The Comparative Enzymology and Cell Origin of Rat Hepatomas

II. Glutamate Dehydrogenase, Choline Oxidase, and Glucose-6-phosphatase*

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SUMMARY

The activities of glucose-6-phosphatase, glutamate dehydrogenase, and choline oxidase were determined in some or all of ten rat hepatomas, including the Novikoff, Dunning L-C18, McCoy MDAB, and the Morris 3683, 392A, and 5123 hepatomas, together with primary hepatomas produced by feeding ethionine or 3'-methyl-4-dimethylaminoazobenzene, and transplanted hepatomas derived from the primary tumors induced with ethionine.

Of these neoplasms, only the Morris hepatoma 5123, the primary and transplanted ethionine-induced hepatomas, and one of the 3'-methyl-4-dimethylaminoazobenzene-induced tumors possessed significant glucose-6-phosphatase activity. These same tumors in addition to the Dunning L-C18 hepatoma had demonstrable glutamate dehydrogenase activity, whereas the other neoplasms tested failed to show significant activity of this enzyme. With the exception of the primary dye-induced neoplasm, which was not tested, only those neoplasms having significant glucose-6-phosphatase activities showed any choline oxidase activity.

Of those neoplasms tested for tryptophan peroxidase activity only the Morris hepatoma 5123, the primary ethionine-induced hepatoma, and some of the Dunning L-C18 hepatomas had any demonstrable activity of this enzyme. In contrast to most of the enzymatic activities reported here, the threonine dehydrase activity of the Morris hepatoma 5123 was of the order of 40 times the level of this enzyme in the livers of animals bearing this tumor.

The significance of these results in relation to the biochemical and morphologic classification of experimental hepatic carcinomas, as well as the importance of studying the biochemistry of slowly growing, highly differentiated experimental neoplasms such as the Morris hepatoma 5123, is discussed.

In the first paper of this series (24) the deoxy-cytidylic acid (dCMP) deaminase activity and the thymine degradative capacity of a number of primary and transplanted rat hepatomas were determined. Of ten hepatomas studied, all but two, the transplantable Dunning L-C18 hepatoma (7) and the transplantable Morris hepatoma 5123 (16), had demonstrable dCMP deaminase activity, but only one of the ten, the Morris hepatoma 5123, could degrade thymine. Normal rat liver had

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1 The following abbreviations are used in the text: dCMP, 5'-deoxy-cytidylic acid; G-6-P, glucose-6-phosphate; G-6-Pase, glucose-6-phosphatase; GDH, glutamate dehydrogenase; CO, choline oxidase; TPO, tryptophan peroxidase; TD, threonine dehydrase; DPN, diphosphopyridine nucleotide; TCA, trichloroacetic acid; 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene.
previously been shown to have no dCMP deaminase activity (15) but did possess the necessary enzyme system for the conversion of thymine $^{2}C_{14}$ to $C^{14}O_{2}$ (5). In view of these findings it was postulated that both the Dunning and the 5123 hepatomas probably arose from cells that closely resembled a hepatic parenchymal cell. On the other hand, those neoplasms having dCMP deaminase activity and no thymine degradative capacity may have been more closely related to biliary epithelium (21).

To gain further biochemical evidence in support of these postulates as well as to look for biochemical differences between the highly differentiated Morris hepatoma 5123 and rat liver, the activities of several other enzymes were determined in many of these hepatomas (24). The results of this survey comprise the subject of this paper.

MATERIALS AND METHODS

The neoplasms used in this study were the same as those employed in the previous paper (24). The tumor-bearing animals were stunned by a blow on the head and their necks immediately dislocated to obtain paralysis of the lower extremities. The tumor and the liver were quickly removed and placed in ice-cold 0.154 M KCl. The tumor tissue was rapidly dissected and any necrotic material removed as completely as possible; 1:4 homogenates of the tissues to be assayed were prepared in 0.154 M KCl homogenizer. The homogenizer was kept in an ice bath during this procedure to dissipate any frictional heat. Samples of each of the tissues to be homogenized were taken for histologic examination. The tissue was fixed for 16 hours in Lesher's fixative (12) and then placed in 70 per cent ethanol until embedded, sectioned, and stained with hematoxylin and eosin.

All enzyme assays described in this paper were performed with the whole homogenate.

