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JPRS: 4621

17 May 1961

CZECHOSLOVAK REPORT ON EXPERIMENTS ON THE CAUSE OF LUNG CANCER

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Distributed by:

OFFICE OF TECHNICAL SERVICES
U. S. DEPARTMENT OF COMMERCE
WASHINGTON 25, D. C.

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WASHINGTON 25, D. C.

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JPRS: 4621

CSO: 1783-S

CZECHOSLOVAK REPORT ON EXPERIMENTS ON THE CAUSE OF LUNG CANCER

[Following is the translation of an article by M. Mestitzova and P. Kossey in Neoplasma, Vol VIII, No 1, Bratislava, 1961, pages 27-38.]

Lung cancers occur with increasing frequency in all population levels in the various countries. This alarming fact has been largely verified by statistics, and it is now necessary to establish its cause. Both in our own and in foreign medical journals the belief is widely held that possibly the growing incidence of lung cancer is to be traced to an increase in air pollution caused by wastes from insufficient coal combustion and motor vehicle exhaust gases. This waste product is essentially made up of aromatic polycyclic hydrocarbons. This is the belief, but it has not yet been proven by experiments which correspond to the actual state of exposure. Perhaps, even more than living surroundings in industrial or other big cities, work surroundings play a part in this respect, since there workers are continually subject to a longer and more intense air pollution by these same wastes. Therefore every operation of production which involves the formation of tar fumes presents a serious source of danger through the presence of cancer-causing substances from the series of the aromatic polycyclic hydrocarbons. The presence of 3:4 benzpyren in a proportion of 0.3 to 12 mg/m^3 air has been established in the atmosphere around the following industries: gas and coke producers, industries where tar, crude oil and its by-products are handled, in vulcanization and impregnation industries, and in the production of aluminum. For purposes of comparison the pollution of air by 3:4 Bp in a couple of large cities amounts to the following: Leningrad 0.0002 -- 0.4 mg/m^3, Manchester 0.00015 to 0.0003 mg/100m^3 (Cooper 1954, quoted according to Truhaut). Therefore it has become a problem of industrial medicine to establish whether there is some factor in work done under these conditions which contributes to the growth of lung cancer.

In the medical journals on experimental cancer investigations, we found no information on adequate model experiments which involved long-lasting effects of tar fumes on the organs of breathing. The

conditions which we suspect of causing lung cancer growth were not met either in the method of application or in the choice of carcinogenic substances by the completed experiments of Pattle et al, Claisse et al, Campbell, Shabad, Shimkin, and Porta. Positive results were achieved only with the use of very large doses. Experiments with exposure to cancer-causing substances contained in exhaust gases and in cigarette smoke usually did not go beyond reporting changes in the epithelium of the breathing organs.

In our present experiment, we duplicated, both in a qualitative and a quantitative way, in our model test the pollution of air with tar fumes which we had found to exist in aluminum production. The effects of the tar fumes on the behavior of 3:4 benzpyren in the lung tissues and in the blood were followed by clinical, anatomical, pathological, and histological, as well as biochemical observations.

Setup for the Experiment

Test Animals: 99 strain A mice and 144 strain C3H mice were used for the experiment. Later mice strains develop mammary tumors but seldom spontaneous lung tumors. They were chosen because it was to be a long lasting test with the smallest single active dose of the noxa. We had to reckon also with the complicating factor that the appearance of carcinogenic effects would occur at the same time as the formation of spontaneous tumors, which usually show up in the second year of life.

