Characterization of Microsomal Epoxide Hydrolase in Hyperplastic Liver Nodules of Rats

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ABSTRACT

Microsomal epoxide hydrolase, a major antigenic marker of putative preneoplastic hepatocytes was studied in hyperplastic liver nodules and in normal liver tissue. Hyperplastic nodules induced by two different protocols contained 2- to 3-fold higher specific activities of microsomal epoxide hydrolase than did normal surrounding tissue. However, no increase in cytosolic epoxide hydrolase determined with typical substrates for the cytosolic enzyme was observed in either nodule or surrounding tissue. No activity for typical substrates of microsomal epoxide hydrolase nor material immunologically cross-reactive with microsomal epoxide hydrolase was observable in the cytosolic fraction.

The increase in activity of microsomal epoxide hydrolase in hyperplastic nodules was similar for two endogenous steroid epoxides, 16α,17α-epoxy-androst-4-en-3-one and 16α,17α-fraction.

The increase in activity of microsomal epoxide hydrolase in hyperplastic nodules was similar for two endogenous steroid epoxides, 16α,17α-epoxy-androst-4-en-3-one and 16α,17α-epoxy-estratrien-3-one and for two xenobiotic substrates, benzo(a)pyrene 4,5-oxide and styrene 7,8-oxide. The effects of several diagnostic modulators, including activators as well as competitive, uncompetitive, and noncompetitive inhibitors of widely varying potencies were not significantly different for nodule microsomal epoxide hydrolase activity as compared to hydrolase from normal tissue.

While no immunological cross-reactivity was observed between cytosolic and microsomal epoxide hydrolase, the microsomal epoxide hydrolase from nodule and normal tissue was immunologically indistinguishable by all criteria tested: Ouchterlony double diffusion experiments with solubilized microsomes of nodule and control tissue and anti-microsomal epoxide hydrolase immunoglobulin G yielded only one precipitin line which was fused with that formed by apparently homogeneous microsomal epoxide hydrolase and the antibody. Immunoprecipitation by adding increasing amounts of antibody to solubilized microsomes precipitated the entire activity of microsomal epoxide hydrolase with dose-response curves not significantly different between nodule and control microsomes.

The available evidence indicates that: (a) The cytosolic epoxide hydrolase is not the microsomal enzyme that has leaked into the cytosol thereby acquiring different properties. It is a distinct entity under different biosynthetic control. (b) The increased diffusibility of microsomal epoxide hydrolase in hyperplastic nodules observed previously is not reflected in a detectable appearance of the enzyme in the cytosol isolated from nodules. (c) No differences between the enzymic or immunological properties of microsomal epoxide hydrolase from nodule and normal tissue were detectable by any of the methods used. The increase in specific epoxide hydrolase activity in hyperplastic nodules appears to be due to a permanent change in the control of the amount of microsomal epoxide hydrolase which is, however, with respect to all criteria tested, an enzyme protein very similar to that found in normal tissue.

INTRODUCTION

Hyperplastic nodules can be induced in the liver of rats by many hepatocarcinogens including 2-AAF, ethionine, nitrosamines, aflatoxin B1, and safrole (7). Such nodules have been found to be one site of origin of hepatocellular carcinomas with ethionine (5, 6), aramite (32), 3'-methyl-4-dimethylaminoazobenzene (10), 2-AAF (33), and DENA (37). Thus, the study of both biochemical and biological aspects of these nodules would seem to be quite important in the sequential analysis of liver carcinogenesis (8, 9).

A biochemical-biophysical marker for these hyperplastic lesions has recently been detected by immunological methods (30, 31) and has been called PNA. PNA readily diffused from liver nodule microsomes into agarose gels, but could not be detected by immunodiffusion in normal nonsolubilized rat liver microsomes (31). Evidence was provided that this antigen was primarily located in the endoplasmic reticulum (18). A PNA was purified and quantitated from hyperplastic nodules by Griffin and Kizer (11) and by Lin et al. (19). These authors (11, 19) presented evidence that a protein with immunodeterminants identical with those possessed by PNA could also be found in normal liver microsomes, when 0.2% deoxycholate was added to the liver microsomes of control animals. These findings are consistent with the hypothesis that an altered membrane state exists in hyperplastic nodule endoplasmic reticulum (11).

