The Glycerophosphatases of the Rat Liver Cancer Produced by Feeding *p*-Dimethylaminoazobenzene

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The metabolism of the liver tumors produced in rats by the feeding of *p*-dimethylaminoazobenzene has recently been studied extensively. In particular it has been shown that the diphosphopyridine nucleotide content of the livers of rats fed this compound is greatly decreased, and that the metabolites of the dye are toxic to fermenting systems in which diphosphopyridine nucleotide is the limiting factor (5–7). In the course of another investigation (13, 14) the author found that the alkaline glycerophosphatase content of some types of human cancer is much greater than that of analogous normal tissues, and that the phosphatases of supposedly normal human livers are somewhat variable. It therefore seemed desirable to determine whether the glycerophosphatase activity of the livers of rats fed *p*-dimethylaminoazobenzene showed any changes that could be related to the other metabolic abnormalities known to be present. The relation of liver phosphatase to the storage of phosphorus was also studied, radioactive phosphorus being used as an indicator. After this work was nearly completed, Greenstein (2) reported a similar study of the phenylphosphatase of liver tumors. A comparison of the action of extracts of liver tumors on two different phosphate esters is thus made possible.

MATERIAL AND METHODS

The rats were young adult albino animals of both sexes, kindly supplied by Dr. K. Sugiura, of this hospital. They were fed a diet consisting of 20 cc. of a 3 per cent solution of *p*-dimethylaminoazobenzene in olive oil mixed with 1,000 gm. of brown rice, and supplemented with carrots. Controls were fed either on the same diet without the dye or on a stock diet of Purina chow and carrots or lettuce. No differences were observed between the two control groups.

After 36 to 272 days on the experimental diet the animals were killed by a blow and the livers were removed and inspected. If the liver was homogeneous, the entire organ was minced with scissors for phosphatase and radioactive phosphorus determinations. If the liver contained both cancerous areas and relatively normal tissue, the different portions were dissected and handled separately. Representative specimens were taken for histological examination before the remainder of the tissue was minced.

Macro-phosphatase determinations were made by the method described in detail elsewhere (14). Microphosphatase determinations were made at pH 4 to 5 and at pH 8.8 to 9.2 by a method based on those of Gomori (1) and of Kabat and Furth (4). Radioactive phosphorus was administered by the subcutaneous injection of a solution containing 5 μc. P^{32} and 0.15 to 0.30 mgm. $Na_2HP^{31}O_4$. The radioactivity of the ashed specimens was determined by methods previously employed in this hospital (9).[1] The total phosphorus of the ash was determined by a modification of the methods of Pregl and Roth (10) and of Scott (11).

TABLE I

Tissue	Number of determinations	Acid P-ase, units per gm. Average	Acid P-ase, units per gm. Range	Alkaline P-ase, units per gm. Average	Alkaline P-ase, units per gm. Range
Liver cancer	11	2.5	0.64–9.8	1.6	0.40–5.0
Precancerous liver	10	2.0	1.1 –3.0	0.39	0.11–0.85
Normal liver	8	1.7	0.45–3.0	0.16	0.10–0.28

RESULTS

The results of the macro-phosphatase determinations are summarized in Table I. In all the liver specimens of the first group there was definite evidence of cancer, but some consisted almost entirely of cancer while others contained cancer nodules mixed with varying amounts of abnormal but noncancerous liver. The specimens of the second group showed precancerous changes with large hyperchromatic nuclei and bile

[1] We are indebted to Dr. John H. Lawrence, of the University of California, for supplying us with the radioactive phosphorus used, and to Mr. L. D. Marinelli, of the Memorial Hospital, for making the radioactivity measurements.

duct adenomas. The specimens in the third group were normal appearing livers from normal animals. Only a few of them were examined histologically.

The average value for acid phosphatase in the liver cancers is a little higher than for the normal livers, but as there is a wide range within each group and considerable overlapping between groups the difference cannot be considered significant. In the alkaline phosphatase, however, significant differences do appear. The average value for the alkaline phosphatase of the liver cancers is 1.6 units per gm., or 10 times the average for the normal livers. The range of alkaline phosphatase readings on the liver cancers is wide, but the lowest is higher than the highest reading in the normal group. The alkaline phosphatase values of the precancerous group occupy an intermediate position, overlapping both the cancers and the normal livers.

