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THE EFFECT OF FENARIMOL ON MARKER ENZYMES IN RAT LIVER IN TWO-STAGES MODEL OF HEPATOCARCINOGENESIS

WPŁYW FENARIMOLU NA ENZYMY WSKAŹNIKOWE W WĄTROBIE SZCZURA W DWUSTOPNIOWYM MODELU HEPATOKANCEROGENEZY

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The effect of fenarimol on the early markers of hepatocarcinogenesis in the two-stage experimental model (initiation/promotion) was studied on the rats which were subjected to partial hepatectomy and received single dose of DEN as an initiating agent, prior to exposure to fenarimol.

INTRODUCTION

Chemically induced liver carcinogenesis is characterized morphologically by the sequential development of phenotypically altered hepatic foci (AHF), hyperplastic nodules and, eventually hepatocarcinomas [4, 6, 7]. Treatment with an initiating carcinogen such as N,N-diethylnitrosoamine (DEN) during a period of rapid growth of young and/or partially hepatectomized (PH) animals may result in the formation of AHF characterized by specific markers. This process may be accomplished by the addition of one of several different promoters [4, 7, 9]. These include many of hepatic cytochrome P-450 inducers of phenobarbital-type (P-4502B) [4, 7, 21]. Fenarimol (alpha-(2-chlorophenyl)-alpha-(4-chlorophenyl)-5-pyrimidimethanol), CAS Reg. No 60168–88–9 widely used as fungicide in crop protection, belongs to the group of cytochrome P-4502B inducers [14]. Our previous studies [18] have shown that fenarimol produces rat liver growth by hyperplasia of the hepatocytes, occurring during the first days of treatment, followed by cellular hypertrophy. Our results have suggested that the hepatomitogenic effect of fenarimol was at least partly related to regenerative hyperplasia. According to the literature data the compensatory proliferation appear to support the initiation [7, 16] and perhaps promotion stages in hepatocarcinogenesis [4].

In this light the studies were designed to asses whether the exposure to fenarimol as a potential promoting agent would cause changes in the activity of enzymes regarded as markers [3, 11, 13, 19, 25] of early stages of hepatocarcinogenesis in the liver of rats following the exposure to DEN and partial hepatectomy as initiation agents. These enzymes include hepatic *gamma*-glutamyltransferase (GTPase), glucose-6-phosphatase (G-6-Pase) as well as alkaline phosphatase (APase).

MATERIALS AND METHODS

Fenarimol (98%) was obtained from Dow Elanco Company.

2-acetylaminofluorene (2-AAF) obtained from Sigma was used as a positive control.

Animals-treatment

Female *Wistar* rats of own breed were used. Prior to use, the rats were housed at controlled temperature of 22 ±1°C and relative humidity of 50 ±10% with a 12 h light/dark cycle. Rats were administered a commercial diet (free from chlorinated hydrocarbons as detected by GLC) and filtered tap water *ad libitum*. When the body weight of rats attained 120 g they were divided into ten groups of five animals each. The experimental protocol is given in Table I and Figure 1.

Table I. Experimental protocol
Schemat doświadczenia

Group of rats	Initiation treatment	Promotion treatment
1	PH + DEN[1]	commercial diet
1a	–	commercial diet
1b	PH	commercial diet
1c	DEN	commercial diet
2	PH + DEN	commercial diet + fenarimol[2]
2a	PH	commercial diet + fenarimol
2b	–	commercial diet + fenarimol
3	PH + DEN	commercial diet + 2-AAF[3]
3a	PH	commercial diet + 2-AAF
3b	–	commercial diet + 2-AAF

PH – partial hepatectomy

[1] PH was performed 24h before DEN administration at the dose 50 mg/kg b.w.

[2] Fenarimol was administered at the dose 250 mg/kg b.w. for 26 weeks

[3] 2-AAF was administered at the dose 10 mg/kg b.w. for 26 weeks

The rats of group 1, 1b, 2, 2a, 3 and 3a were subjected to two-thirds hepatectomy (PH) under anesthesia. 24 h later groups 1, 1c, 2 and 3 were given i.p. injection of DEN in saline solution (50 mg x kg bw^{-1}). 24 h after DEN administration groups 2, 2a, 2b received fenarimol at the daily doses of 250 mg x kg bw^{-1} and groups 3, 3a and 3b received 2-AAF (positive control) in the daily doses of 10 mg x kg bw^{-1}, for 26 weeks.

Fenarimol and 2-AAF were administered orally in an olive oil suspension at 24 h intervals between 8–9 a.m. The respective groups of animals were administered only saline or olive oil vehicle. During the experiments, rats in all groups were given basal diet and tap water *ad libitum*. The animals were observed daily, and body weight and feed consumption were recorded. After 26-weeks of experiment the animals of all groups were sacrificed by decapitation. The livers were immediately excised, rinsed with distilled water, blotted dry and weighed. The representative samples of liver tissue from the left, right, and medial lobe were taken for biochemical, histochemical and histological analysis.