One type of PNA was identified immunologically as microsomal EH (EC 3.3.2.3) (16). A purified PNA and purified microsomal EH had identical minimum molecular weights in SDS-polyacrylamide gels (SDS-gels), and catalyzed the hydrolysis of several arene and alkene oxides (16). Further investigations showed that feeding with 2-AAF and other hepatocarcinogens resulted in an increase of microsomal EH activity and of polypeptides with similar electrophoretic properties to microsomal EH on SDS-gels (16, 35). Significant amounts of microsomal EH diffused from nodule and hepatoma microsomes but not from microsomes from normal liver during an incubation of 2 hr.

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2 To whom requests for reprints should be addressed.

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at 37° (12).

The question therefore arose whether, in these hyperplastic nodules, a fundamentally aberrant microsomal EH was present. In this study, we therefore investigated the enzymic and immunological behavior of the enzyme from each source to determine whether the diffusible enzyme from nodule microsomes is demonstrably different by these criteria from the nondiffusible one from control tissue. The alternative hypothesis, that both proteins are similar but that the differences in diffusibility arise from an altered membrane in nodules, was also considered.

MATERIALS AND METHODS

Chemicals. trans- and cis-2-Methylstereone oxides were synthesized by epoxidation with m-chloroperoxybenzoic acid of the corresponding methylstereones. 2,2-Dimethyl-1-phenylxirane and 1,2,2-trimethyl-1-phenylxirane were made from the corresponding diols by reaction with p-tosylimidazole. cis-stilbene oxide was prepared from trans-stilbene oxide according to the method of Berti et al. (2). The following tritiated compounds were synthesized as described: benzo(a)pyrene 4,5-oxide (4), trans-stilbene oxide (29), styrene 7,8-oxide (27), estroside (38), and 16a,17a-epoxy-androst-4-en-3-one (38). Sources of other chemicals are as follows: 1,1,1-trichloropropene 2,3-oxide and frans-stilbene oxide according to the method of Berti et al. (2). The following epoxides were synthesized as described: benzo(a)pyrene 4,5-oxide hydrolysis (23), and frans-stilbene oxide hydrolysis (15). Protein concentrations were determined as described (20) using bovine gamma-globulin. The samples were kept overnight at 4°.

Animals and Preparation of Microsomes. Male Fischer 344 rats (130 to 150 g) obtained from Charles River Breeding Laboratories, Wilmington, Mass., were fed according to 2 different treatment schedules. The animals received a diet containing 0.05% 2-AAF intermittently with p-tosylimidazole. cis-stilbene oxide was prepared from trans-stilbene oxide according to the method of Berti et al. (2). The following tritiated compounds were synthesized as described: benzo(a)pyrene 4,5-oxide (4), trans-stilbene oxide (29), styrene 7,8-oxide (27), estroside (38), and 16a,17a-epoxy-androst-4-en-3-one (38). Sources of other chemicals are as follows: 1,1,1-trichloropropene 2,3-oxide and trans-stilbene oxide, EGA-Chemie, Steinheim, F. R. G.; a-meste-STO, trans- and cis-2-methylstereone, ICN Pharmaceuticals, Plainview, N. Y.; sodium cholate, Merck, Darmstadt, F. R. G.; Cutscum (isooctylphenoxypolyethanol), Fischer Scientific Co., Pittsburgh, Pa.; Agarose type III, Sigma Chemical Co., St. Louis, Mo.; lyophilized GARGG, Calbiochem, San Diego, Calif.; DEAE-Affigel Blue, Bio-Rad, Richmond, Calif.

RESULTS

Table 1 shows the specific EH activity in nodule and control tissue microsomes. Microsomal EH activity was increased about 3-fold (210 to 370%) in AAF-induced nodules and about 2-fold (130 to 150%) in DENA-AAF-PH (Model 2) as described in “Materials and Methods.”

No effects of storage on enzyme activity or immunological properties were observed.

