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EVIDENCE FOR THE NONENZYMATIC AND IRREVERSIBLE BINDING OF CYTEMBENA TO RAT LIVER MICROSOMES *IN VITRO**

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Abstract—Characteristics of the irreversible binding of cytembena (CYT) to rat liver microsomal proteins have been investigated *in vitro*. Binding of [^{14}C]CYT to rat liver microsomal proteins remained unchanged in the presence of SKF-525A or after heat denaturation and was not dependent upon the presence of pyridine nucleotide (NADPH or NADH). Ethacrynic acid, cysteine, dithiothreitol, and lysine were found to block the irreversible binding of [^{14}C]CYT to microsomal proteins in a dose-dependent manner. The rank order of effectiveness as inhibitors of CYT binding was ethacrynic acid > cysteine > dithiothreitol > lysine. In other studies, CYT was shown to preferentially form adducts with cysteine rather than lysine or glycine. Using structural analogs and metabolites of CYT, it was found that 4-methoxybenzoylacrylic acid and β-benzoylacrylic acid were effective competitors of [^{14}C]CYT binding to liver microsomal proteins. By contrast, 4-methoxybenzoylpropionic acid, 5-(4-methoxyphenyl)dihydro-2(3*H*)furanone and 4-hydroxy-4-(4-methoxyphenyl)butyric acid were ineffective as inhibitors of the irreversible binding of CYT. These data suggest that sulfhydryl groups are involved in the nonenzymatic binding of CYT and that the presence of a carbon-carbon double bond in CYT, in debrominated metabolites or structural analogs, is requisite for an interference of the binding of CYT to microsomal proteins.

Cytembena (CYT; sodium *cis*-β-4-methoxybenzoyl-β-bromoacrylate; NSC 104801) is a derivative of β-crotonolactone which possesses cytostatic activity [1]. This compound has demonstrated valuable antineoplastic properties in the treatment of solid tumors associated with uterine cervic carcinoma and forms of genital carcinoma [2–4]. Studies designed to elucidate biochemical mechanisms using animal models suggested that CYT was an inhibitor of 10-formyltetrahydrofolate synthesis [5], while studies using mammalian cells indicated an inhibitory effect on DNA biosynthesis at the level of DNA polymerase [6]. *In vitro* experiments failed to confirm the direct interaction of CYT with either DNA or DNA polymerase.

It is not uncommon for antineoplastic agents to produce many side effects. While CYT has shown little hematopoietic toxicity [7, 8], there are indications of nephrotoxicity [9, 10]. A consideration of the chemical structure and a study of the metabolic conversion of CYT indicated that this cytostatic agent may interact with endogenous thiol groups [11]. We believe that this interaction, although suggested not to be related to the mechanism of antineoplastic action [11], may play a significant role in the nephrotoxicity associated with CYT administration.

In our preliminary experiments on the metabolism of CYT, we found that a significant fraction of this drug was tightly bound to liver microsomal proteins, *in vitro*. This study was initiated to determine whether the extent of binding was due to an enzymatic formation of bioactivated metabolites of CYT and to further evaluate the nature of this drug–protein interaction in liver microsomes. This communication shows that (a) the binding of CYT to liver microsomes is a nonenzymatic and irreversible process, and (b) the presence of an acrylic acid moiety is a structural feature for the interaction of CYT and structural analogs with microsomes. Evidence is also presented which suggests that CYT may preferentially interact with thiol groups.

MATERIALS AND METHODS

Animals. Male albino Sprague–Dawley (Harlan Laboratories, Cumberland, IN) rats weighing between 200 and 300 g were used. All animals were maintained on Purina rat chow diet and received water *ad lib.*

Chemicals. Cytembena[phenyl-^{14}C (U)] (specific activity: 43.47 μCi/mg) was obtained from the Stanford Research Institute (Menlo Park, CA). Thin-layer ascending chromatography on silica gel GF (250 μm) with benzene–ether–acetic acid (50:30:1) revealed a single radiometric peak corresponding to an $R_f = 0.45$. The radiochemical purity of the [^{14}C]CYT was > 99%. Cysteine was purchased from Matheson, Coleman & Bell (Norwood, OH), SKF-525A from Smith Kline & French (Philadelphia, PA), ethacrynic acid from Merck Sharp & Dohme (Rahway, NJ), β-benzoylacrylic acid from ICN Pharmaceuticals, Inc. (Plainview, NY) and 4-

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methoxybenzoylpropionic acid and 4-methoxybenzoylacrylic acid from the Aldrich Chemical Co., Inc. (Milwaukee, WI). All other biochemicals were obtained from the Sigma Chemical Co. (St. Louis, MO). 4-Hydroxy-4-(4-methoxyphenyl)butyric acid and 5-(4-methoxyphenyl)dihydro-2(3*H*)furanone were synthesized in our laboratories for use in these experiments.

