The particular traits of carcinogenesis induced in Wistar rats by aflatoxin B$_1$. IV. Porphyrins and the activity of gamma-glutamyltranspeptidase in Wistar rats bearing transplantable hepatoma

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In inbred Wistar rats bearing transplantable hepatoma induced by aflatoxin B$_1$ tissue porphyrins and the level of the serum gamma-glutamyltranspeptidase (GGTP) were studied. A liver reaction in carriers of transplants was ascertained, that was expressed especially by an increase in porphyrins, mainly the protoporphyrin fraction, and by morphological changes. The content of porphyrins in lung metastases was higher than in hepatomas and livers. The level of serum GGTP was higher in females up to the 40th passage, and up to the 90th passage higher in males. Hormonal conditioning of the early AFB$_1$-hepatoma passages is supported by a lower level of serum GGTP in females and higher in males, and the liver and tumor porphyrins in animals previously castrated in comparison with uncastrated rats. Comparative data of the quantity and quality of porphyrins in different rat tissues with primary and transplantable AFB$_1$-hepatomas demonstrate that systemic metabolic disorders of porphyrins are greater in rats with primary than in the case of transplantable tumors.

Key words: Transplantable AFB$_1$-hepatoma, porphyrins, γ-glutamyl-transpeptidase, sex dependence.

It has been proven in previous studies that, when hepatocarcinogenesis was induced in inbred Wistar rats with smaller and larger doses of aflatoxin B$_1$ (AFB$_1$), disorders of porphyrin metabolism occurred and there was an increase in the activity of serum and hepatic gamma-glutamyltranspeptidase (GGTP). The porphyrin content and the level of GGTP in hepatomas was higher than in the liver tissue of origin [16, 17].

Karyograms of primary hepatomas induced by AFB$_1$(PR) showed chromosomal destruction, while cytophotometric studies indicate a decreased DNA content of these neoplastic cells [6].

The experiments which constitute the subject of the present study had as their objective the verification of the porphyrin content and GGTP activity in Wistar

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rats, of both sexes, bearing transplantable hepatoma (T), which was previously induced with small doses of AFB$_1$ [15].

**Materials and methods**

Five-hundred-forty-eight, 3-month-old inbred Wistar rats of both sexes were used in the experiment. The animals were kept singly in plastic cages. Room temperature (21—22°C) and relative humidity of 62—65% were automatically controlled. Animals were fed a standard Murigran diet, produced by Bioveterinary Department, Gorzów, Poland; water ad libitum and vitamins weekly. All rats were sacrificed by decapitation.

The T-tumor was derived from a AFB$_1$-hepatoma induced in inbred Wistar rats [15]. The homogenates of neoplastic tissue was taken up by a syringe and injected s.o. into the left side in 0.5 ml doses. The tumor latency and growth period was approximately 2 weeks.

At time of autopsy, serum was obtained for determination of GGTP in all rats, and in certain rats the liver and T-tumors were also taken. In order to determine the porphyrins content fragments of liver, hepatomas, lung and metastases in them were set aside, and in addition fragments of tumors, livers, lung, lung metastases, and sporadically other changed organs were taken for histological studies.

180 males and 180 females were used for 90 passages of AFB$_1$ induced hepatomas whose properties were described previously [15, 17]. The level of serum GGTP obtained immediately after decapitation was determined in these 360 rats. In 14 males and 14 females bearing T hepatomas (1—8 passage) the porphyrin content was assayed in livers and hepatomas. The porphyrin content was also estimated in another 6 males and 6 females who were inoculated successively 6 times with isolated lung metastases from an AFB$_1$ induced hepatoma, which derived from the s. c. inoculated tumor in one of the rats. In 12 rats with successively transplanted hepatomas (passages 1—7) a comparative determination of the porphyrins was carried out on lung metastases and in their adjoining unchanged lung tissue.

A separate group was composed of 48 rats with T hepatomas (passages 3—6). Each passage used 3 males and 3 females which were previously surgically castrated and after 2 weeks inoculated s. c. with AFB$_1$-hepatoma. The same tumor was likewise inoculated in the following passages into 3 males and 3 females with intact gonads. In these 48 rats the serum GGTP and porphyrin content in liver and T tumor were determined.

