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Absence of Pyridoxine- (Pyridoxamine-) 5′-phosphate Oxidase in Morris Hepatoma 7777†

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ABSTRACT: Morris hepatoma 7777 previously has been shown to have no detectable pyridoxine- (pyridoxamine-) 5′-phosphate oxidase activity [Thanassi, J. W., Nutter, L. M., Meisler, N. T., Commers, P., & Chiu, J.-F. (1981) *J. Biol. Chem. 256*, 3370–3375]. In order to determine if this enzyme was missing in the hepatoma, we purified rat liver oxidase and raised antibodies to it in rabbits. Final purification of rat liver oxidase for use as an antigen was accomplished by affinity chromatography and gel electrophoresis. The rat liver enzyme is similar to rabbit liver oxidase [Kazarinoff, M. N., & McCormick, D. B. (1975) *J. Biol. Chem. 250*, 3436–3442] having two noncovalently linked subunits with molecular weights in the range of 25 000–28 000. Evidence indicating that inactive enzyme was simultaneously purified with native enzyme was obtained. The IgG fraction was purified from the serum of a rabbit that had been immunized with rat liver oxidase. This was used in the development of ELISA and immunoblot analyses for the presence of antigenically active pyridoxine- (pyridoxamine-) 5′-phosphate oxidase in cytosolic preparations from normal rat liver and Morris hepatoma 7777. The results indicated that there was no immunologically detectable oxidase protein in the tumor. An alternate pathway of pyridoxal 5′-phosphate synthesis, involving oxidation of pyridoxine to pyridoxal followed by phosphorylation, was ruled out. The implications of these findings with respect to acquisition of nutrients by tumors are discussed.

Pyridoxine- (pyridoxamine-) 5′-phosphate oxidase (EC 1.4.3.5) is responsible for the formation of pyridoxal 5′-phosphate, the coenzymatically active form of vitamin B-6, from phosphorylated precursor forms (Wada & Snell, 1961). This enzyme has been purified from rabbit liver (Kazarinoff & McCormick, 1975), pig brain (Kwok & Churchich, 1980), rat brain (Cash et al., 1980), and bakers' yeast (Tsuge et al., 1979). It has been extensively studied by McCormick and co-workers (Merrill et al., 1979; McCormick & Merrill, 1980; Tsuge & McCormick, 1980; Choi & McCormick, 1981), who have proposed that it plays a regulatory role in vitamin B-6 metabolism (Merrill et al., 1978). Homogeneous oxidase preparations obtained from rabbit liver (Kazarinoff & McCormick, 1975) and pig brain (Kwok & Churchich, 1980) are reported to have molecular weights of 54 000 and 60 000, respectively, and contain one tightly bound FMN per active monomer which is composed of two noncovalently linked subunits.

We have previously found that two poorly differentiated Morris hepatomas lack pyridoxine- (pyridoxamine-) 5′-phosphate oxidase activity (Thanassi et al., 1981). In addition, we have reported that there appears to be an oncodevelop-

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mental correlation between oxidase activity and the degree of differentiation in a series of Morris hepatomas (Meisler et al., 1982). The absence of pyridoxine- (pyridoxamine-) 5′-phosphate oxidase activity in poorly differentiated liver-derived Morris hepatomas suggests that these tumors must have alternate mechanisms for obtaining pyridoxal 5′-phosphate since, unlike liver, they apparently are incapable of making it by the conventional oxidative conversion of phosphorylated precursor vitamer forms (Snell & Haskell, 1971). The observed lack of oxidase activity can result from a variety of causes. Among these are insufficient cofactor, FMN, altered K_m values for substrates and/or cofactor, an alternate pathway(s) for the synthesis of pyridoxal 5′-phosphate, a tumor-specific inhibitor of oxidase activity, and a missing or inactive enzyme. The results of our investigations on the lack of pyridoxine- (pyridoxamine-) 5′-phosphate oxidase in Morris hepatoma 7777 provide the basis of this report.

