On the Mechanism of Action of Carcinogenic Substances*

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In the study of cancer one of the central problems is the elucidation of the pathogenesis of malignant tumors. The direction taken by such studies varies in accordance with prevailing points of view on the etiology of cancer. Since the discovery of carcinogenic compounds by Cook, Kennaway, and others (1, 7), the theory of a chemical etiology of malignant tumors has been advanced. According to this theory the so-called “spontaneous” tumors, which include the majority of the tumors of man, and which are of the greatest interest for practical medicine, develop as the result of the action of endogenous carcinogenic substances engendered in the organism. Recently the hypothesis of endogenous carcinogenic substances has been strengthened by the results of the investigations of Schabad (44, 45), Hieger (17), des Ligneris (9, 10), Steiner (47), and Kleinenberg et al. (19).

A tendency exists also, as indicated by the work of Roffo (43), to invoke a chemical explanation for the carcinogenic action of certain physical factors, such as ultraviolet rays. There is reason to believe that certain endogenous carcinogenic substances may prove to be closely related to some of the known synthetic carcinogenic hydrocarbons.

If the theory of the chemical etiology of malignant tumors be accepted as a working hypothesis, one of the first questions to arise concerns the mechanism of action of carcinogenic substances. What is their mode of action? In what way do these substances affect normal cells to transform them into tumor cells?

Problems of the mechanism of the action of carcinogenic substances have been of particular interest to these laboratories in recent years. The purpose of this communication is to present and discuss the results of some of the studies in this field which I have conducted in association with collaborators.

Oxidation Processes in the Organism

If it is agreed that the cells in malignant tumors possess a special metabolism, the principal characteristic, according to Warburg, being a disturbance between respiration and glycolysis, the surmise arises that carcinogenic hydrocarbons may effectuate these changes in tissue respiration and carbohydrate metabolism at the point of their application. On the other hand, the work of Fischer-Wasels (15) and Neumann (34, 35) indicates that carcinogenic agents may produce a disturbance of respiratory and glycolytic processes in organs, as an essential factor of the so-called “general cancer predisposition.”

To obtain information on these possibilities we investigated the oxidation processes occurring in the animal organism. M. Tchertkova studied the ratios of carbon to nitrogen and of “vacate-oxygen” to nitrogen in the urine, which Müller (31-33) and Bickel (2, 3) regard as ratios characteristic of the state of oxidation processes occurring in the body. The investigations were carried out on 90 mice and 7 rabbits. During a period of 4 months, the skin of mice in the interscapular region was painted every third day with a 0.3 per cent solution of benzpyrene in benzene. Papillomas appeared toward the end of this period and during the next 2 months after the cessation of the painting carcinomas developed. Urine was collected from a group of 10 mice and analyzed. Carbon was determined by the method of Osuca (38, 39). For nitrogen, the determinations were made by the method of Müller (31-33). Urine of normal mice and of mice painted with the solvent, benzene, was used as control. The mean values are presented in Table I.

The data presented in Table I show that throughout the period of painting with benzpyrene, until the appearance of papillomas, the oxidation coefficient remained unchanged and did not differ from the coefficient found in mice painted with benzene and in

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normal mice. The ratio of C:N did not change during the period of painting.

A distinctly different picture came to view after carcinoma had been produced. M. Tchertkova found that in mice, soon after the origination of skin carcinoma, the values for the ratios of C:N and vacate-O:N in the urine increased. The values are presented in Fig. 1. They indicate that the oxidation processes had deteriorated.

From Fig. 1 it is seen that the values of these coefficients in mice with carcinomas exceed the limits of the normal variations, while the values for mice with papillomas lie more frequently within the limits of normal variations. In some of the mice in which higher values of these coefficients occurred, the initial stages of carcinomas existed, together with papillomas, as shown by histological sections of the lesions. Frequently, in the course of applications with benzpyrene, the change from papilloma to carcinoma takes place very rapidly. As a rule our diagnoses of such changes were made macroscopically because we needed the mice for further investigations.