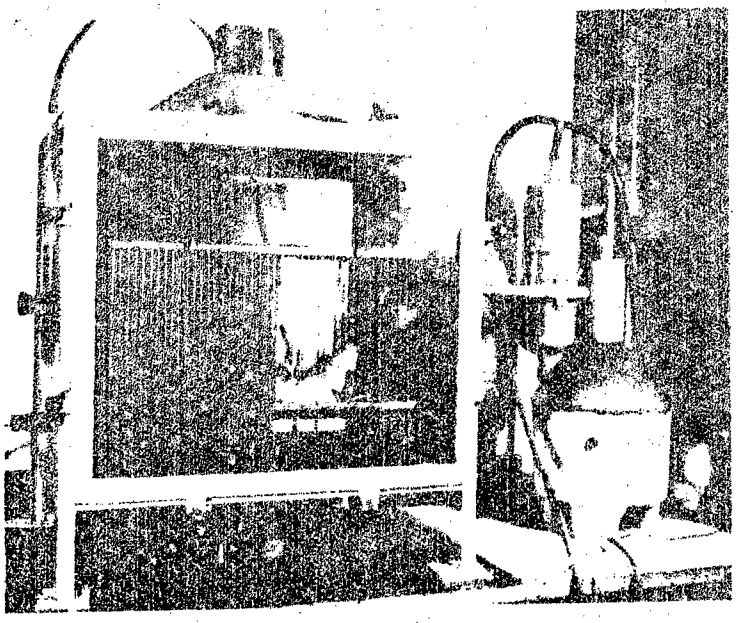


Illustration 1.
Gas chamber with device for production of tar fumes

Gas Chamber: During each test the same amount (40g) of anode material was burned at a temperature of 400° C in a muffle-furnace with a closed air supply. The anode material was the same as that used in aluminum production and consisted of coke dust thickened with coal tar. A controlled system conducted the developing gases and smoke vapors into the test chamber, (Illustration 1.) The supply of fresh air was caused by the ejector effect of the tar fumes to rise in an even stream which flowed into the chamber. During an hour, the air-smoke mixture was renewed five times.

Air Control in the Chamber: The tar fumes were controlled by means of the graphic chromatic paper method, using violet fluorescing hydrocarbon 3:4 benzopyrene according to MALY. The stationary phase of the separation system formed a 10% solution of paraffin oil in gasoline ether, with which the paper used (Whatman 4) had been impregnated. The mobile phase in ascending form produced methanol saturated with paraffin oil. The chromatogram spot of 3:4 benzopyrene with an RF value of 0.28 was evaluated in a planimetric, semiquantitative way with the help of a calibration curve.

Determination of hydrocarbon particles from tar in biological material: Lung tissues were dissected after homogenization with chloroform, put through a centrifuge, evaporated down to the necessary volume, and then treated in the same way as explained above.

Blood was taken from the axillary veins, mixed with a drop of heparin and a five-fold amount of chloroform, shaken thoroughly, put through the centrifuge, and benzopyrene was determined according to the above method.

Histological Preparation: After fixation in thinned-out formaline, the organs were bedded in paraffin and colored with hematoxylin-eosin in the usual way.

Results

Five times a week for the period of a year, strain C3H mice in two groups (I & II) were exposed to the same concentration of tar fumes. Strain A (I & II) were exposed simultaneously to an equal concentration for a period of ten months. For each group of C3H and A mice a separate group (III) served under the same living conditions as a control. Groups I & III received Larsen diets fortified with vitamins. Group II received food to which 0.1% sodium fluoride had been added. In group II we wanted to establish if the simultaneous presence of NaF in the organism would influence in any way the effect of the tar fumes. In aluminum production, fluorine combinations which are present in the air along with tar fumes offer an additional work hazard.

Due to the presence of irritants and also partly to toxic gases in tar, such as phenol, pyridine and others, interrupted exposures proved to be most favorable. For the duration of two

hours, alternately, a mixture of tar fumes and air for ten minutes and then pure air for ten minutes streamed into the chamber. In this way, the mice were exposed daily to sixty minutes of full and sixty minutes of a reduced concentration. The average concentration consisted of 1.4 gamma 3:4 benzopyrene for each liter of air (from 0.3 to 3.4). We have reported in another place on more precise details as well as on the toxicological side of the experiment.

During the fourth test month the first signs of irritation appeared on the skin. They were mostly in the region of the mouth, the eyes, the perianal and perigenital regions and on the ears. These irritations showed one after the other all stages of inflammation, from reddening and swelling to moist eczema with scab formation, and ended with hypertrophy, atrophy, and sclerosis. In the case of the ears it came sometimes to a complete loss of tissue. During later progress, a wart-like formation developed on the eyelids, on the ears, and also in the hairy skin of the back. In the tenth month, the perianal inflammation developed into a flat cell carcinoma. Strain A mice were the only ones hit by the inflammatory changes. Pea-sized tumors with dike-like edges and a central necrose appeared on the backs of the C3H strain only in the eleventh month. The histological picture showed a heavy, horny type of skin carcinoma.