Experimental Procedures

Materials. Pyridoxine hydrochloride, pyridoxal hydrochloride, pyridoxamine 5′-phosphate hydrochloride, 4′-deoxypyridoxine hydrochloride, pyridoxal 5′-phosphate, FMN, DEAE-Sephadex A-50, 6-aminohexyl-Sepharose 4B, *o*-phenylenediamine, phenylmethanesulfonyl fluoride, 2-mercaptoethanol, Tween 20, nitro blue tetrazolium, phenazine methosulfate, Coomassie brilliant blue R, bromophenol blue, amido black, diaminobenzidine tetrahydrochloride, tris(hydroxymethyl)aminomethane (TRIZMA), and fraction V bovine serum albumin were from Sigma Chemical Co. Sephadex G-100 was purchased from Pharmacia. Freund's adjuvant and fetal calf serum were from GIBCO. Nitrocellulose paper (15-μm pore size) was obtained from Schleicher & Schuell. Poly(vinyl chloride) microtiter plates (96 well) were from Costar. Silica gel (Chromagram) sheets with fluorescent indicator were from Eastman Kodak. Enzyme-grade ammonium sulfate was a product of Schwarz/Mann. Horseradish peroxidase conjugated to goat anti-rabbit IgG (heavy and light chains) was supplied by Cappel Laboratories. Pyridoxine tritiated at C4′ and pyridoxine 5′-phosphate were from a previous study (Meisler & Thanassi, 1980). [(5′-Phosphopyridoxyl)amino]hexyl-Sepharose 4B was prepared essentially as described by Cash et al. (1980). All other reagents and chemicals were of the highest commercial grade available.

New Zealand giant rabbits were obtained from a local supplier. Buffalo and Sprague-Dawley Crl:CD(SD)BD strain rats were from Microbiological Associates and Canadian Breeding Laboratories, respectively. Morris hepatoma 7777 was grown in the hind legs of recipient animals as described previously (Thanassi et al., 1981).

Thin-Layer Chromatographic Separation of Pyridoxine and Pyridoxal. Cytosolic extracts of liver and hepatoma were prepared as described previously (Thanassi et al., 1981). Aliquots (15 μL) were incubated in capped 1.5-mL Eppendorf tubes in 0.17 M Tris buffer (pH 8.0) containing 2.75 mM [4′-^{3}H]pyridoxine (1.36×10^6 cpm) and 1 mM 4′-deoxypyridoxine (total volume 50 μL). After a 2-h incubation at 37 °C, 5 μL of 100% trichloroacetic acid was added to the tubes which were then placed on ice for 30 min. After the precipitates had been centrifuged out, the supernatants were extracted 4 times with ether to remove trichloroacetic acid, and 2-μL aliquots of the supernatants were applied to 10 × 18 cm thin-layer chromatography sheets. The solvent system employed was chloroform/ methanol/concentrated ammonia (75:25:0.1 v/v/v); the sheets had been prerun in the same solvent. A parallel line 2.5 cm from the origin was made by using a 2.5% boric acid solution. This retarded the migration of pyridoxine and allowed for better separation of pyridoxine and pyridoxal (Ahrens & Korytnyk, 1970). The solvent front was allowed to migrate to a line 0.5 cm from the top of the sheet. The sheets were air-dried and cut into 10 zones for liquid scintillation counting in order to determine the radioactivity profile. A section of clean sheet that had been run in the solvent system was counted as a blank. Nonradioactive pyridoxine and pyridoxal were run as fluorescent markers. Volatile counts (as ^3H_2O) were determined by distillation and cold-finger trapping of water from reaction mixtures identical with those described above.