Preparation of liver microsomes. Animals were killed by cervical dislocation, and the livers were excised immediately, rinsed in ice-cold buffer, weighed, minced and then homogenized in 0.02 M Tris–HCl buffer, pH 7.4, containing 1.15% KCl using a glass homogenizer equipped with a Teflon pestle. Unless stated otherwise, all steps were carried out at 0–4°. The homogenates (25%, w/v) were centrifuged in a Sorvall RC2-B refrigerated centrifuge at 9000 *g* for 20 min. The microsomal fraction was obtained by centrifuging the supernatant fraction at 105,000 *g* for 1 hr in a Beckman model L5-75 ultracentrifuge. The 105,000 *g* supernatant fraction was recovered, and the microsomal pellet was washed and resuspended in 0.02 M Tris–HCl buffer, pH 7.4, containing 1.15% KCl and centrifuged at 105,000 *g* for 30 min. The final pellet was resuspended in the above buffer such that the final concentration of microsomal protein was 10 mg/ml. Protein content was assayed by the method of Lowry *et al.* [12].

Incubations. Protein binding *in vitro* was determined in an incubation mixture consisting of 10 mg microsomal protein with or without an NADPH-generating system (5 mM $MgCl_2$, 0.44 mM $NADP^+$, 6.4 mM glucose-6-phosphate, and 1 enzyme unit glucose-6-phosphate dehydrogenase) or NADH (0.47 mM) in a final volume of 3.0 ml of 0.02 M Tris–HCl buffer (pH = 7.4). In most experiments, 5 μmoles CYT (0.5 μCi) was used in the reactions. After the addition of CYT, reaction mixtures were incubated under air at 37° with shaking (90 cycles/min) in a metabolic incubator. Unless reported otherwise, the incubation time was 40 min. In other experiments, reaction mixtures were preincubated for 10 min with SKF-525A (5×10^{-4} M), an NADPH-generating system, amino acids (cysteine, lysine, glycine), dithiothreitol, ethacrynic acid or structural analogs of CYT before the addition of [^{14}C]CYT.

Isolation of ^{14}C-bound protein adducts. Reactions were terminated by the addition of 0.5 ml of 1 N HCl, and flasks were transferred to ice. Each reaction mixture was then extracted twice with diethyl ether (7 ml vol.) followed by the addition of 3.5 ml of 95° 10% (w/v) trichloroacetic acid (TCA) to recover a protein precipitate (10,000 *g* for 10 min). A second hot TCA treatment and centrifugation were completed to obtain a protein pellet. This pellet was successively extracted twice each with 3 ml vol. of ethanol–water (1:1), ether, and then chloroform–methanol (2:1). The final pellet was then solubilized in 2 ml of 1 N NaOH by heating at 50° for 8 hr. The resultant protein solution was assayed by the method of Lowry *et al.* [12] using a bovine serum albumin (fraction V) as a standard. Aliquots of the protein solution were dissolved in 10 ml of Thrift-Solv (Kew Scientific, Columbus, OH), and the ^{14}C was measured by liquid scintillation spectrometry using external standardization as the quench monitoring method. Data were expressed as nmoles CYT bound per mg microsomal protein (nmoles/mg protein).

The methods employed for extraction of unbound [^{14}C]CYT were extensive and designed to ensure that covalently bound ^{14}C-conjugates of CYT were assayed. The possibility that a TCA-insoluble analogue of CYT or unchanged CYT itself was being assayed in the recovered protein pellet was unlikely. This suggestion is supported by the near quantitative recovery (≥ 99%) of the [^{14}C]CYT from nonincubated reaction mixtures or samples incubated in the absence of liver microsomes.

^{14}C-Adducts of CYT to amino acids. Cysteine, lysine or glycine (6×10^{-3} M) was dissolved in 0.02 N Tris–HCl buffer, pH 7.4, and incubated with 10^{-3} M [^{14}C]CYT (0.23 μCi/mmole) in a final volume of 2 ml. After 30 min, incubation mixtures were acidified with 2 ml of 1 N HCl and extracted with ether (five times, 5 ml) until no further ^{14}C could be recovered in an ether extract. Aliquots of the residual aqueous phase were spotted on silica gel GF TLC plates (250 μm) and developed with benzene–ether–acetic acid (50:30:1). Unbound [^{14}C]CYT migrated to an R_f of 0.45 while the ^{14}C-bound amino acid conjugates remained at the origin. Location of ^{14}C was achieved through zonal scraping of gel segments and quantitation by liquid scintillation spectrometry as described previously.