Rabbits were painted with benzpyrene every second or third day for a period of 7 months, the solution being applied to the anterior surface of the ears. The only local changes noted were loss of hair and hyperkeratosis. The urinary ratios of C, N, and vacate-oxygen during the painting period varied within the same limits as those determined before the carcinogen was applied. Thus the C:N values varied from 0.5 to 0.66; the ratio of vacate-oxygen to N from 1.3 to 2.0. The numerical values for the same animal were essentially similar.

Oxidation-Reduction Potentials of the Blood

As the oxidation-reduction potentials of the blood may, under certain conditions as shown by Oiwin (37), serve as an index of the state of oxidation processes in the organism, I made measurements of these potentials in an investigation undertaken in collaboration with M. Zalesskaya. The determinations were made with a syringe electrode of bright platinum according to the electrometric method of Kawetzky and Oiwin (18). The blood was drawn by syringe from the heart of the mouse. We used 60 mice, of which 20 were normal and 40 were painted with benzpyrene. We found that in mice painted with benzpyrene during a period of 3½ months the oxidation-reduction potential values varied on the whole within normal limits; namely, from 177 to 198 mv. Lower values were observed in only a few instances.

Mice with carcinoma induced by benzpyrene showed somewhat lower values of the oxidation-reduction potentials of the blood. This decrease occurred especially in the advanced stages of the process. Thus in normal mice the values of the potentials varied from 186 to 196 mv., while in the majority of mice with experimental carcinoma the values ranged from 172 to 184 mv., the difference between the mean values amounting to 7 mv. In the precancerous period, when papillomas developed, the values for the potential lay within the limits of normal variation. The data are presented in Fig. 2.

| Table II: Oxygen Consumption (Values for $O_2$) by Slices of Liver from Mice with Benzpyrene-Induced Papillomas and Carcinomas and from Controls |
|---------------------------------|-----------------|-----------------|-----------------|
| Painted with benzene            | Skin papillomas | Skin carcinomas |
| 0.673                           | 0.661           | 0.491 *         |
| 0.602                           | 0.647           | 0.462           |
| 0.591                           | 0.677           | 0.390           |
| 0.558                           | 0.381           |                 |
| 0.506                           | 0.277           |                 |
| 0.493                           | 0.139           |                 |
| 0.480                           |                 |                 |
| 0.475                           |                 |                 |
| Averages:                       |                 | 0.329           |
| 0.534                           |                 |                 |

* Carcinoma in initial stage.

Oxidation Processes in Tissue Slices

N. Ivashentsova studied the oxygen consumption by slices of liver and brain from mice which had been painted with benzpyrene. The determinations were made with Fenn’s (13) apparatus, using either rabbit or horse serum as the medium for the tissue slices. Each experiment extended over a period of two hours. The control consisted of measurements of oxygen consumption by slices of liver and brain from mice painted with benzene alone. The data obtained for the liver are plotted in Fig. 3.

The data given in Fig. 3 show that there were no significant changes in oxygen consumption by slices of liver from mice painted on the skin with benzpyrene during a period of 3½ months, up to the appearance of carcinomas. The majority of the values obtained lie within the limits of the variations found in the controls, and the values were lower than normal only in a small number of experiments. Similar findings were obtained for the brain.

In Ivashentsova’s study the oxygen consumption in slices of liver obtained from mice with carcinomas induced by benzpyrene was markedly reduced as compared with the normal. The data are presented in Table II.
It may be seen from Table II that in 7 out of the 8 control experiments \( Q_0^2 \) is above 0.480, whereas in all instances in which carcinoma was present, with the exception of the initial stages, the value of \( Q_0^2 \) was below 0.480. On the average the level of this coefficient was 39 per cent lower than normal. Two mice with papillomas had coefficients corresponding to the higher level of values in the controls.

**LOCAL EFFECT OF CARCINOGENIC HYDROCARBONS ON OXYGEN CONSUMPTION OF TISSUES**

According to our findings, carcinogenic hydrocarbons, in the doses sufficient to induce the development of carcinoma of the skin in mice, did not have any appreciable effect upon the oxidation processes of the organism as a whole. Changes occurred only after the carcinomas appeared. On the other hand might not the carcinogen affect tissue respiration at the site of its application?