Purification of Pyridoxine- (Pyridoxamine-) 5′-phosphate Oxidase from Rat Liver. Oxidase activity was measured at 37 °C by the 2,4-dinitrophenylhydrazine procedure developed by Wada & Snell (1961) with modifications as previously described (Meisler & Thanassi, 1980; Thanassi et al., 1981). The purification was based on methods described by Kazarinoff & McCormick (1975) and Merrill et al. (1979) for purification of the rabbit liver enzyme. One unit of activity is defined as that amount of enzyme that catalyzes the formation of 1 nmol of pyridoxal 5′-phosphate/h under the conditions used; specific activities are given as units per milligram of protein. Protein was estimated by the method of Lowry et al. (1951) with bovine serum albumin as standard. Fresh or frozen livers from Sprague-Dawley rats were the source of enzyme.

With minor modifications, the purification of rat liver pyridoxine- (pyridoxamine-) 5′-phosphate oxidase followed the procedures of Kazarinoff & McCormick (1975) through the Sephadex G-100 chromatography step. Modifications worth noting are the following: the proteinase inhibitor, phenylmethanesulfonyl fluoride (50 μM), was added to all buffers, and 0.3 M potassium phosphate buffer was required to elute rat liver oxidase from the DEAE-Sephadex A-50 column in contrast to a buffer concentration of less than 0.2 M required to elute the rabbit liver enzyme.

Further purification of rat liver oxidase for use as an antigen was accomplished by affinity chromatography on 5′-phosphopyridoxyl-Sepharose 4B and polyacrylamide gel electrophoresis under nondenaturing conditions. A typical affinity chromatography purification involved the application of approximately 11 mg of protein (approximately 30 000 units of activity) in 2 mL of buffer to a 1.4 × 6 cm column. The application buffer was 2 mM potassium phosphate (pH 7.4) containing FMN (2 μM), phenylmethanesulfonyl fluoride (50 μM), potassium chloride (100 mM), and 2-mercaptoethanol (0.1 mM). After a 1-h equilibration at 4 °C, the column was eluted with application buffer until the A_{280} of the effluent had approached zero (80 mL). Oxidase activity was then eluted with either 1 mM pyridoxal phosphate (pH 5) or 10 mM pyridoxal phosphate (pH 7) in application buffer without potassium chloride. Five 10-mL fractions were collected and dialyzed against 5 mM hydroxylamine in 2 mM potassium phosphate (pH 7) for 48 h followed by dialysis against 2 mM phosphate buffer (pH 7). Active fractions were lyophilized. The affinity chromatography gave variable results. The average specific activity after this step was approximately 9000 units/mg of protein, and the range was from 3000 to 11 400. The recovery of activity ranged from a low of 20% to a high of 87%.

Preparation of Rabbit Anti-Rat Pyridoxine- (Pyridoxamine-) 5′-phosphate Oxidase. Affinity column purified oxidase (specific activity 9049 units/mg of protein) was run in triplicate lanes (200 μg of protein/lane) on 0.75 mm thick polyacrylamide slab gels (4–12% linear gradient) by using a

Hoefer SE 600 electrophoresis apparatus under nondenaturing conditions (Kazarinoff & McCormick, 1975). One lane was stained for protein with Coomassie blue and another with an activity stain for oxidases (Feinstein & Lindahl, 1973; Kazarinoff & McCormick, 1975). These were used to locate oxidase on the third, untreated lane. The area of the untreated lane containing active enzyme was excised, homogenized in 0.55 mL of 0.9% NaCl, and then mixed with an equal volume of Freund's complete adjuvant. This mixture was injected subcutaneously and into the foot pads of a rabbit (Hartman & Udenfriend, 1969; Boulard & Lecroisey, 1982). Electrophoretic purification of oxidase and subcutaneous injection were repeated 3 times at weekly intervals. A final injection, without Freund's adjuvant, was made directly into an ear vein at week five. The IgG fraction of immune serum and preimmune serum from the same rabbit was purified by chromatography on DEAE-cellulose according to Fahey (1967).