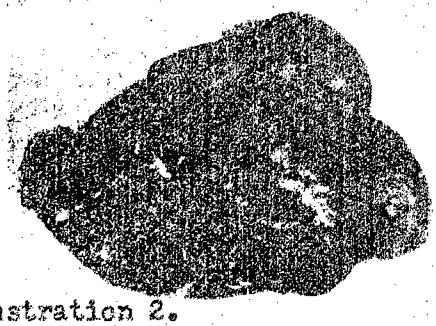


Illustration 2.
Subplural nodules in mice (strain A) after eight months of tar fume inhalation.

In the sixth and seventh months, cherry-sized tumors appeared in two exposed (C3H)mice in the region of the mammary glands; in one case on both sides. From the histological point of view, they exhibited the characteristics of a solid carcinoma, or that of an adenocarcinoma. During the eleventh month in this same strain, tumors appeared in the neck region and in the region of the front and rear extremities. Since, according to the histological picture, these glandular type tumors showed a malignant character, it must therefor have been a case of aberrant mammary gland tumors.

In contrast to the control group, all exposed groups showed a higher rate of mortality. This is significant (Table 1.) Strain A showed in all three groups a higher mortality than strain C3H. We suspect as the cause of death, along with some intercurrent infections, a common toxic effect from the tar fumes. The histological findings in the parenchymatous organs bears this out. After the discontinuation of the exposures, the mortality rate sank to the usual rate for mice.

Table 1.

Condition of the Surviving Mice (A and C3H) after Conclusion of Exposure to Effects of Tar Fumes

Strain & Group	Original Number	Number of Dead Mice Absolute	Percent	Remarks
		In 10th Month		
A/I	35	25	71.3*	*In the fourth month, ten mice died from an intercurrent infection
A/II	32	19	59.4	
A/III	32	4	12.5	
		In 12th Month		
C3H/I	(48) 19*	8	42.1	*29 and 22 mice, respectively, died in the second month from a technical accident
C3H/II	(47) 25*	10	40.0	
CdH/III	49	3	6.1	

Beginning in the fifth month, subpleural nodules developed in the lungs. They were raised, solid, greyish white in color, ranging from pin head to pea in size, and in the later stages coalescent. Since the nodules were numerous and of different sizes, it is possible to infer that they were in various stages of development. For both exposed groups of both strains, their increase in number was directly proportional to the duration of the exposures. This points to a causative connection with the gassing process, (Illustration 2). If we compare the two strains in the ten-month period, it is obvious that in the groups which received fluorine, the nodules appear more frequently. The difference for both strains is most significant when the difference in sensitivities to spontaneous tumors is taken into consideration.

Inflammatory changes of the bronchial mucous membrane and peribronchial tissues were constant accompanying symptoms. Metaplasia of the epithelium in the respiratory organs occurred however, only in the ninth to the eleventh month in both strains. (Illustration 7.)

Completely isolated granuloma type nodules appeared in the control groups. But signs of tumor-like growths could not be found either in their skin or in their lung tissues. After a year,

Illustration 3.

Multiple nodules (strain A) three months after the conclusion of ten months of exposure to the effects of tar fumes.

Illustration 7

Metaplasia of the tracheal mucosus membrane of a mouse (strain A) after nine months of exposure (hematoxylin-eosin x 24)

Table 2.

Number of Lung Nodules in Relation to Length of Exposure

Strain A (I and II)		Number of Mice with Nodules						
Exposure in Months	Number of Dead Mice	Number of Nodules 0	1	2	3-5	6-10	11-20	Nodules per Mouse
1 - 3	5	5	-	-	-	-	-	0
4 - 6	19	12	6	-	1	-	-	0.47
7 -10	20	3	1	-	2	6	8	9.0
1-10	4	3	1	-	-	-	-	0.25
Strain C3H (I and II)								
1 - 3	0	-	-	-	-	-	-	0
4 - 6	0	-	-	-	-	-	-	0
7 -10	12	7	1	1	1	2	-	2.0
11 -12	6	0	-	1	2	3	-	5.8
1 -12	3	2	1	-	-	-	-	0.3

Completely isolated granuloma type nodules appeared in the control groups. But signs of tumor-like growths could not be found either in their skin or in their lung tissues. After a year, the experiment was discontinued, and then after three months of non-exposure, the mice were killed. In the exposed A mice, the lungs in all cases were covered with nodules (Table 3). In the C3H mice this was not true for all cases and then only to a lesser degree. Among both strains of control animals only one case occurred, and this showed only one nodule.