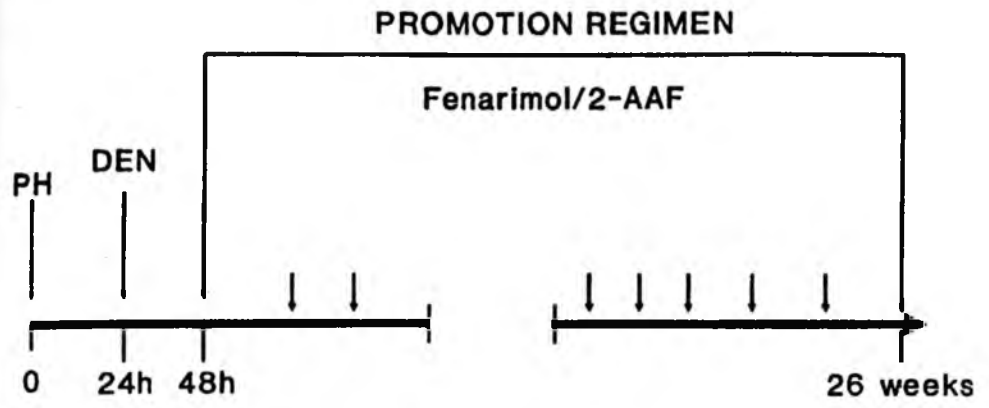


Fig. 1. Experimental protocol: initiation/promotion regimen
PH – two-thirds hepatectomy
DEN – single i.p. injection (50 mg/kg bw)
Promotion regimen: fenarimol – 250 mg/kg bw x day^{-1} or 2-AAF 10 mg/kg bw x day^{-1}.

Biochemical determination

1 g samples of the liver tissue suspended in sucrose solution and homogenized in a *Potter-Elvehjem* homogenizer. Homogenates were centrifuged at 3400 rpm for 10 minutes at 4°C. The postmitochondrial supernatant was used for the determination of marker enzymes. GGTase activity was measured with *gamma*-glutamyl-*alpha*-naphtylamide as a substrate according to the method of *Orłowski et al.* [17] using the enzymatic kit produced by Biomed (Poland). Activity was expressed as nmoles of *alpha*-naphtylamine/g liver x min. G-6-Pase activity was determined by the method of *Harper* [12], and expressed as μM of phosphate/g of liver x min^{-1}. APase activity was measured by the method of *Bassey* et al. [2] and was expressed in *Bassey's* units (mM/g x h^{-1}).

GGTase histochemistry

The GGTase was determined by the method described by *Albert et al.*[1]. Slices of the liver were cut in cryostat (10 nm), air dried, fixed in ice-cold acetone for 30 minutes and incubated in freshly prepared medium containing *gamma*-glutamyl-methoxy-2-naphtylamide as a substrate and Fast Blue BB Salt as coupling agent. Separate sections were stored at 4°C and retained their enzymatic activity unchanged for at least 6 months.

For statistic analysis of the results the two-tailed *Student's* test was used.

RESULTS

The increase in body weight was similar in all groups of tested animals. The relative liver weight (RLW = liver weight/body weight x 100) increased significantly in groups of rats received 2-AAF and in a greater degree after administration of fenarimol for 26 weeks (Fig. 2).

With 2-AAF plus PH and DEN (group 3) there was 1.5-fold ($P< 0.001$) increase in RLW (group 3 versus group 1). 2-AAF alone (group 3b) and 2-AAF plus PH (group 3a) increased RLW, there was a 1.2-fold ($P< 0.001$) and 1.3-fold ($P< 0.001$) increase, as compared with group 1a and 1b, respectively. In fully treated animals i.e. after