Production of Antiserum and Immunological Experiments. Apparently homogeneous EH was prepared according to the methods of Bentley and Oesch (1). Antiserum against this purified enzyme was raised in New Zealand White rabbits (24). The IgG fraction was isolated using DEAE-Affigel blue column chromatography. Serum (25 ml) dialyzed overnight with 20 mM sodium phosphate buffer (pH 8.0) was applied to a 150-ml bed of DEAE-Affigel blue, equilibrated previously with 20 mM sodium phosphate buffer (pH 8.0). The IgG fraction was eluted with the same buffer. Fractions were pooled and the IgG was precipitated by the addition of ammonium sulfate to 50% saturation. The precipitated protein was redisolved in a small volume of 20 mM sodium phosphate buffer (pH 7.4) to a concentration of 20 mg/ml and dialyzed overnight against this buffer. The IgG solution was then frozen until further use.

Ouchterlony double diffusion experiments were performed in 1% agarose gel. When solubilized microsomes were used, the gel contained 0.1% Cutscum. For experiments using cytosol, no detergent was added to the gel. The center well contained 5 µl of the undiluted IgG (100 µg IgG). Apparently, homogeneous EH was always used as a reference protein (3.2 µg in 5 µl).

For immunoprecipitation experiments, microsomes were solubilized in 1% sodium cholate by adding a solution of 10% detergent and stirring for 20 min at 4°. The solubilized microsomes were then centrifuged at 100,000 × g for 60 min. Centrifugation for longer time periods did not lead to detectable further precipitation of microsomes.

The immunoprecipitation experiment was performed as described (13). Increasing amounts of anti-EH IgG were added to the solubilized microsomes. Nonimmune IgG was added to each sample so that the total IgG content was constant. The mixture was incubated for 3 hr at room temperature. For complete precipitation, 12.5 units of GARGG in 225 µl of buffer were added (1 unit is defined by the manufacturer as the amount of GARGG which will completely precipitate 40 µg of rabbit γ-globulin). The samples were kept overnight at 4°. The immunoprecipitate was centrifuged at 20,000 × g for 20 min. The nonimmunoprecipitated EH was determined in this supernatant fraction.

Measurement of Enzyme Activity. Microsomal and cytosolic EH activities were determined according to procedures from the following references: benzo(a)pyrene 4,5-oxide hydrolysis (34), styrene 7,8-oxide hydrolysis (27), estroside hydrolysis (3), 16a,17a-epoxy-androstene-3-one hydrolysis (23), and trans-stilbene oxide hydrolysis (15). Protein concentrations were determined as described (20) using bovine serum albumin as standard.

Table 1

<table>
<thead>
<tr>
<th>Microsomal epoxide hydrolase activity (nmol diol/mg protein/min)</th>
<th>STO a</th>
<th>BPO</th>
<th>EO</th>
<th>AO</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAF-No b</td>
<td>43.8 ± 1.29 c</td>
<td>17.9 ± 1.25</td>
<td>52.7 ± 0.7</td>
<td>69.5 ± 7.09</td>
</tr>
<tr>
<td>AAF-Co</td>
<td>11.9 ± 0.71</td>
<td>8.1 ± 0.51</td>
<td>20.0 ± 2.2</td>
<td>33.9 ± 0.31</td>
</tr>
<tr>
<td>Activity ratio</td>
<td>3.7</td>
<td>2.2</td>
<td>2.6</td>
<td>2.1</td>
</tr>
<tr>
<td>DENA-AAF-PH-No b</td>
<td>37.0 ± 2.29</td>
<td>18.4 ± 0.17</td>
<td>41.7 ± 3.09</td>
<td>60.4 ± 6.68</td>
</tr>
<tr>
<td>DENA-Co</td>
<td>13.5 ± 0.96</td>
<td>8.4 ± 0.22</td>
<td>21.2 ± 0.64</td>
<td>37.2 ± 0.28</td>
</tr>
<tr>
<td>Activity ratio</td>
<td>2.7</td>
<td>2.2</td>
<td>3.6</td>
<td>1.6</td>
</tr>
</tbody>
</table>

a STO, styrene oxide; BPO, benzo(a)pyrene 4,5-oxide; EO, estroside; AO, androstene oxide.
b Microsomes from nodule (No) and control (Co) tissue prepared from rats treated with AAF (Model 1) and DENA-AAF-PH (Model 2) as described in “Materials and Methods.”
c Mean ± S.D.
Microsomal Epoxide Hydrolase in Hyperplastic Nodules