The investigation of this question was undertaken by our collaborator, N. Ivashentsova, who conducted experiments in vitro and in vivo to determine the effect of carcinogenic hydrocarbons upon oxygen consumption by tissues. A study was made first of the oxygen consumption by sections of liver and brain and by thin slices of the skin from the ears of mice, placed in serum containing a saturated solution of 1,2,5,6-dibenzanthracene (concentration about 4 mgm. per cent). Fenn’s method was used. Under these conditions the respiration of tissues exposed to dibenzanthracene did not differ from that of the controls. The data obtained for the liver are given in Fig. 4.

These findings differ from those of Pourbaix (40-42) according to whom the addition of dibenzanthracene or benzpyrene to the medium causes a drop in the oxygen consumption of liver and brain. In her experiments, however, the decrease occurred only under certain conditions and was slight.

It may be objected that in these experiments of Ivashentsova there was not sufficient time for the penetration of the hydrocarbon into the cell and that the period of exposure of the tissue to the hydrocarbon was too short. Ivashentsova, therefore, studied the local effect on oxygen consumption in tissues painted with benzpyrene over a prolonged period. For this purpose ears of mice, which consist essentially of a double layer of skin separated by a thin layer of cartilage, were painted with benzpyrene dissolved in benzene for a period of 3½ months. Ears were amputated at various times during this period and their oxygen consumption was studied in vitro. The results of these tests showed that the oxygen consumption by tissues of the ears of mice subjected for periods of 1 to 3½ months to the action of benzpyrene did not differ from that of the controls. The data are presented in Fig. 5.

Glycolysis in tissue slices was not studied in our investigations.

In Pourbaix’s (40-42) experiments, sections of liver and brain placed in an aqueous colloidal suspension of benzpyrene showed an increase in aerobic glycolysis in some instances, while anaerobic glycolysis remained unaffected. According to de Gaetani (8) benzpyrene is unable markedly to increase tissue glycolysis in vitro. Magat, Lebenson, and Wolkensohn (40) on applying benzpyrene in small doses found no change in the consumption of sugar by fibroblasts in cultures.

**PROTEIN METABOLISM**

It has been our opinion that the primary changes in malignant cells consist in changes in the principal carrier of vital processes; namely, the protein component of protoplasm. For this reason we regarded as momentous Kögl’s (20, 21) discovery of the stereo-chemical peculiarities of proteins of malignant cells.

Applying Kögl’s method we analyzed a number of tumors induced by carcinogens. The first results of the incomplete investigation of A. Braun and N. Schmidt corroborate Kögl’s findings with regard to glutamic acid. The data, presented in Table III, show that the proteins in the tumors produced by the carcinogens used were partially racemized.
Larionow—Mechanism of Action of Carcinogens

Fig. 1 to 5
Effect of Carcinogenic Hydrocarbons on Tissue Cultures

It is generally recognized that the problem of the pathogenesis of tumors would be greatly simplified if it were possible in vitro to change normal cells into malignant cells. Attempts have been made to bring about this transformation in tissue cultures, but the results obtained thus far have not solved the problem.