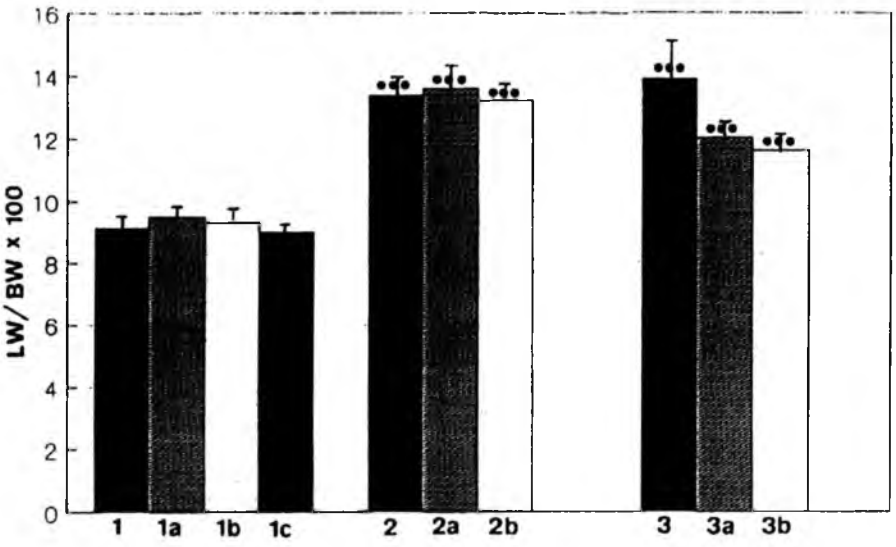


Fig. 2. Relative liver weight (RLW) in control and experimental rats treated with fenarimol or 2-AAF in combination with PH + DEN (groups 2 and 3), PH + fenarimol (group 2a), PH + 2-AAF (group 3a) and with fenarimol (group 2a) or 2-AAF (group 3b) alone. Data are presented as mean ±SEM. Significant difference from control: *** ($p<0.001$).

treatment with PH plus DEN plus fenarimol (group 2) there was 1.5-fold ($P< 0.001$) increase in RLW as compared to group 1. After treatment of rats with fenarimol alone (group 2b) and PH plus fenarimol (group 2a) there was 1.4-fold ($P< 0.002$) and 1.5-fold ($P< 0.001$) increase in RLW as compared to group 1a and 1b, respectively (Fig. 2).

In biochemical investigation it was found that G-6-Pase activity did not show characteristic changes in almost none of rats during the experimental period (26 weeks). Only after treatment of animals with fenarinol (group 2b) and 2-AAF (group 3b) a significant decrease in G-6-Pase activity was noted. However, there was an increase in APase activity. In complete initiation/promotion regimen (group 2 and 3) there was 1.2-fold ($p<0.05$) and 1.3-fold ($P<0.05$) increase in APase activity respectively, as compared with group 1. Administration of fenarimol (group 2b) or 2-AAF alone (group 3b) stimulated significantly APase, being a 2.4-fold ($P<0.01$) and 2.5-fold ($P<0.01$) respectively, as compared to group 1a. After treatment of animals with PH plus fenarimol (group 2a) there was a 1.7-fold ($P<0.001$) increase in APase activity as compared to group 1b. PH plus 2-AAF (group 3a) did not affect the enzyme (Fig. 3).

Initiation alone i.e. treatment of rats with non-necrogenic dose of DEN (group 1c), treatment with DEN plus PH (group 1) as well as after PH alone (group 1b) did not affect the hepatic GGTase in biochemical assay. After 26 weeks there was no differences between rats receiving a commercial diet (group 1a) and animals treated with PH plus DEN (group 1) or DEN alone (group 1c). Although in complete initiation/promotion regimen both fenarimol (group 2) as well as 2-AAF (group 3) stimulated hepatic GGTase activity, there was a 9-fold ($P<0.05$) and 12-fold ($P<0.005$) increase,

respectively, as compared to group 1. Administration of fenarimol alone (group 2b) of treatment of animals with PH plus fenarimol (group 2a) induced significantly increase in GGTase activity, being 16-fold ($P<0.001$) and 13-fold ($P<0.001$), respectively, as compared to groups 1a and 1b. In contrary, treatment of rats with PH plus 2-AAF (group 3a) or with 2-AAF alone (group 3b) did not cause significant increase in hepatic GGTase (group 3a versus group 1b, and group 3b versus group 1a) (Fig. 3).

