endoplasmic reticulum noted by HUTTERER et al.[12] in the liver cells of rats given dieldrin or 3'-methyl-4-dimethylaminoazobenzene. Prolonged treatment with these compounds reduced the initial increase of enzyme activity, without affecting SER hypertrophy. Considering the biochemical data[1-3], SER accumulation in Walker tumor-bearing rats seems to represent a diminished functional capacity of the hypertrophic endoplasmic reticulum. However, further work is needed to establish whether these changes are due to a specific 'toxohormone' secreted by the tumor cells or to some nonspecific toxicity (e.g., protein breakdown products released from the tumor tissue, etc.).

Patients with advanced cancer might exhibit different nonspecific manifestations, including anorexia, weight loss, cachexia, increased susceptibility to infection, abnormal responses to drugs and various endocrine alterations, irrespective of the localization of the tumor. Obviously, not all of these changes are related to hepatic microsomes. However, some of them could result from faulty intermediary drug or hormone metabolism. It remains to be seen whether the structure and function of the endoplasmic reticulum membranes are likewise impaired in these cases[13].

Résumé. L'implantation i.m. de la tumeur de Walker chez les rats provoque une dilatation, une désorganisation et une dégranulation progressives du réticulum endoplasmique granuleux des hépatocytes. Ces changements, qui s'accompagnent en même temps d'une prolifération du réticulum endoplasmique lisse, représenteraient les manifestations ultrastructurales d'une insuffisance microsomale démontrée biochimiquement dans le foie des rats affectés par la tumeur de Walker.

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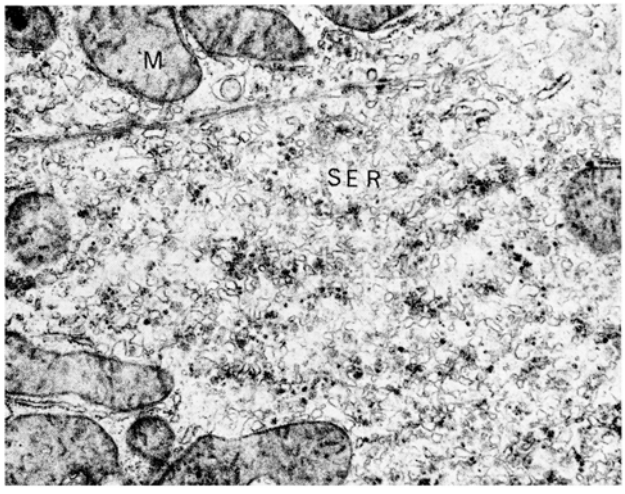

Fig. 3. SER accumulation in a rat hepatocyte 4 weeks after Walker tumor implantation. SER, smooth-surfaced endoplasmic reticulum. ×10,666.

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Interpretation of the Methylene Blue Reduction Test of Human Plasma and the Possible Cancer Protecting Effect of Selenium

Selenium has recently been implicated as a possible cancer protecting agent in humans by SHAMBERGER and FROST[1]. These authors found an inverse relation between the whole blood selenium concentrations from blood bank samples in 20 US cities and the human cancer mortality. They also report data which suggest a lower cancer mortality in areas of Canada where 'selenium indicator plants' are found than in areas where such plants are not found. In the present communication we present data from our laboratory which appear to support this hypothesis, and which explain earlier work on a presumed plasma cancer test based on the measurement of the methylene blue reduction time.

SAVIGNAC et al.[2] and BLACK[3] reported an abnormality in the methylene blue reduction time (MBRT) of plasma of cancer patients. A typical testing procedure consisted in the measurement of the time required for complete decolorization of a sample of plasma to which a known amount of methylene blue solution was added when the mixture was heated under anaerobic conditions on a steam bath[2]. The test was considered positive if the MBRT exceeded the average value of plasma of normal subjects. In systematic studies BLACK[3] reported the plasma of 75% of hospitalized cancer patients to give positive test results. Patients with nonmalignant neoplasia and other diseases gave overwhelmingly normal MBRT values. Subsequent studies[4] indicated practical limitations of the test for cancer diagnosis in that only approximately 50% of cancer cases gave positive test results. Moreover, in other groups of patients with pathological states the percentage of false positives ranged from 7 to 34%. The origin of the abnormal behavior of cancer patient plasma was originally considered to be dependent on the concentration of free sulfhydryl groups

in the plasma protein[2,3]. However, HUGGINS[5] in 1949 observed no difference in the amount of free sulfhydryl groups per mole of normal and cancer plasma. Although the test has since not received further attention, the initial experimental observations remain of some interest and prompted us to search for their chemical interpretation.