With the microscope we observed in the histological sub-pleural sections of mice from the fourth test month a more or less pronounced cell increase in the alveolar septi. These lead to a thickening of the alveoli and contracted them down to small gaps or filled them up completely. The cells were of a relatively large size and round or oval in form. The center was light, round, and usually held a small clear nucleolus. The coarse chromatin structure of the center showed that the cells were not in a calm, healthy state. We found only completely isolated occurrences of clear mitosis. The cytoplasma was acidophile and very finely grained.

In the beginning we considered these cells to be of an alveolar origin, but we were not convinced in this period of the neoplastic character of the growth and thought more in terms of a reactive process. This period presented a hearth-mold shaped stage of proliferation of the aveolar cell type, (Table 4). During further development and within nodules of about 1 mm diameter, the cells did not change their character, to be sure, but in their structural arrangement they were closely joined together, and they showed for the most part a clear azinary formation, (Illustration 5).

Table 3.

Appearance of Lung Nodules in the Individual Strains and Groups in Relation to the Length of Exposure. Expressed by the Index of Percent Affected time ϕ Nodules (Mouse) Exposure Length in Months.

Strain A

Exposure In Months	Group I			Group II			Group III		
	Died	%Struck	Index	Died	% Struck	Index	Died	% Struck	Ind.
1 - 3	2	0	0	3	0	0	0	0	0
4 - 6	16	37.5	8.1	3	33.3	5.5	1	0	0
7 - 10	7	71.4	54.2	13	92.3	112.6	3	33.3	3.3

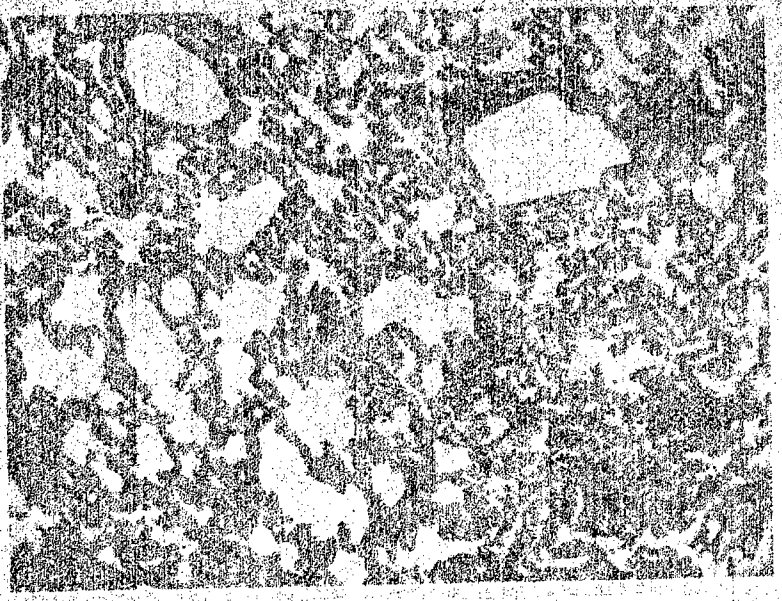
Strain C3H

1 - 3	0	0	0	0	0	0	0	0	0
4 - 6	0	0	0	0	0	0	1	0	0
7 - 10	4	25	5	8	50	27.5	0	0	0
11 - 12	4	100	50	2	100	45.8	2	50	4.1

This arrangement is typical of the so-called lung adenoma. Mitosis seldom occurred in this stage. However, in this period the moderate tumor character of the process is completely clear.