The organ porphyrins were estimated in rats with T hepatoma and compared with the content of these pigments in the same organs of rats with PR tumor which was described in previous paper [16, 17]. The average values of the individual porphyrin fraction were obtained from determination of porphyrin content in different organs, i. e. livers, kidneys, bone marrow, spleen, Harderian glands in males and females with PR or T AFB$_1$-hepatoma.

The porphyrins were isolated from tissues and estimated using the methods described in previous studies [16, 17]. The individual fractions were quantitatively determined by the spectrophotometer using equations according to Remington [13] and the following coefficients: for protoporphyrin (Proto) 1.226, for coproporphyrin (Copro) 0.730, and for uroporphyrin (Uro) 0.832. Next, the porphyrin fractions were placed in a small column filled with talcum and eluated with acetone-amonia.
(7:3). The chromatographic identification was carried out according to ERIKSEN [4, 5] and CHU and CHU [3] methods. The free porphyrins used as markers were obtained from methylers of porphyrins hydrolyzed by 10% HCl (Protoporphyrin IX, dimethyl ester, grade I, crystalline 95% produced by Sigma Chem. Comp. St. Louis, USA, Cat. Nr. P-5880; Coproporphyrin III tetramethyl ester, grade A, Cat. Nr. 23504; Coproporphyrin I tetramethyl ester, grade B, Cat. Nr. 23502; Uroporphyrin I octamethyl ester, grade B, Cat. Nr. 672075 — producer Calbiochem. A. G. Luzerne, Switzerland).

The GGTP activity in serum was measured using gamma-L-glutamyl-alfa-naphthylamine according to the method of ORLOWSKI and SZEWCZUK [11]; the activity of this enzyme in tissues was determined according to the method of the same authors [10].

Histological studies of livers, hepatomas, lungs, and in some animals other macroscopically changed organs, were processed using the paraffin technique and stained with hematoxylin and eosin, and frozen sections were stained with Sudan III.

Results

The T hepatomas were characterized by cellular atypia, numerous pathological mitoses and hemorrhagic changes (Fig. 1). There were many lung metastases (Fig. 2), at times so widespread that normal lung tissue could not be found. With progression of passages such widespread metastases were observed more frequently in females than in males. The metastases sporadically affected the left axillary lymph nodes. The liver of T carriers showed fatty degeneration of varying degrees, small necro-
biotic foci or necrosis of some hepatocytes, activation of Kupffer cells, inflammatory infiltrate around the triads and hemorrhagic changes. In most livers from rats bearing hepatoma passages 1—20, small foci of proliferating hepatocytes with enlarged and hyperchromatic nuclei were seen (Fig. 3). The spleen displayed a hyperplasia with many multinucleated splenocytes.

In castrated rats pituitary hyperplasia was noted.

The average tumor weight in the first 8 passages did not differ significantly in males (4.4 g) from that in females (4.1 g). In further passages the latency period, which up to this time was approximately 14 days, shortened in females about 2—3 days in comparison to males, but in spite of this the average hepatoma weight in females was higher (8.4 g) than in males (6.1 g).

The quantitative ratio of the Proto, Copro and Uro fractions in the 8 times passaged hepatomas and in the livers of their carriers is illustrated in Fig. 4. In all the rats bearing the T hepatoma in passages 1—6, the total content of hepatic porphyrin fractions was greater in females, while in males the porphyrin content of the tumor was greater than in the liver. In passage 7 the difference was less clear, and in passage 8 the content of total porphyrin fractions was somewhat higher in hepatomas than in livers in males and in females. The increase of porphyrins in livers and
Fig. 4. The content of porphyrins in liver and in transplantable AFB1-hepatoma. On the ordinate: μg/g wet tissue. On the abscissa: L — liver, T — tumor, 1—8 — the numbers of successive passages; 1 — uroporphyrin, 2 — coproporphyrin, 3 — protoporphyrin.
The quantitative ratios of porphyrins behaved differently in livers and hepatomas in animal of both sexes carrying s. c. T derived from isolated and prepared lung metastases in a rat with the first inoculation of AFB₁-hepatoma (Fig. 5). Initially the total amount of porphyrins in the liver and tumors was almost identical but in the second passage a slight predominance of porphyrins began in the hepatoma, in the third there was an increase of porphyrins in the female liver but in the male more in the hepatomas, while in the 4—6th passage the pyrrole pigments (PP) were increasingly predominant in the livers irrelevant of rat sex. In the separate passages there was an increase of the Proto fraction in the tumor, and in passages 5—6 Copro also increased.