Evidence obtained from experiments with tissue cultures shows that the carcinogenic hydrocarbons are not growth-accelerating substances in the usual meaning of this term. This point has been brought out by the work of Larionow, Ivashentsova, and Tchertkova (26), Earle and Voegtlin (11, 12), Timofejevsky and Benevolenskaya (48, 49), and Katchka. It still remains to be discovered whether carcinogenic substances change normal cells to tumor cells by a direct and immediate action, or whether this effect is accomplished by means of an intermediate link. The positive results obtained with cultures of chick tissues exposed to tar and arsenic by Fisher (15), Laser (27), and by des Ligneris (9, 10) with dibenzanthracene appear to be doubtful because of the possibility of infection of the material with the virus of chicken sarcoma.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Amount in mgm.</th>
<th>(\alpha) (%)</th>
<th>Percentage d form</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Transplantable dibenzanthracene mouse sarcoma</td>
<td>650</td>
<td>26.8</td>
<td>7.8</td>
</tr>
<tr>
<td>2. Transplantable methylcholanthrene rat sarcoma</td>
<td>115</td>
<td>23.5</td>
<td>13.12</td>
</tr>
<tr>
<td>3. Transplantable o-amidazo-toluol liver carcinoma of mouse</td>
<td>107</td>
<td>15.3</td>
<td>21.4</td>
</tr>
<tr>
<td>4. Normal mouse liver</td>
<td>350</td>
<td>31.1</td>
<td>0.96</td>
</tr>
<tr>
<td>5. Normal rat muscle</td>
<td>276</td>
<td>30.7</td>
<td>1.6</td>
</tr>
</tbody>
</table>

During the past three years in conjunction with my collaborators, M. Tchertkova and A. Samokhvalova (26), I have sought to obtain tumors in tissue cultures with benzpyrene and dibenzanthracene. After numerous failures we finally succeeded in obtaining a peculiar phenomenon in cultures of mouse fibroblasts. For explantation we used skeletal muscle of the hind leg of newborn mice. The cultures were grown in flasks. The first cells which grew were the cells of striped muscle and fibroblasts. In the course of the hind leg of newborn mice. The cultures were introduced into the cultures at the time of passages before coagulation of the plasma of the solid medium had occurred. The longest periods of cultivation were 1½, 2, 4, 4½, and 6½ months, respectively, in 5 series of experiments.

The special feature of these cultures was the appearance of secondary growth centers in the zone of proliferation (Fig. 6). The new daughter culture consisted of cells differing from those of the mother culture. These cells proliferated more rapidly, as a rule, than the elements of the original culture and soon surrounded the initial mass. Possessing greater mobility, these new cells became isolated from each other to some extent at the periphery of their zone of proliferation. The new cells differed also morphologically from the cells in the initial growth. In most instances they did not form the radial strands characteristic of fibroblasts but were irregularly distributed. Some of the cells in some of the secondary centers of growth were distinguished by large size and peculiarity of form. They accumulated lipid substances, often in large amounts, and appeared to differ biochemically from the cells of the primary growth. The characteristics of these cells are shown in Fig. 7.

Even small numbers of cells of some of the secondary growth centers were capable of giving rise to new cultures when transplanted, whereas with normal fibroblasts large numbers of cells were required for successful passages.

Unfortunately, through a series of accidents, nearly all of the secondary growth centers were lost during passages. In one case only was the growth of cells from a secondary center maintained for 2½ months without further addition of the carcinogenic compound.

The periods of time between the first introduction of the carcinogenic substance into the culture and the appearance of secondary centers were: for dibenzanthracene, 14, 24, 36, and 57 days; for benzpyrene 12, 14, and in the last series, 6 days. The concentrations of dibenzanthracene employed in the medium of the cultures varied from 0.3 to 0.6 mgm. per cent. In one series of cultures during a period of 14 days the concentration of benzpyrene was 0.15 mgm. per cent, while in the 6 and 12 days' series the concentration of benzpyrene was 0.6 mgm. per cent.

Our data on the effects of reimplantation of these altered cells into mice are not sufficient to justify conclusions. It is impossible, therefore, for us to say whether or not these cells from secondary growth centers are malignant.

As similar phenomena were observed in tissue cultures by Magat and Lebenson (28-30), Timofejevsky and Benevolenskaya (48, 49), and Earle and Voegtlin (11, 12), it may be surmised with considerable prob-
ability that carcinogenic substances may act directly upon cells in the animal organism.

**Effect of Nervous System on Incidence of Tumors**

The probability of a direct action of carcinogenic substances upon normal cells, rendering them malignant, does not exclude the possibility that in this process other pathogenetic mechanisms may operate through intermediate links. The nervous system, for example, may play no part or a most important one.