Method. 10 ml of fasting blood were withdrawn into a dry syringe and placed in a bottle containing sufficient potassium oxalate to prevent clotting. The contents are mixed by rotation. Care must be taken to minimize hemolysis. As soon as possible the plasma is separated from the cells by centrifugation at 3000*g* for 20 min and the plasma is pipetted of into a separate sample tube. The plasma thus isolated was kept under refrigeration no longer than 24 h before MBRT determination. To 1 ml of plasma in a 10×100 mm Wassermann Tube, 0.2 ml of a standardized solution of methylene blue in doubly distilled deionized water is added by means of a syringe. The dye solution should contain approximately 150 mg of recrystallized methylene blue per 100 ml of solution. One drop of capryl alcohol is added to prevent foaming, and the tube is then placed in a 400 ml beaker containing 200–250 ml of vigorously boiling water. During the measurement the water must boil briskly since the temperature near the surface must always be near 100 °C for sufficient steam generation. If the temperature falls below the boiling point of water, oxygen may seep back into the test tube, introducing errors in the MBRT determination. Without removing the tube from the water bath the time of disappearance of the last trace of color is recorded to the nearest half-minute and noted as the MBRT. The tube must not be removed from the water at any time during the measurement. It is important to treat all samples in exactly the same way to obtain reproducible results. The standard error of a single MBRT measurement is approximately 6.5% of the estimated true value of the reduction time, provided that the pH and oxygen content of the sample are held constant.

Results. The reduction of methylene blue by plasma undoubtedly involves sulfhydryl groups of the plasma protein as the electron donors. However, the reduction of the dye by thiols is slow in the absence of catalysts and is greatly accelerated by ultramicroquantities of certain heavy metal ions and biogenic metal complexes. The most potent catalysts are Cu, Co and Fe-complexes of thiols, iron porphyrins and vitamin B_{12}[6,7]. However, a comparison between the MBRT values and the total plasma levels of Cu, Co and Fe revealed no correlation. Since selenium also catalyzes methylene blue reduction by thiols[7,8], the MBRT of plasma samples was compared with their total selenium content determined by the fluorimetric method of OLSON[9,10]. The results given in Table I demonstrate an inverse relation between the selenium content of human plasma and the MBRT. The relationship was confirmed by adding known quantities of selenium in form of H_2Se to plasma and measuring the MBRT. In view of the approximately linear relation between $[MBRT]^{-1}$ and [Se] it is possible to use MBRT measurements for the approximate determination of plasma selenium levels; the results lie within ±15% of the actual values.

Discussion. The present work shows that abnormally long MBRT values signalize lower than normal plasma selenium levels. The apparent lower concentration of selenium in cancer plasma is unexplained but certainly not specific of the disease. The plasma concentration of selenium is largely determined by the dietary selenium uptake and the rate of excretion. Although injected sodium selenite (Se 75) is accumulated in certain neoplasms[11], we have thus far been unable to observe accumulation of dietary selenium in tumor tissues. For example, the selenium concentration in tissues of gastric cancer and of cancer of the colon was found to be 0.32 and 0.19 ppm (wet wt.), which is still within the normal range. On the other hand, if we follow the ideas of SHAMBERGER and FROST[1], lower than normal plasma selenium levels should be

Table I. Plasma selenium concentration and methylene blue reduction time (MBRT)

Sample No.	Specification[a]	MBRT (min)	Selenium (ng/ml):
1	Hospitalized, non-CA	4.0	120
2	Hospitalized, non-CA	5.0	102
3	Hospitalized, non-CA	6.0	88
4	Hospitalized, non-CA	6.0	80
5	Hospitalized, non-CA	6.0	76
6	Hospitalized, CA	12.0	72
7	Hospitalized, non-CA	12.5	65
8	Hospitalized, non-CA	11.5	61
9	Hospitalized, CA	12.0	60
10	Hospitalized, CA	11.0	55
11	Hospitalized, non-CA	11.0	55
12	Hospitalized, non-CA	14.0	52
13	Control, healthy	4.5	102
14	same, +50 ng of Se/ml	2.5	152
15	same, +95 ng of Se/ml	2.0	197
16	same, +140 ng of Se/ml	1.5	242
17	same, +190 ng of Se/ml	1.0	292

[a] Samples of plasma were selected to obtain large differences in the MBRT values. The selenium in samples No. 14–17 was added as H_2Se under nitrogen to avoid oxidation of plasma sulfhydryl groups. *Tris*-buffer (pH 7.4) was added to compensate for the removal of CO_2 during the addition of H_2Se.