Figure 6 ilustrates the porphyrin content of lung metastases and in their host tissue. With the passaging of the tumor growing in the s. c. tissues, the porphyrin
content increased gradually in lungs and even more in metastases. In rats with the seventh passage, whose lungs were completely involved by metastases, the porphyrin content reached 6 μg per g of tissue; fraction Uro composed 8%, Copro — 23.3%, and Proto — 68.6%.

In comparing the average porphyrin level in rat livers with PR hepatoma, described in previous studies (15—17), with the level of these pigments in the livers of T carriers and control rats (C), the differences of the different PP fractions of the individual group can be seen in Fig. 7. The highest PP content was observed in animals with T, specially in females. It should be noticed that there is an increased level of fraction Uro in precancerous changes in livers in PR, while in livers without hyperplastic lesions in T the fractions Proto predominated. In the T male group the Proto fraction was 4.3 times higher than in the PR group and 1.3 times higher in comparison to C group. The remaining fractions did not quantitatively differ in a characteristic fashion, although fraction Uro was higher in males with PR and T than in the C group. Greater differences appeared in females: the livers of carriers of T contained 1.3 times more Uro, 3.3 times Copro, and a 20 fold increased of Proto.
than the livers of C group and in comparison to livers of group PR 2.2 times more Uro and Copro and 12.6 times more Proto.

The behavior of renal porphyrins (Fig. 8) was the opposite. The average total PP content was highest in rat kidneys with PR, which concerned mainly males and

was expressed as a relatively high Uro level in comparison to remaining groups, reaching values almost 2 times greater than in the T and almost 3 times greater than in the C group. In females the content of Uro was close to that of in the C group but in comparison to group PR it was lower. The remaining renal PR fraction in females had the lowest values, especially the level of Proto, which was 2.2 times lower than in group PR and 2.4 times lower than in the C group.

In the spleen (Fig. 9) the content of all the tested porphyrin fractions, especially Uro and Copro, were the lowest in rats bearing T and also clearly lower in comparison to the level of these fractions in the C group. The highest level of Uro, Copro, and Proto was in the spleens of females with PR.

In the bone marrow (Fig. 10) in males and females with PR and T characterized by a lowered Proto content, slightly decreased Copro and a decrease of Uro in comparison to the C group. The Proto content in the PR and T in males was over 2 times, while in females of PR group 3.5 times and T group 4 times lower in comparison to the corresponding C group.

The increase in average porphyrin content in Harderian glands (Fig. 11) was the result of an increase in Proto and mainly affected the rats with PR; in this group
the level of Uro was slightly elevated. In the animals with T the total amount of porphyrins was less than in the PR group and in females was significantly lower in comparison to the C group. In males of the PR group, the Proto level was 2.12 times higher and in the T group 1.66 times higher in comparison to the C group.

In females of the PR group the Proto content was 1.25 times greater, in the T group 2.1 times lower than in the C group.

Assuming the average total of the Proto, Copro, and Uro fractions in 10 males and 10 females with PR (16, 17) and 17 males and 17 females with T as 100%, a graphic representation was prepared of the proportions of the individual fractions (Fig. 12). As can be seen from this diagram the disorders of PP metabolism are more distinct in the PR than the T. The average Uro content in PR was 41.9% in males and 53.5% in females while in T they were correspondingly 16.5% and 12.0%; Copro in PR was 24.6% in males and 17.0% in females, while in T it was correspondingly 18.5% and 22.5%; and finally Proto in PR was 33.5% in males and 29.5% in females, while in T it was correspondingly 65.0% and 65.5%.