Immunoblot Detection of Pyridoxine- (Pyridoxamine-) 5′-phosphate Oxidase. Immunoblot detection of oxidase was performed essentially as described by Towbin et al. (1979). Denaturing gels containing 0.1% sodium dodecyl sulfate were run on a 5% stacking gel and a 10% running gel. The running buffer was 5 mM TRIZMA/38 mM glycine (pH 8.3). Samples were prepared in complete Fairbanks solution (Fairbanks et al., 1971) with bromphenol blue as the tracking dye. Horizonal electroelution of proteins from polyacrylamide gels to nitrocellulose paper was performed on a Bio-Rad Trans-Blot Cell. The nitrocellulose paper containing transferred proteins was soaked overnight at 4 °C in a blocking solution consisting of 10% bovine serum albumin and 3% fetal calf serum in phosphate-buffered saline (10 mM sodium phosphate buffer, pH 7.2, containing 0.14 M sodium chloride). After several washes with phosphate-buffered saline, the nitrocellulose paper was incubated (1 h, 37 °C) with the purified IgG fraction obtained from the serum of an immunized rabbit; the protein concentration of the IgG fraction was 50 μg/mL in phosphate-buffered saline. The paper was washed 5 times (5 min/wash) with 0.05% Tween in phosphate-buffered saline and then incubated (45 min, 37 °C) with a second antibody (horseradish peroxidase conjugated goat anti-rabbit IgG) that had been diluted 1:10 000 in phosphate-buffered saline containing 3% bovine serum albumin. Excess second antibody was removed by washing with 0.05% Tween in phosphate-buffered saline (five washes, 5 min/wash). Oxidase was then visualized by incubation (room temperature, approximately 30 min) of the paper with substrate for horseradish peroxidase (a freshly prepared nitrogen-gassed, filtered solution of 30 mg of diaminobenzidine tetrahydrochloride in 100 mL of 50 mM TRIZMA hydrochloride buffer, pH 7.5, containing 5 μL of 30% H_2O_2). When crude preparations of oxidase or cytosolic extracts of liver or tumor were analyzed for the presence of oxidase, an additional step to suppress endogenous pseudo horseradish peroxidase activity was incorporated (Farr & Nakane, 1981). This involved incubating (30 min at room temperature) the nitrocellulose paper in 0.3% hydrogen peroxide in phosphate-buffered saline after the initial incubation with blocking solution. The hydrogen peroxide was then removed by washing with phosphate-buffered saline. The rest of the procedure was as described above.

Enzyme-Linked Immunosorbent Assay (ELISA) for Pyridoxine- (Pyridoxamine-) 5′-phosphate Oxidase. The procedures were modifications of those described by Engvall & Perlmann (1972). Liver and tumor cytosols were prepared as described previously (Thanassi et al., 1981) except that 3 instead of 9 volumes of 0.25 M sucrose was used in the preparation of the initial homogenate. Cytosols were dialyzed against 50 mM sodium carbonate buffer, pH 9.6. Dilutions of liver and protein-matched tumor cytosolic preparations were made in carbonate buffer (total volume 100 μL) and added to the wells of 96-well microtiter plates. After 1 h with shaking at 37 °C, the well contents were flicked out, and the wells were completely filled with a solution containing 3% bovine serum albumin and 10% fetal calf serum in carbonate buffer. After 1 h with shaking at 37 °C, the well contents were flicked out and the wells washed 3 times with 0.05% Tween in phosphate-buffered saline (3 min/wash). Rabbit IgG solutions, corresponding to a 160-fold dilution of the original serum concentrations, containing anti-rat oxidase antibodies were added to the wells; dilutions were made in 3% bovine serum albumin and 10% fetal calf serum in phosphate-buffered saline. The control IgG preparation obtained from preimmune serum was matched for protein concentration with the IgG fraction purified from immune serum. After a 1-h incubation with shaking (37 °C), the contents of the wells were removed and the wells washed as described above. Second antibody, diluted 1:5000 in 1% bovine serum albumin plus 2% fetal calf serum in phosphate-buffered saline, was added.