Statistical analysis. Statistical comparisons of independent sample means were made using Student's *t*-test and a 95% level of significance.

RESULTS

Incubation of CYT with microsomal protein resulted in a time-dependent increase in irreversible ^{14}C-protein binding throughout a 2-hr incubation period. As shown in Fig. 1, nearly 50% of the total ^{14}C-bound was complete at 20 min and subsequent ^{14}C-binding was linear for the remaining incubation time. After 2 hr, the total ^{14}C-bound was

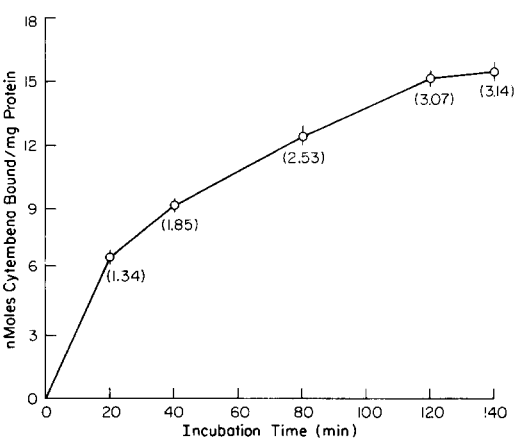

Fig. 1. Time course of the irreversible binding of [^{14}C]cytembena to liver microsomal proteins. Plotted values represent the means ± S.E.M. of N = 3. Values in parentheses indicate the percent of total ^{14}C bound.

Table 1. Influence of various treatments on [^{14}C]cytembena binding to liver microsomal proteins*

Treatment	nmoles CYT bound/mg protein† Control	Treated	% of Control
SKF-525A (0.5 mM)	11.08 ± 0.59	10.09 ± 0.18	91.1
Heat denaturation (100° for 10 min)	12.16 ± 0.43	11.22 ± 0.14	92.3
NADPH-generating system	11.08 ± 0.59	6.07 ± 0.45	54.8‡
Generating system minus NADP	11.08 ± 0.59	7.02 ± 0.53	63.4‡
NADPH (0.83 mM)	11.08 ± 0.59	9.53 ± 0.22	86.0‡
NADH (1.0 mM)	11.71 ± 0.71	9.60 ± 0.26	82.0‡

* Incubation mixtures contained 10 mg microsomal protein, 1.7 mM cytembena (0.5 μCi) and various compounds or cofactors in a final volume of 3.0 ml of 20 mM Tris–HCl buffer, pH 7.4. Time of incubation was 40 min.

† Each value is the mean ± S.E.M. of N = 3.

‡ Significant difference between control and treated preparations ($P < 0.05$).

15.72 ± 0.29 nmoles/mg protein (mean ± S.E.M. of N = 4).

The requirements for the binding of [^{14}C]CYT to microsomal protein are given in Table 1. In these experiments, it was evident that drug-metabolizing enzymes associated with the liver microsomal fraction did not play a functional role in the irreversible binding of [^{14}C]CYT. The effects of the addition of SKF-525A (5×10^{-4} M) or complete heat denaturation (100° for 15 min) as pretreatment supported this view since neither treatment affected binding as compared to the control value. The reduction of CYT binding in the presence of an NADPH-generating system, to 54.8% of the control, paradoxically suggested an enzymatic inactivation of the binding species of CYT. However, the generating system components (minus $NADP^+$) alone were found to reduced binding to a similar level (63.4% of control values). The direct addition of NADPH and NADH also reduced ^{14}C-binding to 86.0 and 82.0% of the control binding respectively. These findings were also qualitatively and quantitatively similar to results observed in the binding of CYT to the liver 105,000 *g* supernatant fraction (unpublished observations).