In the larger nodules (about 2-3 mm in diameter) the tumor cells were tubular in structure and frequently took on a papillary character. The papillae were formed of cubical or cylindrical cells which were closely pushed together. The stroma was very weak. Its arrangement was indicative of the epethelic nature of the tumor (Illustration 6).

Illustration 4



Cell increase in the alveolar septi. (Hematoxylin-eosin X 24)

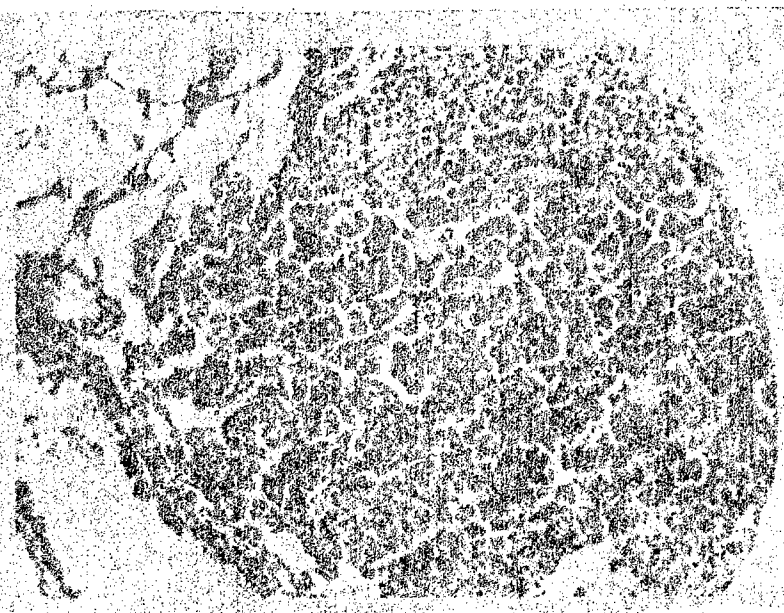


Illustration 5

Azinary tumor in a mouse after a ten-month exposure (hematoxylin-eosin x 24)

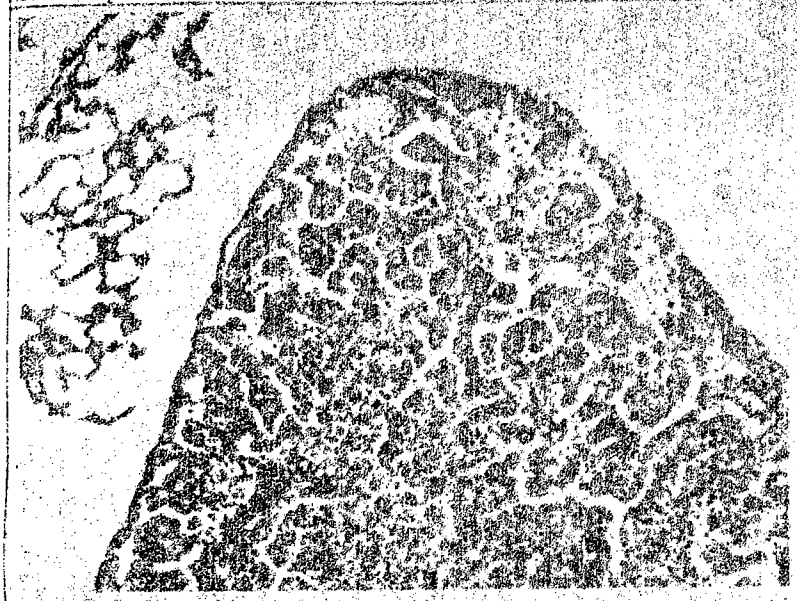


Illustration 6

Papillary lung tumor (hematoxylin-eosin x 60)

After a series of sections made from the whole lobe of one lung, we could not establish whether the tumors originated in the bronchia or in the bronchioles. Most frequently they occurred completely on the subpleural periphery, where there were no more bronchia at all. Our findings agree completely with those of Grady and Stewart. These authors designate these tumors as alveolar carcinomas.

Interesting facts were obtained from biochemical studies of the penetration, the duration, and the dispersal of the tar fumes.