The plate was shaken for 45 min at 37 °C, the contents were removed, and the wells were washed as described above. Substrate solution (100 μL) was added to each well; this consisted of a solution of 37.5 mg of *o*-phenylenediamine and 7.5 μL of 30% H_2O_2 in 0.1 M citrate buffer (pH 5.0). After a 30-min incubation at room temperature, the reactions were stopped by the addition of 50 μL of 2 M sulfuric acid. The intensity of the color at 490 nm was read on a Bio-Tek EIA reader by using the appropriate filter. The sensitivity of the ELISA assay is in the range 4–40 ng with affinity chromatography purifed oxidase preparations as antigen.

Results and Discussion

It is conceivable that Morris hepatoma 7777 has an alternate pathway for the biosynthesis of pyridoxal 5′-phosphate from precursors, specifically, one which involves oxidation of pyridoxine to pyridoxal prior to rather than after phosphorylation of the hydroxymethyl group at C5. To test this possibility, we incubated liver and tumor cytosolic extracts with [4′-^{3}H]pyridoxine in the presence of 4′-deoxypyridoxine, an inhibitor of rat liver pyridoxal kinase (McCormick & Snell, 1961). Analysis of supernatants from the reaction mixtures by thin-layer chromatography indicated that all of the radioactivity remained in the starting material and none could be found in pyridoxal (Figure 1). To confirm these findings, we performed an analysis for volatile counts because, if there had been any conversion of pyridoxine to pyridoxal, there necessarily would have been release of tritium into the medium from the exclusively tritiated C4′ position. In two experiments the volatile counts amounted to 6.2 and 5.4% of the total compared to 7.5% found with a boiled control; the recovery of nonvolatile counts in the residues averaged 96% of the total. Thus, an alternate pathway for the synthesis of pyridoxal 5′-phosphate from pyridoxine does not appear to exist in Morris hepatoma 7777.

Previous studies from our laboratory (Thanassi et al., 1981) indicated that the absence of oxidase activity in the hepatoma was not a result of insufficient cofactor, FMN, the presence of an inhibitor, a change in substrate preference from pyridoxine 5′-phosphate to pyridoxamine 5′-phosphate, or a subcellular relocalization of the enzyme. Accordingly, it became apparent that it would be necessary to establish if the absence of oxidase activity in Morris hepatoma 7777 was a consequence of a defective enzyme or a missing enzyme. Addressing this

Table I: Purification of Rat Liver Pyridoxine- (Pyridoxamine-) 5′-phosphate Oxidase

	step	vol (mL)	units/mL	total units	protein (mg/mL)	sp act.	yield (%)	purification
extract	1	1800	181.7	327 060	27.7	6.6	100	
acid supernatant	2	2000	122.0	243 980	11.6	10.5	75	1.6
ethanol precipitation	3	112	1710	191 543	39.1	43.7	59	6.7
DEAE-A50 chromatography	4	203	438.9	89 089	0.72	610	27	93
G-100 chromatography	5	51	868.6	52 114	0.34	2555	14	389
affinity chromatography	6	1.3	11702	15 213	1.29	9072	4.7	1375

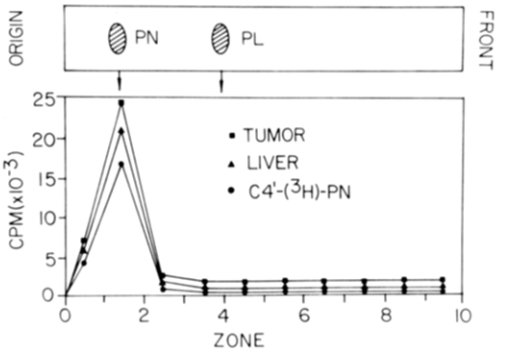

FIGURE 1: Thin-layer chromatography of supernatants obtained from reaction mixtures containing liver or hepatoma cytosolic extracts, [4′-^{3}H]pyridoxine, and 4′-deoxypyridoxine in 0.17 M tris(hydroxymethyl)aminomethane buffer (pH 8.0). (Upper) Positions of authentic pyridoxine (PN) and pyridoxal (PL) determined by fluorescence under ultraviolet light. (Lower) Radioactivity profile of starting material, [4′-^{3}H]pyridoxine, and aliquots of supernatants from reaction mixtures containing cytosolic extracts from liver or tumor.