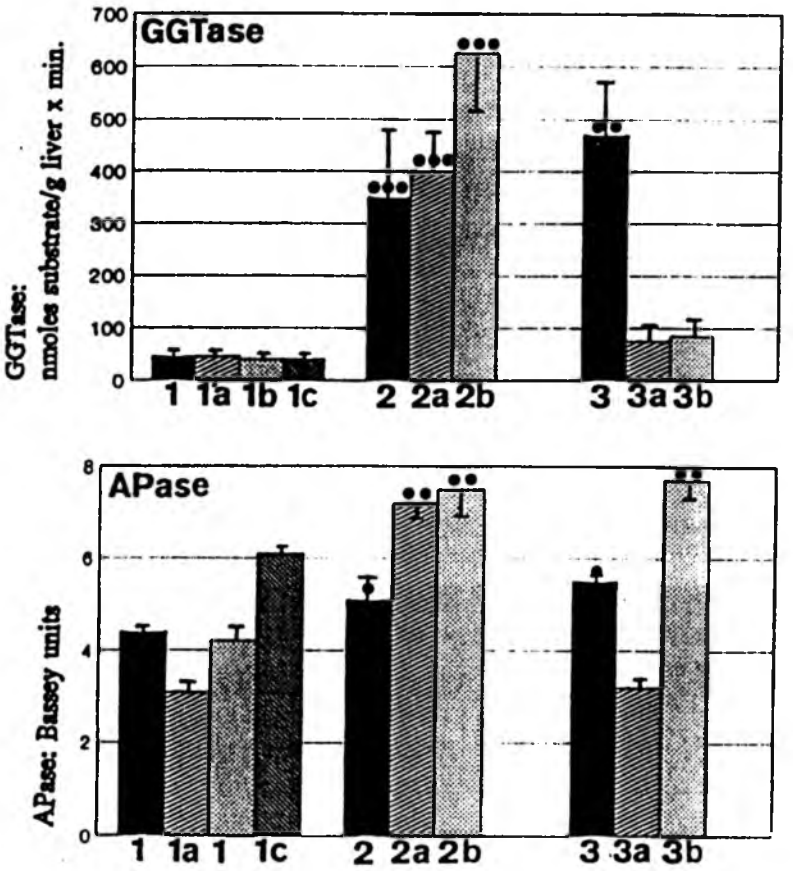


Fig. 3. Activity of γ-glutamyltransferase (GGTase) and alkaline phosphatase (AP) in liver of control and experimental rats treated with fenarimol or 2-AAF in combination with PH + DEN (groups 2 and 3), PH + fenarimol (group 2a), PH + 2-AAF (group 3a), and with fenarimol (group 2b) or 2-AAF (group 3b) alone. Significant differences: *($p<0.05$), **($p<0.01$), ***($p<0.001$).

Biochemical determination of GGTase activity was confirmed by histochemical analysis. Histochemical picture (Fig. 4 A,B,C and D) showed an occurrence of areas

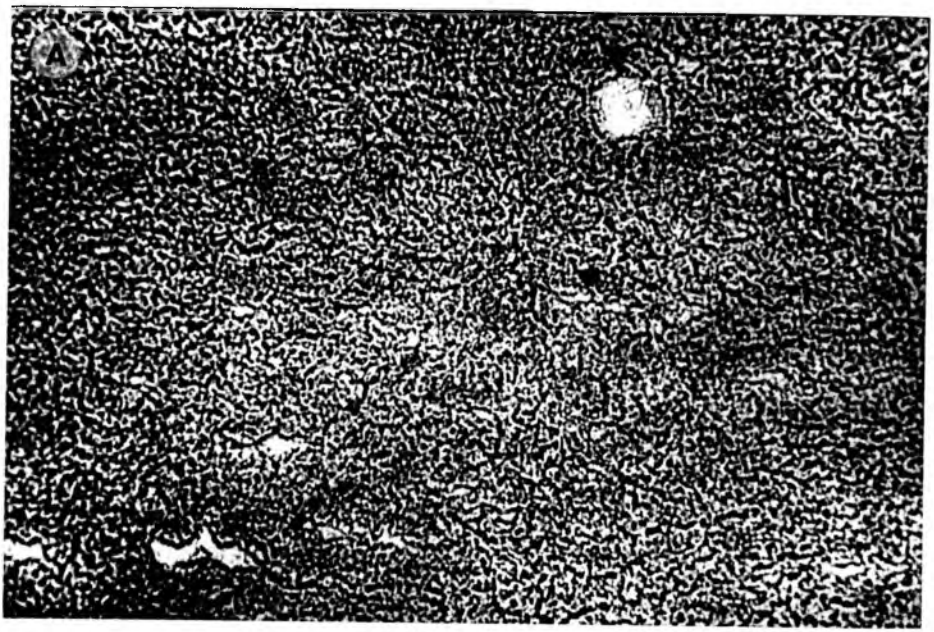


Fig. 4a.