Glucose-6-phosphatase.—The procedure for the assay of this enzyme was essentially that used by Cori and Cori (6); 0.1 and 0.2 ml. of the homogenate was added to a 13 X 100 mm. pyrex culture tube containing 0.3 ml. of 0.1 m potassium citrate buffer, pH 6.8, 0.04 ml. of 0.1 m potassium G-6-P, and sufficient water to make a final volume of 1.0 ml. For each tube containing G-6-P a duplicate tube without substrate was prepared. All tubes were incubated for 30 minutes at room temperature with occasional shaking. At the end of this time 1.0 ml. of 10 per cent TCA was added to each tube. After the precipitated protein had been centrifuged off, 0.5 ml. of the supernatant was used for phosphorus determination by the method of Lowry and Lopez (13). The difference in the inorganic phosphate content of the control tube with no substrate and that containing G-6-P was a measure of the G-6-P dephosphorylation by the enzyme.

Glutamate dehydrogenase.—This enzyme was assayed in the forward reaction with the use of DPN and L-glutamic acid by a slight modification of the procedure of Hogeboom and Schneider (10). The final volume of the components was 3.0 ml., and the pH of the assay was 7.5. The change in optical density at 340 m$\mu$ was read every 2 minutes with the use of a Beckman model DU spectrophotometer and quartz cuvettes. The rate was followed until maximal rate was reached. This maximal rate was used in all calculations.

Choline oxidase.—This enzyme was assayed manometrically by a slight modification of the procedure described by Richert and Westerfeld (26). The substrate was added directly to the reaction chamber in Warburg vessels having no side-arms. After a 5-minute equilibration period, the first reading was taken. Readings were then taken at 10, 30, and 50 minutes. The first 10-minute reading was not included in the calculations; 0.5 and 1.0 ml. of homogenate were assayed with all the tumors.

Tryptophan peroxidase.—This enzyme was assayed by the procedure of Knox and Auerbach (11).

Threonine dehydrase.—The method described by Sayre et al. (27) was used for the assay of this enzyme.

All enzyme assays were carried out within 2 hours of the death of the animal. In particular, best results were obtained with the choline oxidase assay when the homogenate was less than 1 hour old. Unless otherwise specified, all enzyme activities were expressed as $\mu$moles of product formed per hour per gram protein. Protein was determined by the method of Lowry et al. (14).

Dipotassium G-6-P and DPN were obtained from the Sigma Co. L-Threonine, L-tryptophan, and L-glutamic acid were obtained from the California Corporation. Choline chloride was a product of Nutritional Biochemicals Corporation.

RESULTS

Glucose-6-phosphatase.—In Chart 1 are shown the G-6-Pase activities of ten hepatomas expressed as $\mu$moles of phosphorus liberated as inorganic phosphate/gm protein/hr. Enzyme values which were less than 30 $\mu$moles/gm protein/hr were considered to be within the limits of accuracy of the method and of questionable significance. Therefore, by these standards, only the Morris
hepatoma 5123 and the primary and transplanted ethionine-induced hepatomas as well as one of the 3'-Me-DAB-induced tumors contained significant amounts of the enzyme. The histology of the dye-induced neoplasm which showed G-6-Pase activity resembled the primary ethionine-induced neoplasms and cytologically could be classified as a hepatoma of the large-celled variety (25). On the other hand, the other 3'-Me-DAB-induced tumor with little or no G-6-Pase activity was morphologically compatible with a cholangio-carcinoma. Although in the case of primary neoplasms it would be argued that any activity found might be due, at least in part, to contaminating liver, histologic sections of the tumors did not justify such a conclusion in these experiments.

It is evident from these data that, upon transplantation in animals, the ethionine-induced neoplasm lost some of its G-6-Pase activity. Simultaneously, there appeared to be some gain in deoxycytidylic acid deaminase activity upon transplantation of these neoplasms (24). This change was most marked after the tumor had been explanted to tissue culture, grown in vitro for several weeks, and then inoculated into a new host. The resulting neoplasm (H-1) showed almost no G-6-Pase activity but exhibited a high level of deoxycytidylic acid deaminase (24). Histologically, hepatoma H-1 showed a somewhat more uniform cytologic pattern than its parent primary tumor. The morphology and biochemistry of this neoplasm in vivo and in vitro will be discussed more extensively in a further publication.2 The absence of G-6-Pase activity in the Novikoff hepatoma has been previously reported (29).