2-fold (160 to 270%) in DENA-AAF-PH-induced nodules. The increase of the microsomal enzyme activities were similar for the 4 substrates tested, but the highest values were always observed with styrene oxide as substrate (3.7- and 2.7-fold). In the cytosolic fraction of control tissue or hyperplastic nodules, no EH activity was detectable with typical substrates of microsomal EH, benzo(a)pyrene-, styrene-, and estroside. Cytosolic EH activity is exceedingly low in the rat and interaction of these substrates with the cytosolic EH is minimal (14, 26). The assay with benzo(a)pyrene 4,5-oxide as substrate is particularly sensitive (34) and was performed under conditions allowing for detection of activities as low as 8 pmol 4,5-dihydroxybenzo(a)pyrene formed per min per mg protein, so that activities in the cytosol 1,000-fold lower than the specific activity of the microsomal fraction would have been clearly observable (Table 2). These results indicate that no detectable microsomal EH has leaked into the cytosol, whether in control or in nodule tissue. Interestingly, the levels of the cytosolic EH, measured with trans-stilbene oxide as substrate (15), were not increased in nodule tissue above those seen in cytosol from normal tissue (Table 2).

Table 3 represents the effect of diagnostic modulators on microsomal EH activity with styrene oxide as substrate in nodule and control tissue in vitro. The inhibitors chosen were: TCPO, an uncompetitive inhibitor of styrene oxide hydrolysis in control liver microsomes (21); cyclohexene oxide, a noncompetitive inhibitor (21); and cis-β-methyl-1-phenyloxirane, a competitive inhibitor (Chart 1). Other modulators were isomers of methyl-styrene oxides or stilbene oxides. All substances investigated influenced the styrene oxide hydrolysis to the same extent in both nodule and control tissue microsomes: TCPO was a very potent inhibitor, cyclohexene oxide and cis-β-methyl-1-phenyloxirane were moderately potent inhibitors, α-methyl-1-phenyloxirane was a weak inhibitor, and cis-stilbene oxide a very weak inhibitor. trans-Stilbene oxide and trans-β-methyl-1-phenyloxirane had no influence on the enzyme activity in either tissue preparation, and a slight activation of both enzymes was seen when 2,2-dimethyl-1-phenyloxirane and 1,2,2-trimethyl-1-phenyloxirane were added to the incubation.

Figs. 1 and 2 show the double diffusion analysis of anti-EH-IgG towards solubilized microsomes (Fig. 1) and cytosol (Fig. 2) from normal and nodule tissue. All solubilized microsome samples yielded a single precipitin line which was fused to the precipitin line from the purified EH (Fig. 1). Only one protein concentration and one anti-EH-IgG concentration is shown; other protein concentrations ranging from 1 to 1/8 and other anti-EH-IgG concentration ranging from 1 to 1/32 gave similar results.

Table 2

<table>
<thead>
<tr>
<th>Cytosolic epoxide hydrolase activity (pmol product/mg protein/min)</th>
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<tbody>
<tr>
<td><strong>Styrene oxide</strong></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>AAF-control</td>
</tr>
<tr>
<td>AAF-nodules</td>
</tr>
<tr>
<td>DENA-AAF-PH-control</td>
</tr>
<tr>
<td>DENA-AAF-PH-nodules</td>
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</tbody>
</table>

* Mean ± S.D.
ND, not detectable; i.e., <8 pmol product per mg protein per min.
nal tissue and nodule microsomes. The enzymic activity due to proteins immunologically cross-reactive with rat microsomal EH was titrated out. The dose-response curve was parallel for nodule and control tissue microsomes from either AAF- or DENA-AAF-PH-treated animals. Nodule microsome preparations were diluted so that approximately equal amounts of EH activity (measured previously) were added to each antibody incubation. As Chart 2 shows, approximately equivalent amounts of enzyme activity from all sources were precipitated by equal amounts of antibody; on the average, the amount of antibody precipitating a given amount of control tissue hydrolyase activity was capable of precipitating a similar (103 to 125%) amount of nodule hydrolyase activity. Also, the titration curves were similar with 2 substrates of the microsomal EH, the xenobiotic compound benzo(a)pyrene 4,5-oxide, and the endogenous substrate estroside.