These figures suggest that the increased production or retention of alkaline phosphatase began in the damaged livers before definite malignant changes appeared. Unfortunately, there is some uncertainty on this important point, since small cancer nodules may have been included in the large specimens taken for phosphatase determination, but not in the small ones selected for histological examination. In some specimens the appearance of precancerous changes detectable under the microscope is known to have preceded the rise in alkaline phosphatase. One of these is illustrated by Fig. 1, C.

The differences between the phosphatase activities of normal and pathological livers are shown graphically in Fig. 1. In this figure the phosphatase activities are plotted against the pH at which the determinations were made. Curve A shows the behavior of an extract of the liver of a normal rat. There is considerable acid phosphatase with a broad maximum of activity between pH 4.0 and 5.0 and a great diminution below pH 3.0. The alkaline phosphatase activity is low. Curves B and C show two portions of the liver of a rat that had been fed a *p*-dimethylaminoazobenzene diet for 272 days. Specimen B was almost entirely cancer; specimen C showed early precancerous changes. The curves for specimens A and C are very similar. The alkaline phosphatase activity of the liver cancer shown in curve B is 7 to 8 times that of the other specimens, and has a sharp maximum at about pH 9.7.

When it became evident that the liver cancers contained large amounts of alkaline phosphatase, an attempt was made to discover which cellular elements produced this enzyme. Micro-phosphatase determinations were accordingly made on some of the specimens. Dr. F. W. Stewart, of this hospital, kindly reviewed the slides and reported that acid phosphatase was present in the nuclei and in the cytoplasm of the tumor cells and in the nuclei of the connective tissue cells. Alkaline phosphatase was present in the intercellular spaces of many specimens, though this probably did not represent tissue phosphatase but rather the phosphatase activity of small amounts of bile included in the preparation. Alkaline phosphatase was not demonstrated in any of the normal liver cells, as was to be expected from the very low alkaline phosphatase readings on extracts of normal liver. In the liver cancers, alkaline phosphatase was present mainly in the endothelial cells of the sinuses, but could also be seen in the nuclei and possibly also in the cytoplasm of the tumor cells.

As the function of the alkaline phosphatase in the metabolism of liver cancer is unknown, it seemed desirable to determine whether this enzyme promoted the storage of phosphorus. This was done by making subcutaneous injections of a solution of radioactive phosphorus into normal and *p*-dimethylaminoazobenzene-fed rats and measuring the radioactivity of their livers at various times thereafter. It is known from the work of numerous investigators that when radioactive phosphorus is administered to animals the isotope is taken up very rapidly by the liver, but that a considerable portion is lost rather promptly. The initial rapid uptake is probably due to the incorporation of the radioactive phosphorus in various phosphorus-containing compounds, especially phospholipids, which are elaborated in the liver. These are not stored permanently at the site of formation, but are transported to other parts of the body. The smaller amounts of radioactive phosphorus remaining in the liver after the initial rise and drop probably represent true intracellular phosphorus. In order to study both types of compounds, some of the animals were killed 48 hours after injection, and the remainder 96 hours after injection. The average differential absorption ratios at 48 hours were 1.21 to 1.24, and at 96 hours were 0.81 to 0.96. There were no significant differences between the normal livers, the livers that had undergone precancerous changes, and the liver cancers. Only three animals were included in each group, as it was evident early in the work that large differences between the groups would not be observed.

For comparison with the livers, radioactive phosphorus determinations were also made on the kidneys, thigh muscles, and femurs of the same animals. The total phosphorus (P^{31}) content of the ash of the specimens was also measured. Results for the total phosphorus of liver, kidney, and muscle, and for the radioactive phosphorus of kidney and muscle were rather variable, so that it was not apparent whether there were significant differences between the normal and the dye-fed animals. There was some evidence that

the total phosphorus content of the bones was less, and the uptake of radioactive phosphorus by the bones was slower, in the rats with damaged livers than in the normal animals. This may have been due to the poor nutritional state of the dye-fed animals, with diversion of the available phosphorus supplies from the bones to carbohydrate cycle that is inactivated by such split products. Dimethyl-*p*-phenylenediamine is a probable metabolic product of *p*-dimethylaminoazobenzene whose inhibiting effect on fermenting systems has been demonstrated clearly (6). This substance interferes with the analytical methods for phosphatase only