The average values of serum GGTP in carriers of T (Fig. 13) as determined in 2 males and 2 females in each passage, differed depending on the sex of the animals. In females, passages 1—30, the level of GGTP was raised and at the same time showed a tendency to decline, in 30—65th passage the level was within norm, and in the next 10 passages it gradually increased and became almost 2 times greater than
normal values; in the passage 75—90 the level of GGTP dropped. In males the level of this enzyme was increased in the passages 1—10 but was lower than in the females. In males with the passages 10—40 the level was within norm, next up to the 45th passage the values increased gradually and were higher than the corresponding

values in females. Finally in the 65—75th passage the values grew even higher, reaching values similar to those in the initial passage, after which there was a drop in the values of serum GGTP. As it can be seen from the curves in Fig. 12 the level of serum GGTP in T carriers was lower in males up to the 40th, and in the 40—90th passage the level of this enzyme was higher in males than females.

The influence of animal sex on the level of GGTP and porphyrins is seen clearly in comparative studies on castrated and normal rats (Table 1) inoculated successively with AFB
-hepatoma (passages 3—6). In castrated males the level of serum GGTP was generally higher, while in castrated females the level was significantly lower than in intact rats. The influence of castration on porphyrin content in livers and T hepatoma was seen more clearly in females. In castrated females all porphyrin fractions, in the liver and hepatoma, were lower than in the same tissues of uncastrated females. In castrated males the Uro and Copro fractions showed quantitative

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**Fig. 11.** The average porphyrin values in Harderian glands in rats with primary AFB-hepatoma (PR), transplantable tumor (T), and in control rats (C). The designations as in Fig. 4.

**Fig. 12.** The summarized values of tissue porphyrins in rats with primary AFB-hepatoma (PR) and transplantable tumor (T). The designations as in Fig. 4.
wavening, while the Proto fractions in the liver was significantly lower and in hepatomas significantly higher than in males with intact gonads.

Paper chromatography of porphyrins obtained from all the tissues studied derived from carriers T and healthy control rats, showed Uro, Copro, and Proto spots. In experimental rats the Uro fraction, obtained from livers, contained additionally 7- and 6-COOH porphyrins, fraction Copro was divided into isomer III and more intensively fluorescing isomer I, and fraction Proto contained 5- and

![Graph](image)

**Fig. 13.** The average values of GGTP activity in serum in rats bearing successive passages of AFB$_1$-hepatoma (in each passage 2 females and 2 males were used). On the ordinate: the numerical values of GGTP in J — μM/1000 ml/min. On the abscissa: the numbers of passages; 1 — females, 2 — males.

3-COOH porphyrins. The fraction Uro isolated from hepatomas did not contain other porphyrins, and the composition of the remaining fractions was the same as in hepatic Copro and Proto fractions. The Uro fraction obtained from different tissues, i.e. lung, lung metastases, kidney, bone marrow, spleen, and Harderian glands, did not display the presence of other porphyrins, fraction Copro divided into isomer I and III; in Harderian glands the Copro fraction showed the intensively red fluorescing spot of isomer III, and in kidneys isomer I. The Proto fraction isolated from the above mentioned tissues did not contain other porphyrins.
The period of growth and the macro- and microscopic characteristics of T in our experiments were in accordance with the observation of WARD et al. [14].

The main problem, which demonstrate the results of our experiments is the reaction of the liver of T carriers. The reaction is seen histologically as moderate degenerative and inflammatory changes, and in initial passages as small foci of proliferating hepatocytes. The hyperplasia of liver cells in Wistar rats carrying the T AFB1-hepatoma is described by BEDNARZ et al. [2], and they are of the opinion that this hyperplasia could be the result of tumor AFB1 metabolite influence on the carrier's liver.