Except at the beginning of the exposure, normal lung tissue showed no fluorescence with the chromatographical paper method we used. However, after only a single two-hour exposure fluorescence showed up in the lung tissue and in the blood, although in the blood this was not always clearly defined. After an exposure of three days, easily recognizable spots of tar fumes were already present in the lungs. The positive value of these spots increased in proportion to the duration of the exposure (Illustrations 8, 9).

It appears that a moderate accumulation is built up under a concentration of 1.4 gamma Bp/l air in spite of the fast elimination through the blood. A twenty-two hour interruption of the exposure was apparently not enough to eliminate the inhaled quantity completely from the lungs. A one-week exposure was enough to show, by a semi-quantitative evaluation, the amount of 3:4 benzpyrene in the lungs of three mice. To be sure, the quantity of proven benzopyrene is only a fraction of the gross computed quantity inhaled. In theory, under the given conditions of the experiment, 10.1 gamma Bp should enter one lung. We found only 0.19 gamma for each lung,

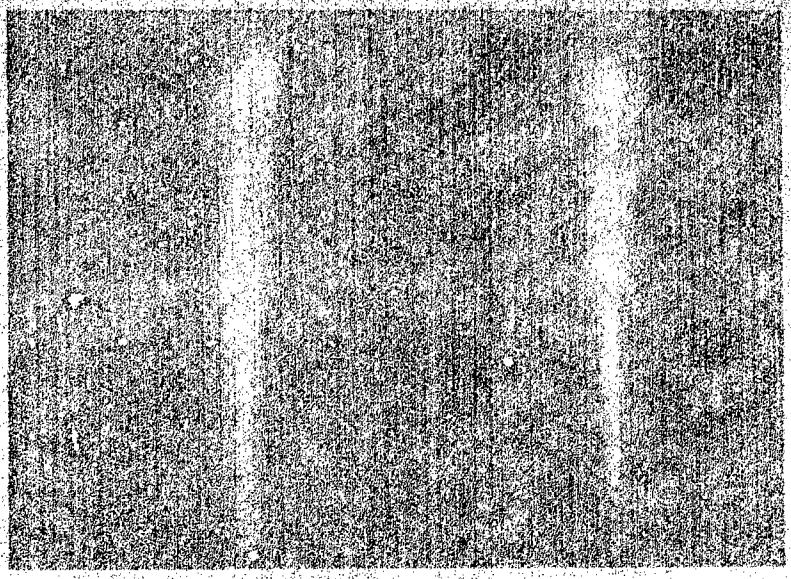


Illustration 8

Chromatogram of tar fumes from the test chamber (1 & 3) and from three mice lungs after a three-day exposure (2).

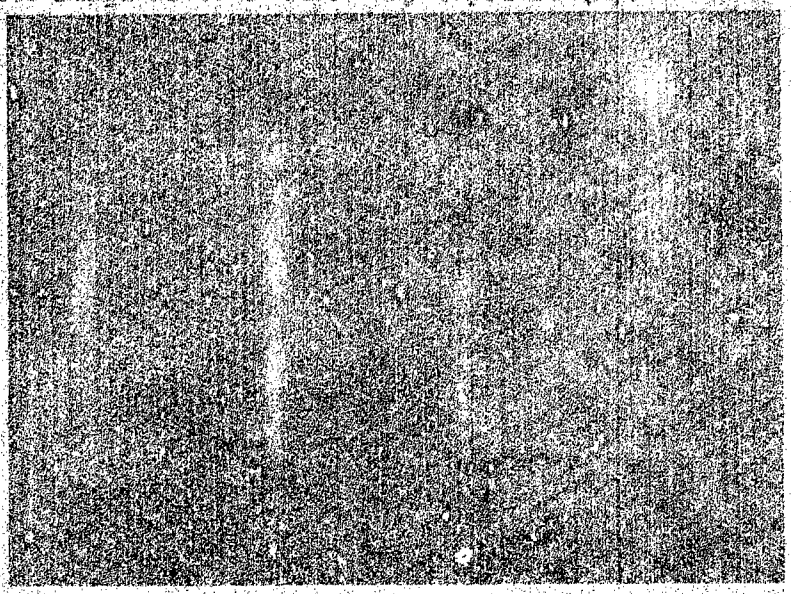


Illustration 9

Chromatogram of three mice lungs after seven months of exposure (1 & 2) and after one week of exposure (3), and out of the chamber (4).