Glutamate dehydrogenase.—The activities of this enzyme in the same series of hepatomas are seen in Chart 2. As with the G-6-Pase, the Morris hepatoma 5123, the ethionine-induced primary and transplanted hepatomas, and one hepatoma induced by 3'-Me-DAB consistently showed significant amounts of the enzyme. In addition, the Dunning hepatoma also has been found to possess GDH activity (20). The single 3'-Me-DAB-induced hepatoma with a high GDH activity was the same neoplasm that possessed a significant level of G-6-Pase. Two dye-induced hepatomas of the "small-cell" type (25) showed little if any GDH activity. There was no significant loss of

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2 H. E. Swim and H. C. Pitot, unpublished.
steps must be taken to make all the GDH of the cell available for assay. In view of this, the very low or negligible GDH activities of six of the hepatomas probably represent low activities of the enzyme. In particular, Novikoff (17) has reported the presence of GDH in acetone powder extracts of the Novikoff hepatoma; however, Allard et al. (1) were unable to show GDH activity in homogenates of the Novikoff hepatoma.

**Choline oxidase.**—Chart 3 shows the CO activity of seven of this series of hepatomas. Only the CO activity of the Morris hepatoma 5123 was of the same order as that found in liver. The primary and transplanted ethionine hepatomas exhibited about one-fifth the activity of liver. The presence of the enzyme in the Dunning hepatoma as well as its virtual absence in the Novikoff hepatoma has been previously reported (20). As with the other mitochondrial enzyme in this study, GDH, the CO activity did not appear to differ in the first generation transplanted compared with the primary hepatomas produced by ethionine feeding.

**Tryptophan peroxidase and threonine dehydrase.**—In Charts 4 and 5 are, respectively, the specific activities of the TPO activity of rat liver and four hepatomas and of the TD activity of the Morris hepatoma 5123 as compared with the activity of the livers in animals bearing hepatoma 5123. TPO activity was low in primary ethionine-induced hepatomas and in hepatoma 5123, and low as well as variable in the Dunning hepatoma. The presence of the enzyme in primary tumors induced by ethionine, as well as in the Dunning hepatoma, has been previously reported (18, 20). The absence of TPO activity in the Novikoff hepatoma has been reported by Auerbach and Waisman (2). Pitot *et al.* (20) have also shown that primary hepatomas, possessing a low TPO activity, as well as the Dunning hepatoma, do not have the capacity to increase their TPO in response to tryptophan administered in vivo or in slices incubated with tryptophan in vitro. This failure of the hepatoma to respond is not due to the presence or absence of an inhibitor or to the absence of formylase in the tumor cell (19). The capacity of the enzyme-forming system in the Morris hepatoma 5123 to synthesize new TPO in response to tryptophan in vivo and in vitro is presently under investigation in this laboratory.

In contrast to almost all the other enzymes...
discussed. The TD activity of the Morris hepatoma 5123 was about 40 times the activity of this enzyme in the livers of animals bearing this neoplasm. The TD activity of livers of normal Holtzman rats was in the neighborhood of 10–20 μmoles α-keto butyrate/hr/gm protein. Greenberg et al. (9) have previously shown that the total TD activity of the livers of rats bearing Walker carcinosarcoma 256 is essentially unchanged as compared with the livers of normal rats. Whether or not the high level of TD activity in the 5123 hepatoma is merely a unique feature of this tumor or reflects some basic mechanism of the neoplastic process is a matter which must await further study.

DISCUSSION

A comparison of the GDH, CO, and G-6-Pase activities with the dCMP deaminase and thymine degradative activities (24) of these hepatomas lends further support to the concept that experimental hepatic carcinomas in the rat can be divided into at least three distinct classes with regard to their enzymology. These classes have been designated D+T-, D-T-, and D-T+ (24).

The first type, D+T-, as exemplified by the Novikoff hepatoma and Morris hepatoma 3924A, has readily demonstrable amounts of dCMP deaminase but little or no G-6-Pase, GDH, CO, and the thymine degradative enzyme system. G-6-Pase, CO, and the capacity to degrade thymine are enzymatic properties which are found only in the liver and kidney of the adult rat and may thus be considered as markers of hepatic or renal origin. In contrast, dCMP deaminase does not occur in normal adult rat liver but probably does occur in proliferating bile duct cells or in some related epithelial cell of the liver (21). Thus, neoplasms of this first type are probably more closely related to biliary epithelial cells than to hepatic parenchymal cells. Histologically, these neoplasms are almost always composed of small basophilic cells corresponding to the "small cell" type of hepatoma (25).

The second type, D-T-, of which the Dunning hepatoma is the only clear-cut example in this series, has no dCMP deaminase or capacity to degrade thymine and little or no G-6-Pase activity, but does have GDH and CO activity. This neoplasm has at least one of the enzymes characteristic of normal liver and lacks the deaminase but it also lacks several of the other enzymes of normal liver.

The third type of hepatic carcinoma, D-T+, is uniquely demonstrated by the Morris hepatoma 5123. This neoplasm possesses demonstrable activity of all hepatic enzymes studied and lacks significant dCMP deaminase. Whether or not this classification will retain its validity with further investigations will be decided in the future. The D-T- and D-T+ hepatomas may be classified as "large cell" hepatomas (25).