Experiments were undertaken by L. Notik (36) to determine whether the nervous system influences the origination of experimentally induced cancer. For this purpose 114 mice were used. Of these, 62 had the skin in the lumbosacral region painted with tar during a period of 6 months, and 52 had similar areas of the skin painted with a 0.3 per cent solution of benzpyrene in benzene for 3½ months. In the precancerous period, at the time when papillomas appeared at the sites of painting, each series of animals was divided into two equal groups. One group served as control while in the animals of the other group the nervous system was "traumatized." In these mice the sciatic nerve was transected and its central end was treated with formalin or croton oil. Some of these animals developed trophic lesions of the joints of the foot on the side operated upon.

In mice subjected to traumatization of the nervous system in this manner papillomas which had developed following the application of the carcinogenic hydrocarbon regressed more frequently than in the controls and the incidence of carcinomas was less than it was among the controls. The data are presented in Table IV.

<table>
<thead>
<tr>
<th>Carcinogen used</th>
<th>Period in months after painting</th>
<th>Regression of papillomas</th>
<th>Incidence of carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tar</td>
<td>3</td>
<td>2</td>
<td>19</td>
</tr>
<tr>
<td>Benzpyrene</td>
<td>3½</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>36</td>
<td>31</td>
</tr>
</tbody>
</table>

The results of these experiments showed that a pathological process evoked in the nervous system may influence the incidence or origination of cancer induced by carcinogenic hydrocarbons. Traumatization of the nervous system during the precancerous period inhibited the development of carcinomas. In the course of subsequent observations of the benzpyrene series evidence was obtained that this inhibitory nervous influence gradually abated with the subsidence of the pathological process in the nervous system. Carcinomas finally appeared, but at a considerably later period.

In another series of investigations, L. Notik applied the same method of traumatization of the nervous system much earlier, as soon as the benzpyrene painting was started. The lesions of the nervous system were thus produced before the onset of the pre-
Canceromas appeared earlier than in mice of the control group. The results of these experiments are presented in Fig. 8.

The fact that an injury of the nervous system may have a different effect on the development of a pathological process according to the time of its application has been noted frequently by Speransky (46) and his collaborators. Although we cannot as yet say in what way the process of origination of experimental carcinoma is influenced by traumatization of the nervous system, the facts brought out by these experiments must be considered in the light of Speransky's theory that in the pathogenesis of pathological processes there is always a "nervous component."

![Graph of data of observations of the effect of the nervous system on the incidence of carcinomas induced by painting the skin of mice with benzpyrene.](image)

**DISCUSSION**

Although comments have been made on the results of some of the experiments reported in this communication, more extended discussion of certain aspects of the problem is appropriate.

The biochemical data on oxidation processes and oxidation-reduction potentials of the blood correspond with the results of my investigations of the morphology of the endocrine glands in mice in which carcinoma of the skin was induced by painting with benzpyrene (24). Previous to the appearance of carcinoma no obvious morphological alterations could be detected in any of the endocrine glands. In particular the thyroid gland, which is one of the regulators of the oxidation processes in the organism, showed no morphological changes. On the other hand, I found that, after the appearance of carcinoma induced by applications of tar or benzpyrene, changes could be detected in the thyroid, parathyroid, thymus, suprarenal cortex, hypophysis, and ovary. A decrease in the function of the thyroid was especially significant in view of the important relation of this gland to processes of oxidation in the organism.

The mechanism of the development of the observed changes in metabolism requires special study. It may be that metabolism is affected by intoxication of the body by the products of destruction of portions of the tumors and by products of bacterial action in infected tumors.

The conception that carcinogenic agents primarily produce changes in the cellular metabolism in the organism, particularly in the respiratory processes of various organs, and that this is an indispensable condition for the development of tumors, was based in part on experiments with coal tar and arsenic. It is well known, however, that tar contains many toxic substances in addition to carcinogenic hydrocarbons and that arsenic is likewise a toxic substance. Our experiments with pure carcinogenic hydrocarbons indicate that this conception needs to be revised.