which does not come to a complete 2%. The difference is explained by the fact that on the one hand the larger part of the tar-air mixture does not penetrate beyond the large bronchia, and on the other hand the very smallest particles which penetrate into the alveoli are partly breathed right out again and in part are quickly metabolized and eliminated by the blood. After seven months of exposure, the quantity of benzopyrene remaining in the lungs increased to 0.34 gamma. According to this, the accumulation was very slow. We would like to stress, however, that here it is only a question of an evaluation for orientation purposes. The value was ascertained with the help of semi-quantitative methods, which refer to the damp weight of the lungs and are subject to the inaccuracy which is characteristic of every biological test.

We are convinced in later tests that the accumulation is reversible. After a six-day period of gas exposure, and then following interruption of the exposure, the hydrocarbon amounts from tar fumes gradually disappeared from the lung tissues. After a forty-eight hour interrutpion, the fluorescence decreases. However, it is still clearly distinguishable. But after seventy-two hours the single spots merge, and after 120 hours, completely disappear. Even after a ten-month exposure, the fluorescent quality decreases in the lungs. After a five day interruption we could no longer establish the fluorescence of benzopyrene in the lung region.

Fluorescence in the blood can only be intimated, and because of this we could not identify single spots under low concentrations. It was only immediately after the exposure that we were able to establish fluorescence. After twenty-four hours not even traces of it were present.

Review

In this communication we limit ourselves to opinions expressed on the given problem from a purely oncological viewpoint.

It has been proven in numerous experiments that the carcinogenic potency of the single aromatic polycyclic hydrocarbons develops in a definite sequence, but differs according to the method of application.

These observations have been collected from percutaneous, subcutaneous and intravenous applications of cancer-causing substances, and with the use of large doses. This fact shows a certain relativity of carcinogenic materials, depending upon a series of circumstances.

Using small concentrations and with our inhalation method, we could not produce lung tumors in a sensitive strain before the fifth month. In those test arrangements which we quoted in our introduction the tumors showed up in a short time. With the intratracheal application of carcinogenic hydrocarbon in fluid or in solid dispersed form, some authors were able to produce lung tumors

in a short time, others could not. Using a high exposure to coal tar dust as a causative agent, found lung tumors in 74% of the cases, and after removal of the carcinogenic components, in 45%. Shabad found only a pseudo carcinoma-like formation. After a two month inhalation test of tobacco smoke Lupu and Velican [22] obtained in rabbits and guinea pigs chronical bronchitis and a fibrose reaction of the alveolar interstitums, which led to sclerosis of the lungs. After a one-year exposure to the effects of cigarette smoke, Lorenz et al could not establish any increased tumor formation. In the same way, other authors found only irritation and metaplastic changes of the bronchial mucous membrane. After a single intratracheal use of 0.1 mg 1:2:5:6 - Dibenzanthracene, Shimkin found lung cancers which could be identified by the histological method. Andervont and Shimkin found them after the use of 0.25 mg.

Except for the experiment with tobacco smoke, the various ways of introducing the carcinogenic materials into the organism can not be compared to our model test. In each previous case it was a question of introducing large doses into the lungs. This has the added application interference of adding the toxic effect of the solvent for the cancer causing substance, as well as the automatic irritating components in the dust particles, or in some cases causing a local reaction in the tissues to a thread soaked in benzopyrene. Apparently, metabolism takes place in a different way when either a colloidal suspension of benzopyrene blocks the capillaries and thus leads to a fixing of the cancer causing substance, or when it is compounded with aluminum hydroxide and introduced by intratracheal methods, (PLOC at the Health Conference in Prague 1959). The metabolism is different than under the physiological conditions of direct inhalation of the harmful materials.

Inhalation in aerosol mixture offers the best condition for dissolving the carcinogenic fractions of tar fumes in the body fluids, for their quick diffusion through the alveoli walls, and for their metabolism. Feldman and Fuhrman (quoted according to Lazarev) give the solution rate for 3:4 benzopyrene in rabbit serum as 4 mg/100 ml in comparison to 0.24 micromol/l in water.