**DISCUSSION**

With respect to all criteria tested, the microsomal EH in the nodules was not distinguishable from the enzyme in normal liver. The basis for this conclusion rests on the similarities of the relative activities of EH from nodules and control liver with respect to different substrates, activators and inhibitors, and on the ratio of catalytically active enzyme to the amount of protein precipitated with antibody. Obviously, final proof of full identity depends upon analyses of the content, sequence and possible modifications of the amino acids of the enzyme from each source.

In hyperplastic nodules produced by either intermittent prolonged treatment with 0.05% AAF or by treatment with one dose of DENA plus brief exposure to dietary 0.02% AAF plus PH, microsomal EH was increased about 2- to 3-fold. The increase in specific EH activity was similar for several substrates including benzo(a)pyrene 4,5-oxide and styrene oxide. Levin et al. (16) investigated, in hyperplastic nodules produced by intermittent prolonged treatment with 0.05% AAF according to Epstein et al. (5), EH activity for several alkane and arene oxides and reported a 6- to 7-fold increase for benzo(a)pyrene 4,5-oxide and styrene oxide as substrates. In these experiments, the specific activity of the control microsomes was 2-fold lower than in our control rats; however, the specific activity of nodule microsomes was approximately the same as we report here. In the present studies, we included 2 steroid epoxides as substrates, since the active site of foreign compound-metabolizing enzymes is expected to be more exactly tailored for endogenous substrates for which the enzyme may be evolved. It is possible that subtle changes in the active site of a foreign compound-metabolizing enzyme may therefore be most delicately monitored by endogenous substrates for which the steric fit may be most strictly defined. The relative substrate specificity remained similar in the microsomes from hyperplastic nodules generated by either of the two procedures used as in control microsomes, in that in all cases the specific activity of hydration was androstene oxide > estroside > styrene oxide > benzo(a)pyrene 4,5-oxide.

The present immunoprecipitation studies show that specific EH activity in nodule microsomes is on an average 2.4-fold higher than in control microsomes. A given amount of antibody precipitated similar amounts of enzyme activity (103 to 125%) from nodule compared to control microsomes. Thus, the increase in EH activity in the hyperplastic nodule compared to control tissue is exclusively or predominantly (more than 80%) due to an increase of enzyme protein. This agrees with the observation of Levin et al. (16) that the EH band in SDS-gel electrophoresis is more intense in hyperplastic nodule compared to control tissue and to the observation of Thomas et al. (39) that, in hyperplastic nodules, the catalytic activity towards octene oxide as substrate was increased 7.0-fold concomitantly with an increase in the enzyme protein immunologically determined by radial immunodiffusion by a factor of 4.4-fold. Furthermore, the immunoprecipitation dose-response curves of added antibody and decreasing EH activity remaining in the supernatant fraction after centrifugation of the immunocomplex had identical shapes for EH from normal tissue and nodules. The effectiveness of the antibody was not significantly different towards microsomal EH from hyperplastic nodules prepared by 2 different induction schemes and from normal tissue microsomes. Further evidence of the immunological relatedness of microsomal EH from nodules and normal tissue is given by double diffusion analysis in which a single precipitin line, totally fused and therefore immunologically very similar or identical to proteins immunologically cross-reactive with rat microsomal EH, was seen in all cases. This was already observed by Levin et al. (16) after intermittent feeding of AAF according to the protocol of Epstein et al. (5). It is noteworthy that, in the present study, this and a quite different regimen, initiation by DENA followed by AAF and PH to select for resistant cells which in contrast to the intermittent AAF feeding very rapidly generates nodules (36, 37), led to the same results. This is also true for other parameters and is of special significance as discussed below.

On the other hand, the cytosolic EH activity assayed with
trans-stilbene oxide, a good substrate for this enzyme (14, 25), was not increased in hyperplastic nodules compared to control tissue (Table 2). This indicates that cytosolic EH, which is different from microsomal EH in substrate specificity (14, 21, 22, 25, 26) and in immunological properties (13), is not microsomal EH which has leaked into the cytosol, thereby acquiring these different properties. It is apparently a distinct entity under different biosynthetic control, for otherwise it should be increased in hyperplastic nodules where microsomal EH is increased. Furthermore, no EH activity was detectable in the cytosolic fraction of nodules using typical substrates of microsomal EH such as benzo(a) pyrene 4,5-oxide, styrene 7,8-oxide, and estroside. Also, no precipitin line was formed between the cytosol and anti-EH- IgG at any concentration used. These results indicate that no microsomal EH is detectable in the 100,000 × g supernatant fraction.