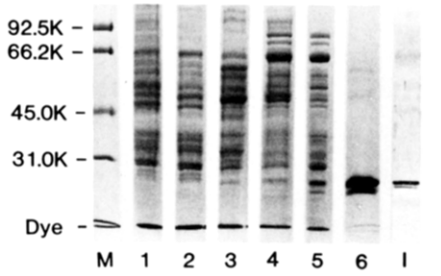

FIGURE 2: NaDodSO$_4$/polyacrylamide gel electrophoresis of pyridoxine- (pyridoxamine-) 5′-phosphate oxidase fractions. Lanes 1–6 correspond to the fractions obtained from steps 1–6 in Table I and were stained with Coomassie blue; 35 μg of protein was applied to each well. The lane designated M contained marker proteins of known molecular weights. The lane designated I shows an immunoblot detection of affinity chromatography purified rat liver pyridoxine- (pyridoxamine-) 5′-phosphate oxidase.

question required an immunological approach which in turn required purified rat liver oxidase. The purification of rat liver oxidase was carried out as described under Experimental Procedures and was mostly patterned after procedures developed by McCormick and his colleagues for the purification of rabbit liver oxidase (Kazarinoff & McCormick, 1975; Merrill et al., 1979). The results of a representative purification are provided in Table I. The highest specific activity for oxidase that we have obtained after the affinity chromatography step was 11 400 units/mg of protein which is approximately half of the highest specific activities reported for the rabbit liver enzyme (Kazarinoff & McCormick, 1975; Merrill et al., 1979). The subunit molecular weights as determined by denaturing sodium dodecyl sulfate (NaDodSO$_4$)/polyacrylamide gel electrophoresis were determined to be in the range 25 000–28 000 when compared to marker proteins of known molecular weights. The Coomassie blue staining patterns obtained after NaDodSO$_4$/polyacrylamide gel electrophoresis at various stages of purification are provided in Figure 2. As shown in Figure 2, there were two major Coomassie blue staining bands (25 000–27 000 and 27 000–28 000 daltons) evident in oxidase preparations after purification by affinity chromatography. Densitometric analysis indicated that these two bands comprised more than 98% of the total protein applied to the gel. These two bands were also seen with immunoblot detection methods (Figure 2). As noted under Experimental Procedures, affinity chromatography purification of rat liver oxidase yielded variable results. The use of an AffiGel 501 column as a final step in the purification of rabbit liver oxidase also has been reported to yield variable results (Merrill et al., 1979). We have observed that the relative intensities of the two bands found after NaDodSO$_4$/polyacrylamide gel electrophoresis of purified oxidase preparations vary among preparations and believe that the faster running band arises from enzymatically inactive oxidase, possibly caused by proteolytic degradation in spite of the presence of a protease inhibitor throughout the purification procedure. Sensitivity of both rabbit liver and yeast oxidases to proteolysis has been noted by others (Merrill et al., 1979; Tsuge et al., 1979). In addition, inactive apoenzymic subunits of purified rabbit liver oxidase having slightly lower molecular weights (26 000–27 000 instead of 27 000–28 000) have been observed after NaDodSO$_4$/polyacrylamide gel electrophoresis by Horiike et al. (1979). In contrast to the double bands observed in denaturing gels, purified rat liver oxidase, when subjected to electrophoresis on polyacrylamide gels under nondenaturing conditions, as described below, yielded a single band, suggesting that the single band observed on nondenaturing gels is a mixture of active and inactive oxidase.