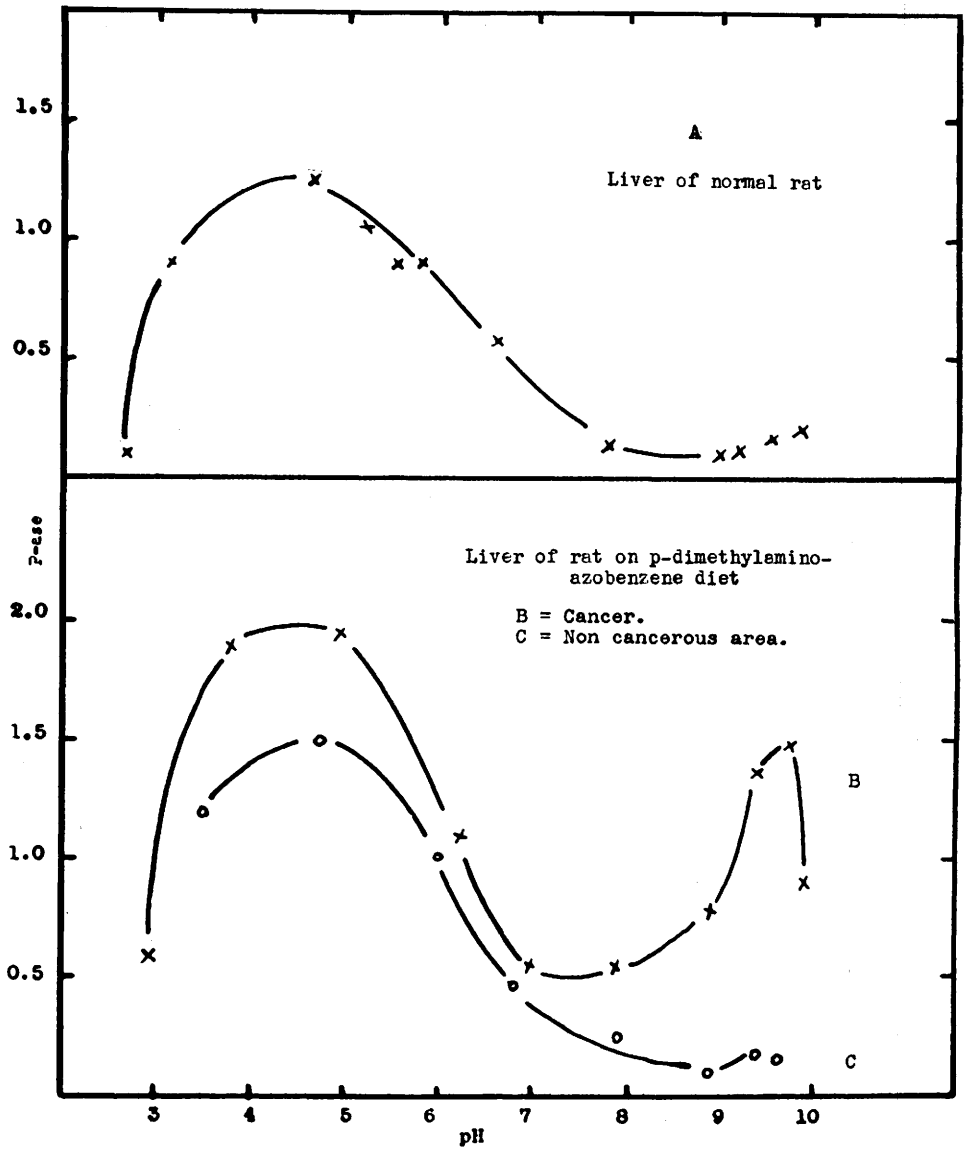

FIG. 1.—Phosphatase of extracts of normal and pathological rat livers. Tissue phosphatase in units per gram plotted against the pH at which the determinations were made.

other tissues where it is needed more urgently. Similar findings on the bones of tumor-bearing animals have been made by other investigators (8).