The conditions for the dispersion of carcinogenic hydrocarbons in the lungs under the aerosol state favor their metabolization and prevent to a certain degree their accumulation. The experiment shows clearly the relation between the early appearance of nodules and the exposure to the effects of tar fumes. The change to a malignant degeneration appears to be dependent on the intensity of the concentration of tar fumes and the possibility of its accumulation in the lungs.

Spontaneous or induced lung tumors in mice have been studied since the end of the last century (Livingood quoted according to Stewart). Various authors describe these tumors, using various names, such as adenoma, papillary cystadenoma, or adeno carcinoma. The morphological picture described by several authors is uniform

and also agrees with our own findings. In contrast to this, the various authors do not agree as to the histogenesis. The opinion prevails, however, that the tumors originate in the alveolar epithelium. The question of histogenesis was thoroughly investigated by Grady and Stewart. They brought about such tumors with subcuteneous injections of 1:2:5:6 - dibenzanthracene and 2P-methylchelanthren.

Mostofi and Larsen arrived at completely identical histological results with tumors caused by urethane. Gricjute maintained likewise that these lung tumors were alveolar in origin in most cases, although he did not exclude the possibility that in individual cases these tumors might be bronchial in origin. We also observed a proliferation of alveolar cells, which gradually turned into obvious tumor growths. For this reason we are inclined to believe that the tumors found by us are alveolar in origin.

At the start, as previously mentioned, we were not convinced of the neo-plastic nature of the process. In this regard Gricjute quotes Glazunov and Larionov, who regard these tumors not as actual new formations, but as irritant proliferations, or as "nodule-like reactive hyperplasia".

These, however, are only isolated opinions, and the great majority of Soviet and English authors consider these tumors to be genuine new formations. Not only the histological picture, but metastases, as well as successful transplantings bear this out. It has been mentioned that in the case of transplants, these tumors lose epithelic nature and take on a sarcoma-like character.

The biochemical results show that 3:4 benzopyrene as well as other fractions of tar fumes penetrated very quickly into the blood. The assertion found in the literature that these metabolites do not remain very long in the blood was also confirmed by us. Under continuous absorption of these materials by the lungs, their elimination remains behind the intake, and benzopyrene and other condensation products are accumulated in the lungs. This behavior explains the negative results from experiments which tried to cause lung tumors in early periods. Only after a longer exposure is it possible to accumulate a sufficient quantity of carcinogenic material which leads to a qualitative change in the cell process.

In any case, our results stand in opposition to the experimental findings that the cancer process is more marked after the repeated action of small concentrations of aromatic polycyclic hydrocarbons during a longer period of time. This is true of percutaneous and subcutaneous applications. Our results could not confirm this for the lung tissues when application was through inhalation. They strengthen, however, the observations of Shimkin et al, which state that under intravenous application the number of induced tumors is directly proportional to the size of the dispersed particles and to the thereby conditioned fixation in the capillaries.

The parallel experiment with NaF fed mice is tied to the fact that fluorine inhibits glycolysis. Its abnormal increase is one of the most prominent peculiarities of the metabolism in tumor cells. Our experiment also recalls the work of Flamming who followed the favorable effect of fluorine on transplanted sarcoma. In our experiment we could not confirm the favorable influence of fluorine. In both strains which were fed with NaF, we could only establish after the ten-month period that the lungs were covered to a greater degree by nodules than in the exposed comparison groups. In the advanced stages of chronic fluorine poisoning, a common debility, and a reduced capability of the organism to equalize metabolism disturbances are added to the otherwise pathological happenings. The right conditions for an investigation of the question as to whether fluorine inhibited glycolysis in vivo were not given until the tenth month, and then the reaction of fluorine could not come to full recognition. This means, in a practical way, that the risk of exposure to the influence of fluorine will be superimposed on that of the tar fumes.

In spite of a large number of questions which are still open, a definite direction for preventive medicine may be derived from our results for industries which work with tar fumes. Along with technical methods of prevention, it is well to consider a suitable division of work time and work rest through a corresponding limiting of daily, weekly, and yearly work periods.

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