Immunoblot detection and ELISA determinations of rat liver pyridoxine- (pyridoxamine-) 5′-phosphate oxidase required antibody to purified rat liver oxidase. We purified the IgG fraction of serum from a rabbit that had been immunized with rat liver oxidase. The antigen preparation used in the immunization protocol described under Experimental Procedures was affinity chromatography purified rat liver oxidase that was further purified by electrophoresis on polyacrylamide gels under nondenaturing conditions. The enzyme was located on such gels by staining marker lanes on either side with Coomassie blue and an oxidase-specific activity stain. Immunoblot detection of rat liver oxidase after NaDodSO$_4$/polyacrylamide gel electrophoresis (Figure 2) or after electrophoresis on nondenaturing gels (Figure 3) involved initial electrophoresis, horizontal electroelution of the electrophoretically resolved proteins to nitrocellulose, incubation of the nitrocellulose paper with rabbit anti-rat oxidase followed by incubation with horseradish peroxidase conjugated second antibody (goat anti-rabbit IgG), and incubation with horseradish peroxidase sensitive staining solution. Figure 3 shows the banding patterns obtained after affinity chromatography purified oxidase had been run on gels under nondenaturing conditions. The Coomassie blue stain and activity stain both yielded single bands. The amido black pattern obtained after

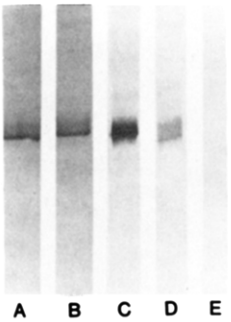

FIGURE 3: Polyacrylamide gel electrophoresis of affinity chromatography purified rat liver pyridoxine- (pyridoxamine-) 5′-phosphate oxidase under nondenaturing conditions: (lane A) stained with Coomassie blue; (lane B) stained for activity. Amido black and immunoblot detection of oxidase after electroelution from polyacrylamide gels run under nondenaturing conditions to nitrocellulose paper: (lane C) stained with amido black; (lane D) stained by immunoblot detection; (lane E) same as lane D but preimmune rabbit serum was used as the source of the first antibody. For details, see Experimental Procedures.

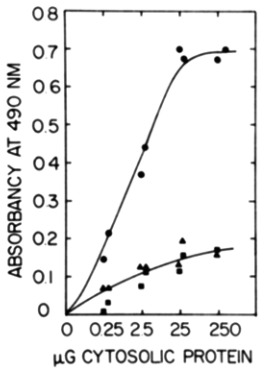

FIGURE 4: ELISA analysis for the presence of immunologically detectable pyridoxine- (pyridoxamine-) 5′-phosphate oxidase in cytosolic extracts from normal rat liver (circles) and Morris hepatoma 7777 (squares). The control (triangles) was run with liver cytosolic extracts by using the IgG fraction obtained from preimmune serum. For details, see Experimental Procedures.

electroelution of proteins to nitrocellulose paper also revealed only one band as did the horseradish peroxidase dependent immunological detection method. As expected, when the IgG fraction obtained from preimmune rabbit serum was used as first antibody, there was only background staining. As noted previously, immunoblot detection of affinity chromatography purified oxidase after denaturing $NaDodSO_4$/polyacrylamide gel electrophoresis invariably yielded two bands whose relative intensities varied from preparation to preparation (Figure 2).

With the availability of rabbit anti-rat pyridoxine- (pyridoxamine-) 5′-phosphate oxidase, it became possible to test extracts of Morris hepatoma for the presence of antigenic material reactive toward the rabbit antibody. We did this by ELISA and immunoblot detection methods.

The conditions for the ELISA detection of oxidase were initially established with purified oxidase and are described under Experimental Procedures. The IgG fractions from immune and preimmune rabbit sera were used at a concentration equivalent to a 160-fold dilution of their original serum concentrations. Figure 4 provides the results of ELISA analyses for pyridoxine- (pyridoxamine-) 5′-phosphate oxidase in rat liver and Morris hepatoma 7777. Cytosolic extracts from normal rat liver are shown to contain antigenically active protein which is proportional to concentration and which, at higher concentrations, can saturate a fixed concentration of antibody. The IgG fraction from preimmune serum shows only a nonspecific response in the ELISA determination. Tumor