There is a fourth biochemical type of hepatic carcinoma described in this paper. This is the ethionine-induced carcinoma of the liver which possesses both dCMP deaminase activity as well as CO, GDH, G-6-Pase, and TPO activities. The degradation of thymine was not studied in the primary neoplasms of this group; however, the transplanted tumors showed no thymine degradative activity (24) and a lowered G-6-Pase activity (Chart 1). The simplest explanation of these findings is that these neoplasms are mixtures of the D+T- and the D-T- type of hepatoma in our classification. Histologic studies of these tumors indicate that they are indeed composed of at least two distinct cell types comparable to the small and large cell hepatomas of Richardson et al. (25). When one of these tumors was stimulated to rapid growth by explantation to tissue culture and inoculated back into the animal, the small-cell hepatoma predominated and the enzymology progressed to a D+T- hepatoma. From these findings it would appear that, on continued transplantation, a tumor of mixed-cell type will eventually select out the most rapidly growing cell, of which the neoplasm will eventually be almost entirely composed. Such a phenomenon is certainly one form of the much discussed "dedifferentiation" of neoplasms.

However, when the primary neoplasm is of only one cell type, then subsequent transplants will result in neoplasms homogeneous with respect to cell type unless genetic mutations or deletions change the cell population. In the case of the Dunning hepatoma repeated transplantation since 1947 has not resulted in any major morphologic change in the tumor. After explantation to tissue culture and subsequent inoculation into the animal, the resulting tumor is biochemically and morphologically essentially the same as the original explant.

The Morris hepatoma 5123 is a very slowly growing neoplasm requiring several months to kill the host, whereas the Dunning hepatoma grows somewhat more rapidly, killing the host in about a month when implanted subcutaneously. However, both tumors grow more slowly than the Novikoff hepatoma, which when implanted in an animal can lead to the death of the host in 7–10 days. As emphasized previously (24), it is of interest that both...
the 5123 and Dunning tumors can be carried only in inbred strains (7, 16). A possible explanation of this is that slowly growing, highly differentiated neoplasms will be able to maintain their growth only when host resistance is at a minimum. Anaplastic, rapidly growing neoplasms such as the Novikoff hepatoma will tend to overcome host resistance by their rapid rate of growth. Since many, if not the majority, of human neoplasms are relatively slowly growing and highly differentiated, the more highly differentiated experimental neoplasms of animals may offer model systems for study and comparison with their human counterparts.

Weinhouse has recently suggested that rapid growth, i.e., cell division, may actually be the primary cause of deletion of many enzymes in cancer tissues (30), and it appears probable that growth per se is neither a unique characteristic nor a necessary prerequisite of the neoplastic cell. The growth rate of embryonic tissue equals or surpasses that of most tumors, and the elegant experiment of Fisher and Fisher (8), as well as considerable clinical evidence (28), indicates that the neoplastic cell need not grow in order to retain its malignant potential.

The important characteristic of neoplasia as emphasized by many authors (22) and summarized by Burnet (4) is the "...change in the character of the cells rendering them in one way or another susceptible to the normal control." That certain controlling mechanisms are faulty in the neoplastic cell has been previously suggested (18). Neoplastic growth may be only one expression of a more basic abnormality in some controlling mechanism of the cell. The promotion and growth of tumors are considerably influenced by host and environmental factors, but there is little available evidence indicating an effect other than that of the carcinogenic agent itself in the initiation of a neoplasm (3).

This may suggest that some basic change is required to produce a neoplastic cell, but the expression of the malignant potential of this cell, i.e., uncontrolled and invasive growth, is dependent on environmental conditions, host factors, or other as yet unknown circumstances (3). Indeed, the induction of rapid growth in a neoplasm may in itself represent an enzymatic change or deletion in the latent or dormant tumor cell (23). In view of the above considerations any attempt to define the essential biochemical characteristics of neoplasia may be aided by the use of a slowly growing, highly differentiated neoplasm when comparing a normal tissue with its neoplastic counterpart. In this way, the investigator may at least eliminate the distinct possibility that the process he is measuring is a result of rapid growth rather than the neoplastic process itself. In view of the biochemical (24), biologic, and morphologic (16, 17) characteristics of the Morris hepatoma 5123, this neoplasm offers perhaps the best system, at present, for the elucidation of any alterations in enzymatic and controlling mechanisms between the normal and malignant liver cell. Whether or not any difference that is found between the two can be generalized to other neoplasms must await careful experimental investigation.

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