). Roberts described an increase in β-globulin and a very slight increase in α-globulin in rats carrying transplantable lymphosarcoma (15). V5 carcinoma in rabbits has been found to produce an elevation of the β- and γ-globulins (7). In working with pooled plasma of white Swiss mice, Wharten et al. found no significant changes upon growth of Sarcoma 37 (28). By means of paper electrophoresis, a decrease of albumin and of β-globulin in spontaneous mammary gland carcinoma in C3H mice and a lowering of γ-globulin in spontaneous mammary gland carcinoma in C3H SP mice have been observed (9).

In contrast to the lack of uniformity among the above-mentioned results, our finding of a substantial increase in the α-globulin component of the plasma of mice during the period of 12 days following implantation of Sarcoma 180 appears to be significant and rather uniform. This may be owing to the fact that in the present work only plasma from individual animals was used, that a large number of animals was included in this study, and that all animals under investigation were of one and the same pure and inbred strain.

The fact that the α-globulin again decreased upon further growth of the tumor is difficult to interpret. The observation is somewhat analogous in its time relationship to tumor growth with the finding that the lymphoid hyperplasia caused by Sarcoma 180 in white Swiss mice is greater 6 days after tumor implantation than after 12 days of tumor growth (10).

A phenomenon similar to the increase and decrease of α-globulin described in this paper has been observed by Thompson (20) in mice following infection with Salmonella typhimurium. This author concludes that these fluctuations of plasma proteins during the Salmonella infection are possibly related to immune reactions. If this were also the case for the α-globulin changes produced by the growth of Sarcoma 180, this phenomenon would have to be specific for the growing tumor cell or for its insoluble constituents, because it has been demonstrated that the injection of a liver cell suspension or of the soluble constituents of the tumor and the growth of a nonmalignant nodule (embryonic tissue) produce no significant plasma protein alterations.

The Antweiler micro-electrophoresis apparatus only yields the protein patterns from the ascending boundaries and not those from the descending boundaries. Electrophoretic anomalies that appear mainly in the ascending boundaries have been described (5), and they have been attributed to the presence of small amounts of polyelectrolytes in the plasma, rather than to the appearance of new proteins therein (9). Electrophoretic analyses of pooled plasma from Sarcoma 180-bearing C57BL/6 mice in a 2.5-ml. cell of a Perkin Elmer instrument have shown that there is an increased α-globulin peak in both descending and ascending parts of the cell. It appears likely, therefore, that the increased α-globulin peak reflects an actual increase in the concentration of this protein.

It is evident from our data that the concentration of α-globulin increased as early as 4 days after implantation of the tumor. The average increase of α-globulin during this period, as calculated from the data of Chart 2 and Table 3, amount-
ed to 8 mg. for an animal of 20 gm. If the increase of a-globulin in the plasma were due to the passage of tumor proteins into the bloodstream, the size of the tumor nodules should be in a reasonable proportion to the amount of protein released by it. If one supposed for this calculation that the dry matter of the tumor nodules were solely a-globulin and that these nodules contained about 25 per cent dry material, it would follow that a mouse of 20 gm. should have two nodules weighing at least \( \frac{2}{3} \times 221.02 = 6 \) mg. each, being at least 2 mm. in diameter. It was observed, however, that the tumor nodules were still so small on the 4th day following transplantation that they were barely palpable; i.e., they were certainly less than 1 mm. in diameter.

It therefore appears unlikely that the increase in plasma a-globulin is due to the passage of tumor proteins into the bloodstream. The above considerations also indicate that the appearance of increased plasma a-globulin precedes the formation of the tumor nodules.

Furthermore, factors related to food intake do not appear to be responsible for the alterations in plasma proteins observed.

SUMMARY
Plasma protein changes during the growth of Sarcoma 180 in C57BL/6 mice have been studied by means of boundary electrophoresis. The data obtained with 126 tumor-bearing mice and 83 normal mice show that the tumor growth significantly increased the a-globulin component of the plasma, while there was a decrease in plasma albumin. Four days after the implantation of tumor, the a-globulin had already increased considerably, while the tumor nodules were still hardly palpable. There was a maximum in a-globulin concentration between the 8th and 14th day after implantation, after which time the a-globulin concentrations again decreased. Neither the injection of a liver cell suspension nor of the soluble constituents of embryonic tissue, produced significant plasma protein alterations. This suggests that these a-globulin changes are actually associated with the neoplastic growth.

ACKNOWLEDGMENTS
We should like to express our thanks to Miss Norma Inglis for technical assistance and to Mr. John Baker for preparing the histological sections.

REFERENCES
The Influence of Tumor Growth on the Plasma Proteins in Mice

Peter Bernfeld and F. Homburger


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/15/6/359

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.