Table II. Cancer morality rates (1967) and selenium concentrations in grain and forage crops in the 50 States of the U.S., including the District of Columbia

Selenium content[12] in grain and forage crops	Cancer mortality rates per 100,000 deaths[13] 160–190	125–160	90–125
Low to very low (⩽ 0.05 ppm)	17	4	0
Low to adequte (0.05–0.10 ppm)	0	5	5
High, locally excessive, median (0.26 ppm)	5	10	5

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observed with higher probability among cancer patients. Since the body pool of selenium is essentially determined by the dietary selenium intake, the published selenium concentrations in grain and forage crops of the US[12] provide an approximate indication of the dietary selenium supply. If selenium has a cancer protecting effect an inverse correlation between the cancer mortality and the selenium content of forage crops and grains may be expected. Using published[13] cancer mortality rates and the selenium distribution map of the US one finds that 21 States are located in low to very low selenium areas. Of these 17 or 81% show the highest cancer mortality, the remaining 4 are in the second highest rank. Of 30 states situated in areas with adequate to high or excessive supply of selenium 10 or 33% show the lowest cancer mortality rate, 15 or 50% the second highest and only 5 or 17% the highest mortality rates (Table II). It would seem for this reason that the possible cancer protecting effect of selenium merits further attention. The role of selenium as a biological antioxidant may be connected with this function[14].

Zusammenfassung. Der früher zur Krebsdiagnose vorgeschlagene Plasma-Methylenblau-Entfärbungstest nach SAVIGNAC[2] und BLACK[3] spricht im wesentlichen auf die Plasma-Selenkonzentration an. Daraus ergeben sich Hinweise für die möglicherweise krebsschützende Wirkung des Selens, die durch statistische Daten gestützt wird.

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The Potency of N-acetylaminofluorene in the Production of Cytokinin Autonomous Tobacco Tissues in vitro

In a previous communication[1] we reported the induction of cytokinin autonomous nodules in cultured tobacco tissues by several substituted fluorenes. The experiments showed small amounts of substituted fluorenes in the presence of 0.1 to 12.5 μM kinetin and 10 μM indoleacetic acid (IAA) produced significant quantities of cytokinin autonomous nodule tissue. The nodules upon subculture behaved like the hormone-dependent plant tumours described by BRAUN[2]. These tissues have lost their exogenous cytokinin requirement, but still need an auxin such as IAA for growth in vitro. The purpose of this study was to quantitatively define the levels at which one of the more potent fluorenes, acetylaminofluorene (AAF), induced the formation of cytokinin independent tobacco tissue under standardized conditions.

Materials and methods. The medium and general procedures were those described for use in cytokinin bioassays[3]. The tobacco stock callus requiring both an auxin and a cytokinin for growth in vitro was obtained from the pith of *Nicotiana tabacum* Wis. No. 38 and grown on medium containing < 0.15 μM kinetin and 10 μM IAA. Some stock callus was routinely subcultured on kinetin and/or IAA free media to detect any spontaneous occurrance of auxin and/or cytokinin independent tissues. No hormone autonomous tissues were observed in stock or control cultures. The experimental series contained kinetin at a concentration of 0.5 μM with IAA maintained at 10 μM. The AAF solution was filter sterilized and added to the autoclaved medium at concentration between 0.02 and 12.5 μM. 3 small (about 20 mg fresh weight each) pieces of friable stock callus were planted on 50 ml medium solidified with 1% agar in 125 ml erlenmeyer flasks. 4 replicate flasks of each treatment were incubated at 28 °C. Illumination was furnished by standard cool-white fluorescent lamps producing 4500 $\pm$ 500 ergs $cm^{-2} sec^{-1}$ incident upon the tissues. After 58 days the tissues were harvested. The firm white nodules were counted, separated from the moderately friable callus and weighed independently.

Results and discussion. The appearance of the 2 kinds of tissues produced in the presence of AAF is illustrated in Figure 1. The tissue shown consists of a white compact nodule composed of cytokinin autonomous cells and darker green friable callus which is still cytokinin dependent upon subculture. The potency of AAF in producing these nodules and its effect on the growth of callus are presented in Figure 2. The yield of the cytokinin independent tissue increased with increasing concentrations of AAF between 0.02 and 2.5 μM. Concentrations of AAF above 5.0 μM showed a strong inhibitory effect on the growth of both callus and nodule tissues. The greatest yield of nodule tissue was obtained at concentrations of 0.5 and 2.5 μM

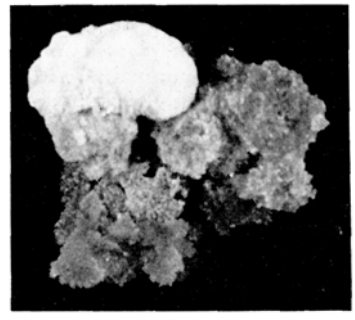

Fig. 1. Cultured friable tobacco callus with a large nodule composed of cytokinin-independent cells.

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