The Effect of Dietary 2-Acetylaminofluorene on the Uracil-degrading Enzymes in Rat Liver

P. FRITZSON AND J. EFSKIND
(Departments of Biochemistry and Pathology, Norsk Hydro's Institute for Cancer Research, Montebello, Oslo, Norway)

SUMMARY

The activities of the uracil-degrading liver enzymes, uracil reductase (EC 1.3.1.2), dihydrouracil hydrolase (EC 3.5.1.5), and carbamoyl-$\beta$-alanine hydrolase (EC 3.5.1.6) have been determined in rats fed 2 different basal diets supplemented with the carcinogen 2-acetylaminofluorene for various lengths of time. A fall in the activities of all 3 enzymes occurred in the preneoplastic liver during carcinogen feeding.

It was most pronounced in the case of uracil reductase, the enzyme which limits uracil breakdown in rat liver. The activities of uracil reductase and carbamoyl-$\beta$-alanine hydrolase reached the minimum level in about 30 days. The dihydrouracil hydrolase activity decreased still more slowly. The enzymes remained at the low activity level or increased slowly in activity during continued carcinogen feeding.

Removal of the carcinogen after 90 days resulted in almost complete recovery of the activities of all 3 enzymes.

With dietary supplements of 0.03 and 0.05% 2-acetylaminofluorene, the decrease in uracil reductase activity was proportional to the amount of carcinogen in the diet, the minimum level being about 50% of the control. The decrease in enzyme activity was the same with the 2 different basal diets, even though the rate of occurrence of malignant transformation was strikingly different with the 2 diets.

The results are discussed in relation to uracil incorporation into RNA, and to liver carcinogenesis. Possible factors responsible for the decrease in enzyme activity are considered.

Preneoplastic livers of rats fed the carcinogen 2-acetylaminofluorene for 90 days have a 10-fold increased capacity to incorporate uracil into the ribonucleic acids in vivo (2, 16). According to current views (1, 6), the incorporation is determined by the activity of the anabolic as well as the catabolic enzyme systems involved in uracil metabolism. The increased incorporation of uracil might thus be due, at least in part, to a decreased capacity of the liver to degrade the compound.

In order to shed light on the mechanism underlying the increased uracil utilization, it was of interest to study the activity of the uracil-degrading system in the precancerous liver. The present work is concerned with the effect of dietary 2-acetylaminofluorene on the activities of the 3 uracil-degrading enzymes, uracil reductase, dihydrouracil hydrolase, and carbamoyl-$\beta$-alanine hydrolase.

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Abbreviations used are: AAF, 2-acetylaminofluorene; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; DHU, dihydrouracil; CBA, carbamoyl-$\beta$-alanine; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form.

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**MATERIALS AND METHODS**

Female black-and-white rats from the Institute colony, about 60 days old and weighing about 150 gm, were used. The initial experiment was conducted with the stock diet commonly used in this laboratory for the maintenance of rats. The composition of the diet, termed Diet A in the present work, has been described previously (4). When the diet was supplemented with 0.03% AAF, malignant transformation or significant morphologic alterations did not occur. Accordingly, the content of AAF was increased to 0.05%, and we also introduced the basal diet (here termed Diet B) customarily used in the laboratory of Miller and Miller (11) for the induction of tumors with AAF. Both diets were obtained in powder form and were thoroughly mixed with the proper amount of AAF (Hoffman-La Roche). The special basal diet used by Rutman et al. (15) had a high content (26%) of corn oil, and for practical reasons we preferred to use a diet which could be obtained as a dry powder.

The experiments were started by placing groups of 12–30 rats on the particular AAF-containing diet. Control animals were fed ad libitum the identical diet without the carcinogen.

The diet was kindly produced for us by Felleskjøpet, Oslo, which also manufactures the ordinary stock diet.
signs of malignancy. Even the livers from rats killed at 434 days appeared normal apart from the presence of a few small cysts.

When the content of AAF was increased to 0.05%, histologic abnormalities occurred progressively during the experimental period. The rate of occurrence was strikingly increased when the basal Diet B was used in place of Diet A. But even with Diet B the livers were not significantly different from the control livers during the 1st month, apart from a slight inflammatory response at the end of the period. During the next 2 months, most of the parenchymal cells were somewhat enlarged, possibly due to hyperplasia. Small regeneration nodules were seen. But the proportion of the different cell types seemed still to be almost as in normal liver, although toward the end of the period signs of bile duct proliferation appeared. At later stages, cyst formation was a predominant phenomenon in the livers.