The microsomal EH was characterized with several modulators of diverse structures (17, 28), including an uncompetitive inhibitor, TCPO (28); a noncompetitive inhibitor, cyclohexene oxide (28); and a competitive inhibitor, cis-β-me-STO (Chart 1), as well as closely related compounds, which varied widely in their effectiveness from potent to weak inhibitors, inactive isomers, or activators. No differences in the interaction of these inhibitors with microsomal EH with styrene oxide as substrate were detectable in nodule compared with control microsomes. In all cases, styrene oxide hydrolysis was inhibited potently by TCPO, moderately by cyclohexene oxide, very weakly by cis-stilbene oxide, and not observably influenced by trans-stilbene oxide. In contrast to these two 1,2-diphenyloxiranes, the cis- and trans-isomers of the two 1-phenyl-2-methyloxiranes showed greater differences in inhibition. cis-β-me-STO was a relatively potent inhibitor, while trans-β-me-STO had no effect on styrene oxide hydrolysis. The third monomethyl styrene oxide derivative used (α-me-STO) caused a weak inhibition. The di- and trimethyl-substituted styrene oxides activated EH with styrene oxide as substrate equally in microsomes from nodules and control tissue. These results suggest that the catalytically active site of microsomal EH exists in a similar conformation in hyperplastic nodules and in control microsomes.

It is noteworthy that experiments using liver nodules induced by 2 quite different regimens yielded results which were very similar if not identical. This suggests that, in respect to the properties examined, rapid emergence of nodules induced by the brief exposure to dietary AAF plus PH following an initiating dose of DENA were comparable to the slowly emerging nodules induced by prolonged exposure to dietary AAF. These findings are consistent with the suggestion that the changes in EH reflect the cell populations in the nodules rather than the method of induction or selection.

From the data presented in this study, it follows that the earlier observed differences in diffusibility of EH from nodule and normal tissue microsomes are not likely due to major structural differences in the respective protein molecules. Other possible explanations for this difference in diffusibility include alterations in overall membrane structure and/or assembly in hyperplastic nodules or changes in a small portion of the microsomal EH molecule which affect its association with the membrane but not its catalytic or antigenic sites. Whether these changes may be a cause or effect of hyperplastic growth remains an intriguing question.

ACKNOWLEDGMENTS

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Fig. 1. Ouchterlony double diffusion analysis of microsomal or cytosolic epoxide hydrolase from various tissues. Microsomes were solubilized in sodium cholate. The gel (1% agarose) contained 0.1% Cutscum. The center well contained 100 μg anti-EH-IgG. The outer wells contained: at 11 and 5 o'clock, 3.2 μg apparently homogeneous microsomal EH; at 1 o'clock, 15 μg AAF-nodule microsomes; at 3 o'clock, 40 μg AAF-control microsomes; at 7 o'clock, 17 μg DENA-AAF-PH-nodule microsomes; at 9 o'clock, 30 μg DENA-control microsomes.
Fig. 2. Ouchterlony double diffusion analysis of cytosolic fractions from various tissues versus antibodies which were raised against microsomal epoxide hydrolase. **Left-hand side,** the center well contained 100 µg anti-EH-IgG. The outer wells contained: at 11 o'clock, 3.2 µg apparently homogeneous microsomal EH; at 1 o'clock, a mixture of 3.2 µg apparently homogeneous microsomal EH plus 50 µg of cytosol from (a) AAF-nodules, (b) AAF-control, (c) DENA-AAF-PH-nodules, (d) DENA-AAF-PH-control tissue. From 3 to 9 o'clock, the wells contained cytosol, diluted from 50 µg (3 o'clock) to 1/16 (5 o'clock), 1/4 (7 o'clock), 1/8 (9 o'clock). **Right-hand side,** the center well contained 50 µg of cytosolic protein from each tissue preparation. The outer wells contained 100 µg anti-EH-IgG (11 o'clock) or serial dilutions from 1/8 to 1/32.
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