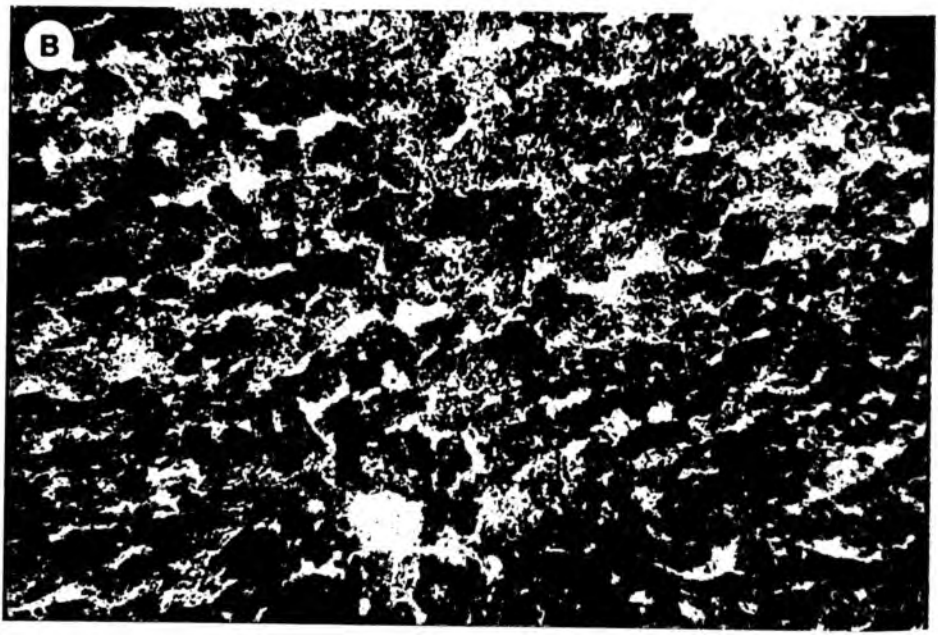


Fig. 4b.

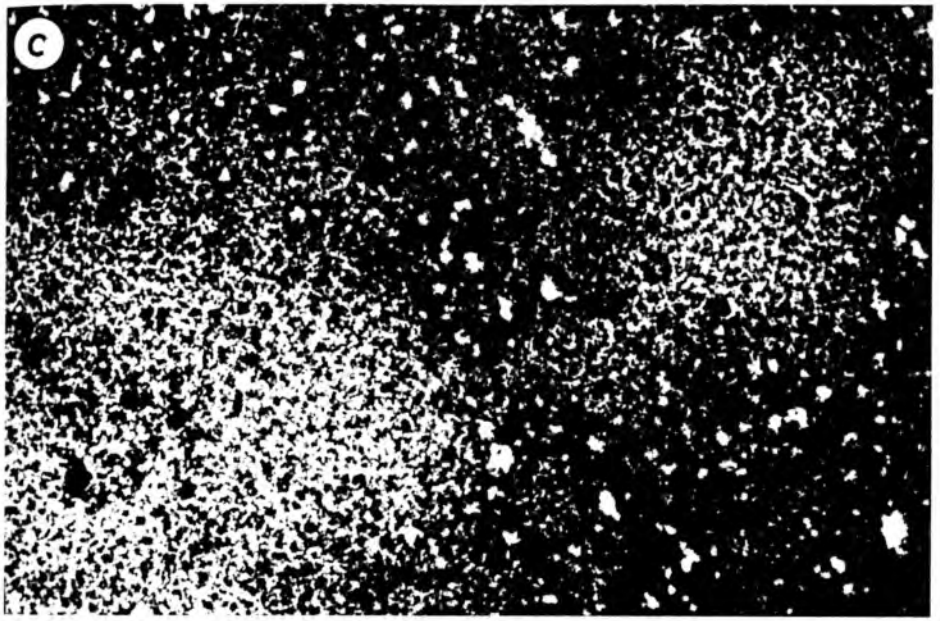


Fig. 4c.

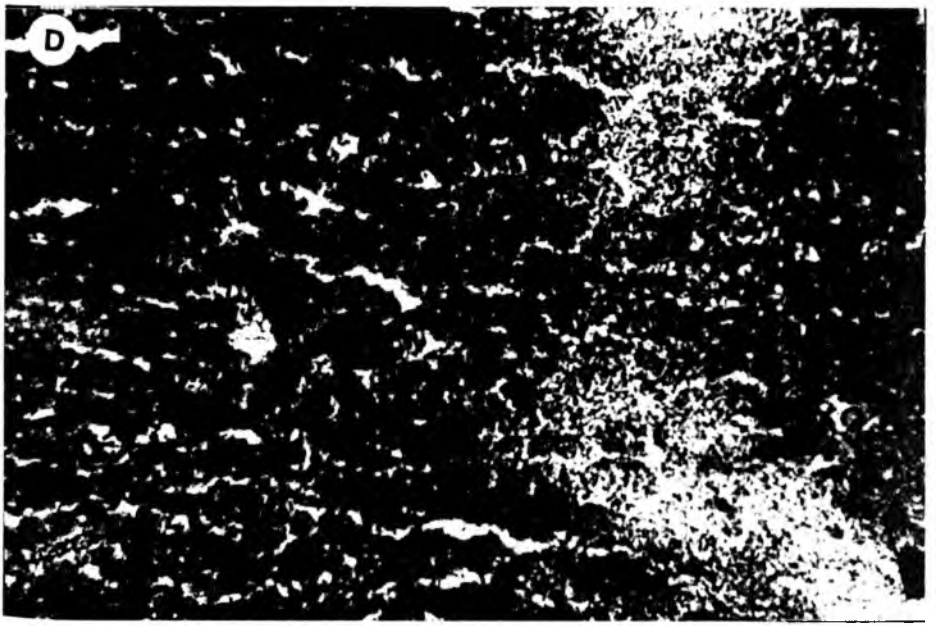


Fig. 4d.