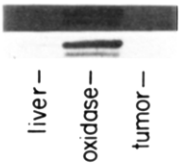

FIGURE 5: Immunoblot detection of pyridoxine- (pyridoxamine-) 5′-phosphate oxidase in cytosolic extracts obtained from rat liver and Morris hepatoma 7777. Denaturing polyacrylamide gels, run in the presence of 0.1% $NaDodSO_4$, were heavily loaded with protein (500 μg/well). Proteins were horizontally electroeluted to nitrocellulose paper. The position of oxidase protein on the nitrocellulose paper was established with affinity chromatography purified oxidase. Two different photographic exposures of the appropriate area of the nitrocellulose paper are provided.

cytosolic extracts, matched for protein concentration to liver extracts, react toward immune serum in ELISA determinations in a nonspecific manner indistinguishable from that observed with liver cytosolic extracts and preimmune serum. Thus, within the limits of detection, the ELISA method clearly indicates that the absence of oxidase activity in Morris hepatoma 7777 is not due to the presence of antigenically active, enzymatically inactive enzyme but rather to missing enzyme protein.

The results of immunoblot analysis (Figure 5) for the presence of oxidase in hepatoma extracts qualitatively confirm the results obtained with ELISA methods. Polyacrylamide gels, heavily overloaded with protein (500 μg/well) obtained from liver and tumor cytosolic extracts, were run under denaturing conditions, the proteins were transferred to nitrocellulose paper, and immunoblot detection of oxidase was performed as described under Experimental Procedures. As shown in Figure 5, liver extracts contain immunologically reactive protein that migrates identically with purified oxidase whereas hepatoma extracts do not contain immunologically detectable oxidase. It is worth noting that freshly prepared, unfractionated cytosolic liver extracts show only one of the two immunologically reactive bands found in purified oxidase preparations. This finding lends support to the supposition that the faster of the two bands observed on denaturing gels is an inactive degradation product arising during purification.

The absence of immunologically detectable pyridoxine- (pyridoxamine-) 5′-phosphate oxidase in Morris hepatoma 7777 clearly indicates that there has occurred during transformation a loss of the expression of the genetic information necessary to synthesize this particular enzyme. This could result from a variety of causes such as gene deletion, faulty mRNA production or processing, translational errors, etc. The absence of an enzyme essential for the conversion of B-6 vitamer forms to pyridoxal phosphate must be compensated for, or there would be expected multiple deleterious metabolic consequences owing to the numerous and vital coenzymatic functions of pyridoxal phosphate. One can assume that Morris hepatoma 7777 becomes dependent on the direct or indirect acquisition of pyridoxal phosphate, the latter process most likely involving uptake and phosphorylation of pyridoxal. In marked contrast, normal liver not only is active in the synthesis of pyridoxal phosphate from precursors such as pyridoxine but indeed is an exporter of pyridoxal phosphate to extrahepatic tissues (Lumeng & Li, 1980). It is known that transformed hepatocytes can become more efficient in the transport of nutrients such as amino acids (Kelley & Potter, 1979; White & Christensen, 1982). It is likely that the shedding of a vital metabolic pathway may require that there be a lessened dependency on the metabolite, that there be compensatory increases in alternate pathways or in existing transport systems,

or that there be expression of information that allows for transport systems not normally found in the untransformed terminally differentiated cell.

The manner in which Morris hepatoma 7777 obtains its pyridoxal phosphate and the utilization of this essential micronutrient are intriguing questions in tumor nutrition. Recently, monoclonal antibodies to various B-6 vitamer forms have been shown to be capable of identifying cellular pyridoxal phosphate binding proteins in a variety of tissues (Viceps-Madore et al., 1983). These antibodies may be useful in examining vitamin B-6 utilization by tumors.

Registry No. Pyridoxine-5′-phosphate oxidase, 9055-72-5; pyridoxal 5′-phosphate, 54-47-7; pyridoxine, 65-23-6; pyridoxal, 66-72-8.

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