Alkaline glycerophosphatase thus is present in large quantities in liver cancers produced by feeding *p*-dimethylaminoazobenzene, but is not concerned in the storage of phosphorus in the tissue. Possibly this enzyme is substituted for some mechanism in the when it is kept long enough in alkaline solution to develop an intense purple color. Its effect on phosphatase preparations can therefore be determined readily, except when the alkaline phosphatase activity is so low as to require 2 hours or more of incubation. Accordingly, we studied the effect of $M/1{,}000$ dimethyl-*p*-phenylenediamine on the acid and alkaline β-glycerophosphatases of one normal rat liver and of two rat

liver cancers. For comparison we also studied its effect on the acid phosphatase of normal and hypertrophied prostate glands, and on the alkaline phosphatase of a low grade chondrosarcoma. The apparent effect of dimethyl-*p*-phenylenediamine ranged from 7 per cent inhibition to 15 per cent activation. Such differences are within the experimental error of the method for tissue phosphatase, which, under unfavorable conditions, may equal ±25 per cent. We may therefore conclude that dimethyl-*p*-phenylenediamine in a concentration of *M*/1,000 has no effect on the phosphatases examined.

DISCUSSION

The evidence presented above shows that the alkaline β-glycerophosphatase activity of rat liver cancers produced by the feeding of *p*-dimethylaminoazobenzene averages 10 times that of normal rat livers. The appearance of excess enzyme probably precedes that of definite malignant changes, but does not take place until degeneration is advanced. The enzyme is present in the nuclei of the tumor cells. It is not inactivated by a probable split product of *p*-dimethylaminoazobenzene that has a strong inhibitory effect on one phase of the carbohydrate cycle.

The demonstration of the presence of large amounts of alkaline glycerophosphatase in primary rat liver tumors induced by feeding *p*-dimethylaminoazobenzene is in harmony with the observations of Greenstein (2) on the phenylphosphatase of transplanted rat liver tumors of a strain originally induced by a diet that included *p*-dimethylaminoazobenzene. The enzyme is thus not specific for any one phosphate ester. The inability of our tumors to store more radioactive phosphorus than normal livers is not in harmony with the observation of White, Dalton, and Edwards (12) that rat hepatoma 31 frequently calcifies. It is possible that this characteristic of the tumor may become altered somewhat on transplantation.

This occurrence of much larger alkaline β-glycerophosphatase activities in cancer than in analogous normal tissue is not an isolated phenomenon. We have shown elsewhere (14) that the alkaline phosphatase activities of embryonal carcinoma of the testis and of myogenic sarcoma may be much higher than those of normal testis and normal muscle respectively. The high alkaline phosphatase activity of osteogenic sarcoma is well known, and is associated with the rapid storage of phosphorus. We have shown, however, that the alkaline phosphatase of osteogenic sarcoma is not nearly so effective per unit of activity in promoting the storage of phosphorus as is that of normal bone, and that the more malignant the tumor the less effective is the enzyme (13). Recently Gutman, Warrick, and Gutman (3) have reported that calcifying cartilage contains a mechanism for phosphorylative glycogenolysis, and that the ester so produced probably serves as the substrate for bone phosphatase. It is possible that in osteogenic sarcoma this mechanism is inactivated, and that excessive amounts of alkaline phosphatase are formed in an attempt at compensation. While comparisons between different tissues are not necessarily valid, it is permissible to speculate whether a similar situation may exist in liver cancer. Some mechanism which normally furnishes substrate for the small amounts of alkaline phosphatase in normal liver may become inactivated by the metabolic products of *p*-dimethylaminoazobenzene. Since the activity of the phosphatase itself is unaffected by these toxic products, the production of phosphatase may be increased as a compensatory mechanism.

SUMMARY

The alkaline β-glycerophosphatase activity of the liver cancer produced in rats by the feeding of *p*-dimethylaminoazobenzene averages 10 times that of normal rat liver.

Alkaline β-glycerophosphatase can be demonstrated in the endothelial cells of the sinuses of the liver cancers and in the nuclei of the tumor cells.

The storage of radioactive phosphorus in liver cancer is the same as that in normal liver 48 and 96 hours after injection of the isotope.

Neither the acid nor the alkaline β-glycerophosphatases of normal rat liver or rat liver cancer are inhibited by dimethyl-*p*-phenylenediamine, one of the probable metabolic products of *p*-dimethylaminoazobenzene.

The possible significance of these findings is discussed.

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