Fig. 4. Histochemical localization of GGTase-positive hepatocytes in liver of control and experimental rats treated with fenarimol in combination with PH + DEN (group 2), PH + fenarimol (group 2a) and with fenarimol alone (group 2b).
A – control animals (group 1a); B – rats treated with PH + DEN + fenarimol (group 2); C – rats treated with PH + fenarimol (group 2a) and, D – rats treated with fenarimol alone (group 2b).

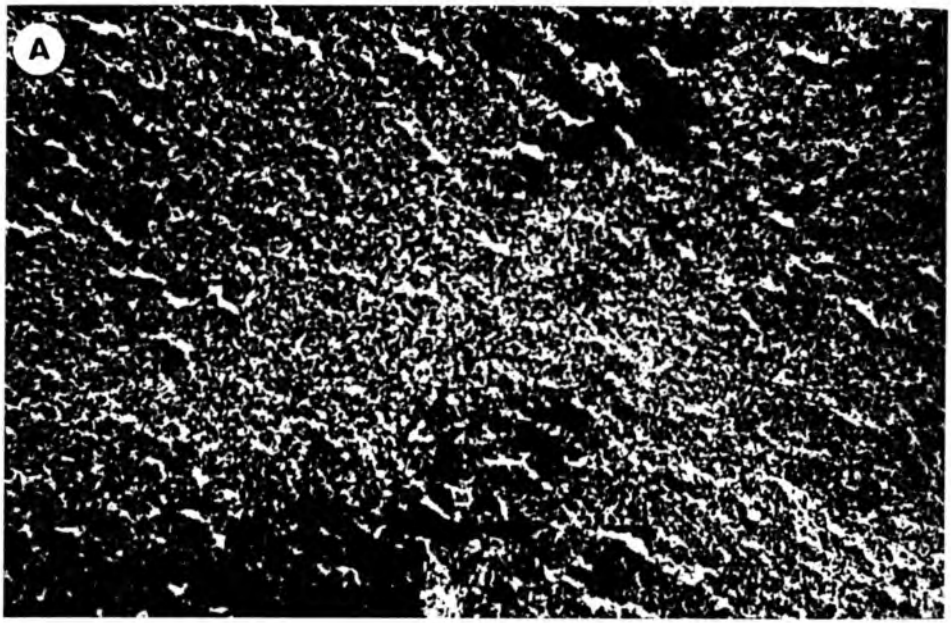


Fig. 5a.

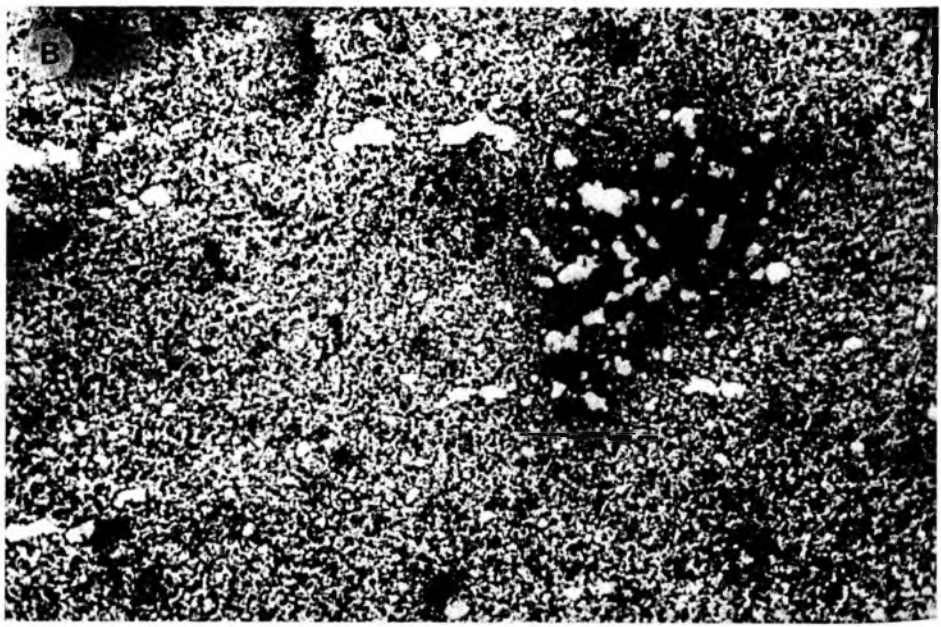
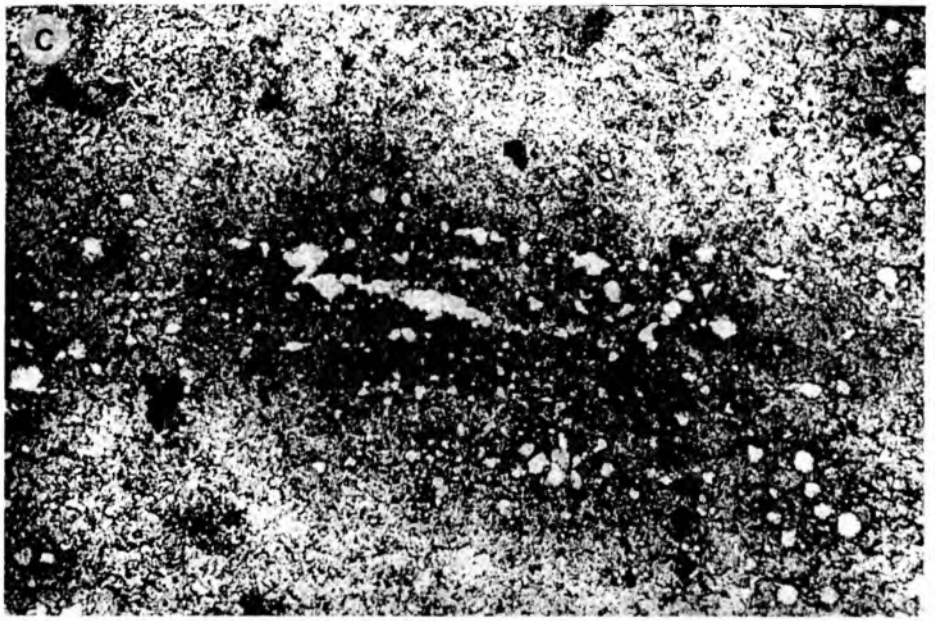