While coinciding with Neumann's (34, 35) data on the decrease in oxygen consumption by the liver when a tumor is present, our findings differ from his in respect to the respiration of the cells of internal organs previous to the development of tumors produced by carcinogenic hydrocarbons. Neumann obtained a slight drop in oxygen consumption and a rise in anaerobic glycolysis in liver and kidney previous to the appearance of sarcomas produced by carcinogenic hydrocarbons. Neumann, however, injected these substances in solution in sunflower oil. His own experiments showed that the injection of sunflower oil alone produced an almost identical decrease in oxygen consumption and increase in glycolysis in liver and kidney.

The results of our experiments furnish ground for the inference that in the case of the local action on tissues by carcinogenic hydrocarbons, under conditions that lead to the appearance of tumors, these substances are barely, if at all, able to affect oxygen consumption and carbohydrate metabolism of tissues. This does not mean, however, that under no conditions can changes in oxygen consumption and glycolysis occur. Different methods of tumor production or different carcinogenic compounds might perhaps have other effects. Thus, in the experiments of Hayashi and Tomita (16), on the application of o-aminooazotoluol, changes in oxygen consumption in the liver could be detected previous to the appearance of carcinomas. The questions are: to what degree are these changes characteristic of the action of carcinogenic agents; to what extent is their appear-
ance necessary; and are they essential for the process of transformation of normal cells into malignant cells? Our own data lead us to infer that a modification of oxygen consumption and glycolysis, which since Warburg’s investigations have been considered characteristic of malignant cells, is less an immediate effect of the action of carcinogenic substances than a secondary phenomenon following upon some other primary changes in the cells, suffered while these cells undergo a transformation into malignant cells. It is to be noted in this connection that the recent work of Boyland (5, 6), Berenblum (4), and others has raised doubts as to the specificity of the changes in carbohydrate metabolism of tumor cells.

**Summary and Conclusions**

General systemic effects of carcinogenic hydrocarbons (benzpyrene) applied to the skin of mice were investigated by determinations of the ratios of partially oxidized substances excreted in the urine, the oxidation-reduction potentials of the blood, and the oxygen consumption of slices of organs. Local effects upon oxidation processes were studied in tissue slices and in skin to which the hydrocarbons had been applied. The oxidation processes were not disturbed during the precancerous period of papilloma formation, but supervened in a secondary manner after the appearance of carcinomas.

Incomplete investigations of proteins of induced tumors indicated that they contained an abnormally large proportion of d-glutamic acid.

The inclusion of the carcinogenic hydrocarbons, benzpyrene and dibenzanthracene, in the medium of tissue cultures of mouse fibroblasts produced changes in the morphological, biochemical, and proliferative characteristics of the cells. The data were not sufficient to indicate whether these cells had been transformed into malignant cells.

Traumatization of the nervous system by section of the sciatic nerve and treatment of the central end with formalin or croton oil affected the incidence of tumors indicated that they contained an abnormally large proportion of d-glutamic acid. The inclusion of the carcinogenic hydrocarbons, benzpyrene and dibenzanthracene, in the medium of tissue cultures of mouse fibroblasts produced changes in the morphological, biochemical, and proliferative characteristics of the cells. The data were not sufficient to indicate whether these cells had been transformed into malignant cells.

The following conclusions are suggested:

1. Carcinogenic hydrocarbons applied to the skin of mice do not affect the oxidation processes during the precancerous period of papilloma formation.
2. Changes in the oxidation processes of the organism of some organs, and of tissues occurring in connection with carcinoma induced by carcinogenic hydrocarbons, are of a secondary nature.
3. The primary change caused by carcinogenic hydrocarbons may be an alteration of protein metabolism.
4. Carcinogenic hydrocarbons produce changes in cells by action directly upon the cells.
5. In the organism the nervous system seems to function as an intermediary link in the production of carcinomas induced by carcinogenic hydrocarbons.

**REFERENCES**

On the Mechanism of Action of Carcinogenic Substances

L. Th. Larionow