Malignant transformation occurred much earlier in rats on Diet B than in those on diet A. Thus, livers from rats killed after 188 days on Diet B containing 0.05 % AAF, showed large regeneration nodules and nodules of the hepatoma type, the latter containing in part atypical cells as the 1st sign of malignancy. Rats killed at 285—306 days were found to exhibit large liver tumors which, on microscopic examination, were hepatocarcinomas and

carcinogen. At intervals throughout the experimental period, 2—4 animals from each group were killed by decapitation, the livers were excised, placed in cold 0.25 M sucrose, and weighed. A small wedge of liver was removed for histologic examination. The remainder of the liver was washed with several portions of 0.25 M sucrose, coarsely minced with scissors and homogenized for 2 min in 1.5 volumes of 0.25 M sucrose, using a Teflon-glass homogenizer of the Potter-Elvehjem type. The homogenate was diluted with 0.25 M sucrose to give 1 gm of liver/3.3 ml of homogenate. The homogenate of the pooled livers was used for the assay of uracil reductase, dihydrouracil hydrolase and carbamoyl-β-alanine hydrolase, as described previously (4). Protein was determined according to the method of Lowry et al. (8), with aliquots of the homogenates, using crystalline bovine serum albumin as the reference standard.

RESULTS

Chart 1 shows the body weights and liver weights of rats fed Diet B with and without AAF. Figures in parentheses denote number of animals. The decreasing number of animals (upper curve) is due to the removal of rats for enzyme determinations. The points in the lower diagram represent individual livers, except the 0 time point which is the mean value of the livers from 14 animals fed the ordinary stock diet. —— O, 0.05% AAF in Diet B; O—O, Diet B without carcinogen; O—O, AAF removed from the diet after 90 days. For further details see “Materials and Methods.”

Microscopic Observations

The livers from rats fed 0.03% AAF in Diet A for 90 days showed no significant morphologic alterations or signs of malignancy. Even the livers from rats killed at 434 days appeared normal apart from the presence of a few small cysts.

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DHU hydrolase, and CBA hydrolase in the liver when rats were fed 0.03% AAF in Diet A for various periods of time. It can be seen that the uracil reductase activity decreased to about 75% of the control level, whereas DHU hydrolase and CBA hydrolase were only slightly affected. When the amount of AAF was increased to 0.05% (Chart 3), all 3 enzyme activities decreased considerably, particularly the activity of uracil reductase, the enzyme which limits uracil breakdown in rat liver (4). The decrease in this activity was apparently proportional to the amount of carcinogen in the diet. The minimum level was about 50% of the control. Chart 3 further shows that the fall in the enzyme activities was slow. The minimum levels of uracil reductase and CBA hydrolase were reached in about 30 days. The DHU hydrolase activity decreased still more slowly. The uracil reductase and CBA hydrolase activities approached normal levels during the continued carcinogen feeding. Apparently, a close similarity existed between the variation patterns of these 2 enzymes. It can also be seen that withdrawal of the carcinogen from the diet after 90 days led to the recovery of normal activities of all 3 enzymes. Chart 2 indicates that the time of recovery was about 30 days.

Chart 4 shows the activities of the enzymes when rats were fed Diet B containing 0.05% AAF. The change from Diet A to Diet B did not appreciably affect the control activities.

cholangiocarcinomas. In contrast, livers from rats killed after 288 days on Diet A containing 0.05% AAF, showed no signs of malignancy, although pronounced histologic abnormalities were present.

If the carcinogen was removed from the diets after 90 days, a reversal of the histologic changes occurred, as seen in the livers of rats killed about 1 month later. In spite of this fact, signs suggesting the development of malignancy were found at a later stage. Thus, 340 days after removal of the carcinogen from Diet A, the livers showed large regeneration nodules with histologic characteristics indicating that malignant hepatomas and cholangiomas would form.

The livers of control rats on the basal Diet B appeared normal apart from a very slight shrinkage of the cells.

ENZYME STUDIES

Chart 2 shows the activities of uracil reductase, DHU reductase, and CBA hydrolase in the liver when rats were fed 0.03% AAF in Diet A for various periods of time. It can be seen that the uracil reductase activity decreased to about 75% of the control level, whereas DHU hydrolase and CBA hydrolase were only slightly affected. When the amount of AAF was increased to 0.05% (Chart 3), all 3 enzyme activities decreased considerably, particularly the activity of uracil reductase, the enzyme which limits uracil breakdown in rat liver (4). The decrease in this activity was apparently proportional to the amount of carcinogen in the diet. The minimum level was about 50% of the control. Chart 3 further shows that the fall in the enzyme activities was slow. The minimum levels of uracil reductase and CBA hydrolase were reached in about 30 days. The DHU hydrolase activity decreased still more slowly. The uracil reductase and CBA hydrolase activities approached normal levels during the continued carcinogen feeding. Apparently, a close similarity existed between the variation patterns of these 2 enzymes. It can also be seen that withdrawal of the carcinogen from the diet after 90 days led to the recovery of normal activities of all 3 enzymes. Chart 2 indicates that the time of recovery was about 30 days.