Fig. 5b.



Ryc. 5c.

Fig. 5. Histochemical localization of GGT-positive hepatocytes in liver of experimental rats treated with PH + DEN + 2-AAF (group 3), PH + 2-AAF (group 3a) and with 2-AAF alone (group 3b).
A – rats treated with PH + DEN + 2-AAF (group 3), B – rats treated with PH + 2-AAF (group 3a) and C – rats treated with 2-AAF alone (group 3b).

of GGT-positive hepatocytes in the liver of animals receiving fenarimol alone (group 2b), fenarimol plus PH (group 2a) and fenarimol plus PH plus DEN (group 2) as compared to control (group 1a). The strongest response was observed in the case of fenarimol treated animals (group 2b and 2a). In contrary to fenarimol, in the livers of 2-AAF treated animals the areas of GGPase-positive hepatocytes were best marked in fully carcinogen treated rats i.e. PH plus DEN plus 2-AAF (group 3). In the remaining groups these changes were less marked (Fig. 5 A, B and C).

DISCUSSION

The promoting potency of fenarimol appears to be of particular interest, since as stated in the introduction this fungicide (structural analogue of DDT) belongs to the group of cytochrome P-4502B inducers, many of which have been demonstrated to be non-genotoxic hepatocarcinogens [10] and effective liver tumor promoters [4, 7, 9, 21]. Moreover, in our earlier studies [18] we have found that hepatomitogenic effect of fenarimol, simulated regenerative hyperplasia. Therefore, the *in vivo* assay model on female rats, used in the present experiment included administration of fenarimol immediately after initiation with non-necrogenic dose of DEN and PH followed by chemical administration. As a positive control 2-acetylaminofluorene (2-AAF) was used, which in sub-carcinogenic doses is one of the most effective promoters of liver carcinogenesis [9, 23, 24].

The increase in GGTase activity in the liver is regarded as dominant marker during the early stages of carcinogenesis [3, 4, 13, 19]. Therefore, it seems safe to conclude that 2-AAF could be regarded as a promoter of rat liver carcinogenesis under experimental conditions applied in our study. Evidence for this fact was adduced by a significant increase in hepatic GGTase activity as well as GGT-positive hepatocytes against the negative background in animals treated with initiating agents (PH, DEN) and promoter (2-AAF) as compared to animals treated with 2-AAF plus PH and 2-AAF alone. Promotion by 2-AAF does not seem to involve selective cytotoxicity, since neither 2-AAF alone nor 2-AAF plus DEN have any inhibitory effect on post-regenerative liver growth. The hepatectomized liver showed significant weight increase, regardless of whether 2-AAF was given alone or after DEN pretreatment. Unexpectedly, the hisptopathological analysis performed in the Immunology Department of the National Institute of Hygiene did not show occurrence of hyperplastic nodules nor hepatocellular carcinomas at the end of the experiment. In contrary, according to the literature data, in pretreated with DEN *Wistar* rats, 2-AAF promoted the early (6-weeks) appearance of phenotypically altered (GGTase-positive) cells, after 2–4 months appearance of nodules and after 4–8 months hepatocarcinomas were observed [23].

It was found that fenarimol likewise 2-AAF failed to suppress of post-regenerative liver growth. There was even a significant increase in RLW in all groups of animals receiving fenarimol, which induced a typical growth by cellular hypertrophy [18].