The liver reaction to the s.c. hepatoma passaging was above all a disorder in porphyrin metabolism, which manifested itself mainly as an increase in fraction Proto in distinction to livers with PR in which there was a relative increase of fraction Uro in comparison Copro and Proto [16, 17].

The quantitative difference of the individual porphyrin fractions in liver in comparison with the AFB1-hepatoma was expressed differently in PR and T tumors. During primary AFB1 carcinogenesis a higher level of porphyrins in the tumor than in the liver was a constant phenomenon [16, 17]. In rats with initial AFB1-hepatoma passing a similar but less clear difference was seen only in males and later was lost in successive passages. The opposite results occurred in rats inoculated s. c. with a homogenate of lung metastases: in the 3 initial passages the difference of porphyrin content in liver and tumor was not significant, but in the next 3 passages the level of porphyrins, especially Proto, in males and females was constantly higher in the transplantable hepatoma. The studies done by Polish authors [7, 8] prove the influence of the transplantable neoplasm on liver porphyrinogenesis. They observed that 24 hours after introducing i. p. in Syrian hamsters pigmented melanoma homo-

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**Table 1. GGTP in serum and porphyrins in livers and**

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<th>Successive passage with division of rats into castrated (A) and those with intact gonads (B)</th>
<th>GGTP in serum expressed in ( \text{J} \cdot \mu \text{M/1000 ml/min} )</th>
<th>Uroporphyrin in ( \mu \text{g/g of tissue} )</th>
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Carcinogenesis induced by aflatoxin B₁, IV.

The tissues of PR was characterized by a significant disproportion among the fractions under investigation, mainly as a result of the elevated Uro level, while the T displayed a well maintained Uro, Copro, and Proto proportions. It may be
assumed then, the metabolic block of PP biosynthesis concern in PR an early, and in T an later step of porphyrinogenesis.

The influence of sex in animals carrying the T appeared in the different GGTP activity and in the content of porphyrins in livers and hepatomas. During 90 passages of hepatoma, a higher level of serum GGTP was observed in females up to the 40th transplant, and in further passages it was higher in males. Probably in 1—40 passages the enhanced values of GGTP in serum was influenced by estrogens, as described [2], during hormonally conditioning growth of T. This assumption is supported by the lower GGTP activity in serum in castrated females and a raised level in castrated males in comparison to intact rats bearing T.

A similar relation in castrated and unastrated animals concerned porphyrins in the liver and T of females and in T in males. ALBERT [1] observed a decrease of serum GGTP activity in females Buffalo rats with the transplantable Morris hepatoma 5123D after administration of Sustanon containing 4 testosterone esters. Further passages of AFB<sub>1</sub>-hepatoma most likely became independent of the host hormonal stimulation and the GGTP level reached values even higher in males than in females.

The tumor weight in passages 40—90, which was greater in females, did not correlate with the level of serum GGTP despite the fact that such a correlation has been established histochemically in primary AFB<sub>1</sub>-hepatomas [9] and biochemically in primary AFB<sub>1</sub> hepatocarcinogenesis in serum, liver, and hepatomas [17]. Ultra-structural studies of the AFB<sub>1</sub>-hepatoma from the 66th passage [18] did not show differences associated with rat sex. Two types of cells were seen in animals of both sexes: well differentiated and undifferentiated neoplastic hepatocytes with the presence of virus particles type C and mycoplasmas.

In accordance with accessible literature, the properties of transplantable AFB<sub>1</sub>-hepatomas have not been studied, especially in the aspect of porphyrinogenesis and GGTP activity in the carriers. The correlation between the porphyrin level in the liver and tumor and the activity of serum GGTP allows the suspicion to arise of the eventual interdependence of the metabolism of this enzyme and PP; such a suggestion is also supported by the results of primary AFB<sub>1</sub> carcinogenesis studies [17]. The increased hepatic porphyrin content in animals bearing transplantable AFB<sub>1</sub>-hepatoma may attest to influence of the transplantable tumor on the organ from which it originally arose.

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References

CARCINOGENESIS INDUCED BY ALFATOXIN B₁. IV.