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trol activities of uracil reductase and DHU hydrolase. The CBA hydrolase activity decreased by 20%. It can be seen that the enzyme activities in the presence of the carcinogen were not significantly different from those in Chart 3 when compared at corresponding times. This fact indicates that the effect of AAF on the enzymes was independent of the composition of the basal diet.

In Charts 3 and 4 is also recorded the total liver uracil reductase activity per 100 gm of body weight. This quantity is almost equal to the capacity of the animal in vivo to degrade uracil (4, 5). The finding that this quantity was at all times decreased relatively less than the activity per mg of protein reflects the fact that the ratio of liver weight to body weight increased during AAF feeding.

A few experiments were carried out to study the possible effect of AAF on the enzymes in vitro. Suspensions of 10⁻² m AAF were included in the standard incubation mixtures for assay of the enzymes and were preincubated for 15 min in the presence of normal liver homogenate. No significant effect on the enzyme activities could be determined. Furthermore, a mixture of equal amounts of liver homogenates from normal and AAF-treated rats (64 days with 0.05% AAF in Diet A) was incubated under conditions for uracil reductase assay. The fact that the activity was equal to the sum of the activities of the individual homogenates indicates that no inhibitor of the enzyme was present in the liver from AAF-fed rats.

**DISCUSSION**

The decreased activity of the uracil-degrading enzymes, particularly of uracil reductase, may bring about increased cellular concentration not only of uracil but also, through the mediation of uridine phosphorylase, of uridine. It has been shown by Canellakis (1) that the rate of incorporation of uracil and uridine into rat liver RNA is highly dependent on their concentrations. This phenomenon has been ascribed to the low affinity of uridine kinase for uridine (13). It is thus apparent that the increased incorporation of uracil into liver RNA of AAF-fed rats (2, 16) may be the result of the decreased activity of the uracil-degrading system in such livers. In this connection it is of importance to note that the actual decrease in the uracil-degrading activity might be greater than that found by measuring enzyme activity under optimal conditions, since the concentration of NADPH₃, the cofactor necessary for uracil reductase activity, may change from a nonlimiting to a limiting level during carcinogenesis (3). Determination of the level of uridine kinase in AAF-treated rats has not been reported, and it cannot at present be stated whether the increased ability to incorporate uracil is caused only by the decreased activity of the uracil-degrading system. In livers from rats fed carcinogenic azo dyes the level of uridine kinase is somewhat increased (14).

The feeding of AAF to rats does not generally lead to decreased enzyme activities in the liver. Trams et al. (17) found that the activity of glucuronol transferase, glutathione reductase, and uridinediphosphoglucose dehydrogenase increased significantly during AAF-feeding. It is thus evident that widely different variation patterns of enzyme activities exist, indicating that the levels of various enzymes are under specific influence of the carcinogen. The decrease in activity of the uracil-degrading enzymes, however, showed no correlation to the rate of occurrence of malignant transformation. This lack of a relation to neoplasia is consistent with findings in other laboratories that minimal deviation hepatomas (12) and primary liver tumors (14) retained normal uracil-degrading activity.

The mechanism underlying the decrease in activity of the uracil-degrading enzymes is at present obscure. But a few points related to observations in the present work may be discussed. The decreased enzyme levels apparently reflect actual enzyme variations in the cells, since the numerical proportion of cell types seemed to be unaltered for 90 days or more, as revealed by microscopic examination. Moreover, the studies in vitro showed that the presence of AAF as such did not influence uracil reductase activity, nor was there any evidence for the presence of an inhibitor of the enzyme in the livers from AAF-treated rats. The possibility exists, however, that the decreased activity of the enzymes is due to a reduced dietary intake, caused by the presence of AAF in the food. It was shown elsewhere (6) that starvation of normal rats for 4 days brought about a decrease in enzyme activity similar to that observed in rats fed 0.05% AAF for 90 days. The reduced gain in body weight of the AAF-treated rats (Chart 1) might indicate that conditions similar to starvation occur in these animals. On the other hand, the variations in body and liver weights were very different in starved and AAF-fed rats. Thus, during the short period of starvation the body weight of the rats decreased by 19% and the weight of the livers decreased by about 39% whereas a weight gain occurred in the AAF-fed rats, and excessive increase in the liver weight was observed. It is therefore doubtful whether the starved rats are comparable to those fed the carcinogen.

Other observations might suggest a more direct influence of AAF or its metabolites on the uracil-degrading enzymes. It was found that the variation patterns of carbamoyl-β-alanine hydrolase and uracil reductase were similar in both AAF-treated rats and regenerating rat liver (4). These observations may be indicative of a common site in the control system for the synthesis of these enzymes. Should this be so, AAF or its metabolites might have combined with this site. It is tempting to speculate, in terms of the hypothesis of genetic control of protein synthesis (7), that the carcinogen formed transitory linkages with the DNA of a regulator gene or an operator locus common to both enzymes. Evidence has recently been presented that AAF becomes bound to liver nucleic acids in vivo (9).

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