In these studies we also report that in contrary to 2-AAF, fenarimol stimulated hepatic GGTase activity as well as the development of GGT-positive hepatocytes, regardless of whether it was given alone or after PH and DEN pretreatment. Thus, the interpretation of the elevation in GGTase observed in all groups of rats received fenarimol (both by biochemical and histochemical findings) is difficult, but the results should be carefully related to the type of cellular response. One possibility is that the treatment with fenarimol, likewise observed by *Rooni et al.* with phenobarbital [15] in the absence of an initiator, may cause the development of foci of altered cells of similar properties to the foci which occur after treatment with inducing agents. However, in this case the possibility of elimination of initiated preneoplastic hepatocytes by the apoptosis should be also regarded [22]. On the other hand, more probably conclusion that could be drawn from our experiments is that the over expression of GGTase might be associated with an adaptative response of the liver [13]. This could be confirmed by finding of others who observed several-fold increase in GGTase activity induced by phenobarbital and other cytochrome P-4502B inducers in both *in vivo* and *in vitro* conditions [5, 20].

It should be noted that under the conditions of our experiments, hepatic G-6-Pase did not show characteristic changes in almost all groups of animals throughout the observation period. The increase in APase activity was moderate and correlated in most cases with GGTase activity in livers.

In summary, the present results suggest that fenarimol did not affect the rat hepatocarcinogenesis induced by PH and DEN, if it was given to rats at the same time as genotoxic carcinogen. These results are in agreement with the earlier observations by *Flodström et al.* [8], who showed that fenarimol (1000 mg x kg^{-1} in the diet) had no

promoting potency in the livers of rats exposed to this compound for 13 weeks subsequent to DEN pretreatment.

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Summary

The two-stage model for the development of early markers of hepatocarcinogenesis was applied to assess the potential of fungicide fenarimol (α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pirimidinemethanol) as a possible promoter in this process. In this experiment the rats were subjected to partial hepatectomy (to induce proliferation), followed by the single (50 mg/kg bw) dose of diethylnitrosoamine (DEN) (initiator) and then, followed by the 26 weeks exposure to fenarimol administered in the olive oil suspension (250 mg/kg daily). The activities of *gamma*-glutamyltransferase (GGTase), glucose-6-phosphatase (G-6-Pase) and alkaline phosphatase (APase) regarded as markers of early stages of hepatocarcinogenesis were measured biochemically and histochemically in the livers of exposed rats as well as in the respective positive and negative controls. Rats exposed to 2-acetylaminofluorene (2-AAF), instead of fenarimol, served as positive controls. It was found that in the full initiation/promotion regimen both 2-AAF and fenarimol induced the increase of GGTase activity in the liver and formation of GGTase-positive hepatocytes. However the exposure to fenarimol alone also increased GGTase activity, although this response was not observed in rats exposed to 2-AAF alone. The possible mechanisms and explanation for such types of responses were discussed, and conclusion has been drawn that fenarimol did not affect the rat hepatocarcinogenesis induced by PH and DEN.

D. Palut, J. K. Ludwicki, B. Wiadrowska

WPŁYW FENARIMOLU NA ENZYMY WSKAŹNIKOWE W WĄTROBIE W DWUSTOPNIOWYM MODELU HEPATOKANCEROGENEZY

Streszczenie

Zbadano wpływ fenarimolu na enzymy wskaźnikowe wczesnych zmian nowotworowych z zastosowaniem dwustopniowego modelu hepatokancerogenezy inicjowanej PH i DEN. Szczury, samce rasy *Wistar* poddane PH otrzymywały jednorazowo DEN (50 mg x kg mc.$^{-1}$), a następnie fenarimol (250 mg x kg mc^{-1}) przez 26 tygodni. Jako kontrolę pozytywną zastosowano 2-AAF (10 mg x kg mc^{-1}), który wg piśmiennictwa w stosowanej dawce oddziaływuje w procesie hepatokancerogenezy na etapie promocji. Aktywność enzymów wskaźnikowych, tj. GGTazy, G-6-Pazy i APazy oznaczano metodami biochemicznymi i histochemicznymi w wątrobie szczurów badanych w porównaniu z grupami zwierząt stanowiących kontrolę pozytywną i negatywną. Stwierdzono, że zarówno fenarimol, jak i 2-AAF wzmagały aktywność GGTazy jak również powstawanie „pozytywnych" wysypek tego enzymu. Jednakże w odróżnieniu od 2-AAF podawanie szczurom samego fenarimolu wywoływało wzrost aktywności GGTazy potwierdzony następnie badaniami histochemicznymi. Uzyskane wyniki sugerują, że w opisanych warunkach doświadczalnych fenarimol w odróżnieniu od 2-AAF nie wzmagał na etapie promocji procesu hepatokancerogenezy inicjowanej u szczurów PH i DEN.

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