Since the mixed-function oxidase system was not required to facilitate the binding of CYT to microsomal protein, it was of interest to examine the chemical nature of the sites implicated in this non-enzymatic binding process. Various amino acids and dithiothreitol were incubated with [^{14}C]CYT and microsomal protein and were found to compete with this binding process. In our preliminary studies, cysteine and dithiothreitol, which contain sulfhydryl groups, were found to significantly ($P < 0.05$) reduce CYT binding to liver microsomes by 25–30% at concentrations of $\geq 4 \times 10^{-2}$ M. Lysine was found to be an inhibitor of [^{14}C]CYT binding but required much higher concentrations ($> 10^{-1}$ M). These data are consistent with a suggestion that CYT could directly bind to the nucleophilic groups of these compounds. To assess this possibility, [^{14}C]CYT was incubated with cysteine, lysine and glycine, in the absence of microsomal protein, and the extent of ^{14}C bound to the amino acids was measured (Table 2). Under these conditions, [^{14}C]CYT was found to preferentially bind to cysteine with 20.7% of the total CYT being associated as a ^{14}C-adduct of this amino acid. Lysine bound about twice as much ^{14}C as glycine, and the formation of these ^{14}C-adducts was only slightly greater than the incubated control.

The logical question that arose was what structural characteristics of CYT are required for this irreversible binding process? Various analogues of CYT were compared as competitors of the ^{14}C-binding process in liver microsomes (see Fig. 2 for chemical structures). Of the analogs tested, 4-methoxybenzoylpropionic acid (MBPA) was the least effective inhibitor of [^{14}C]CYT binding. In fact, ^{14}C-binding of CYT to microsomal protein was not reduced by MBPA at any concentration tested (Fig. 3). By contrast, 4-methoxybenzoylacrylic acid was the most effective inhibitor of ^{14}C-binding. This analogue exhibited a concentration-dependent reduction in total CYT binding. The addition of ethacrynic acid also produced a concentration-dependent inhibition similar to that of 4-methoxybenzoylacrylic acid and which was considerably more effective than the addition of CYT on the blockade of ^{14}C-binding to microsomal proteins (Fig. 3).

An examination of structural derivatives and metabolites of CYT and of their influences on ^{14}C-binding to liver microsomes is given in Fig. 4. At 10^{-3} M, ethacrynic acid and β-benzoylacrylic acid were effective inhibitors of [^{14}C]CYT binding to microsomal proteins ($P < 0.05$). Each of these compounds was more effective as an inhibitor of ^{14}C-binding than unlabeled CYT. The relative order of effectiveness was 4-methoxybenzoyl acrylic acid > β-benzoylacrylic acid > ethacrynic acid > CYT. 5-(4-Methoxyphenyl)dihydro-2(3*H*)-furanone, 4-hydroxy-4-(4-methoxyphenyl)butyric acid and 4-methoxybenzoylpropionic acid were not significant inhibitors of the [^{14}C]CYT binding at 10^{-3} M.

Table 2. Binding of [^{14}C]cytembena to various amino acids*

Conjugating agent	Percent of [^{14}C]cytembena bound to amino acid†
None	1.4 ± 0.20
Cysteine	20.7 ± 0.84‡
Lysine	2.7 ± 0.12‡
Glycine	1.1 ± 0.41

* Incubation mixtures contained 1.7 mM cytembena (0.5 μCi) and 6×10^{-3} M amino acid in 3.0 ml of 0.2 M Tris–HCl buffer, pH 7.4. Incubation time was 1 hr. Aliquots of the reaction mixture were applied to TLC plates (silica gel) and separated by ascending chromatography in benzene–methanol–acetic acid (50:30:1). R_f values of cytembena and conjugate were 0.45 and 0.1 respectively.

† Each value is the mean ± S.E.M. of N = 3.

‡ Significant difference ($P < 0.05$) between control and amino acid treated preparations.

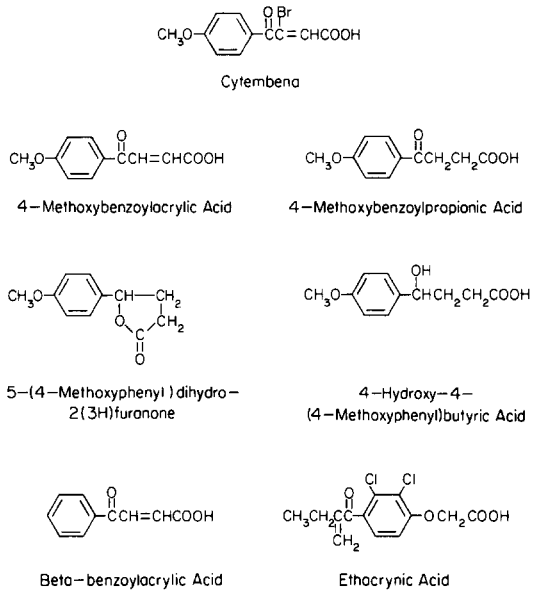

Fig. 2. Chemical structures of cytembena and related analogs.

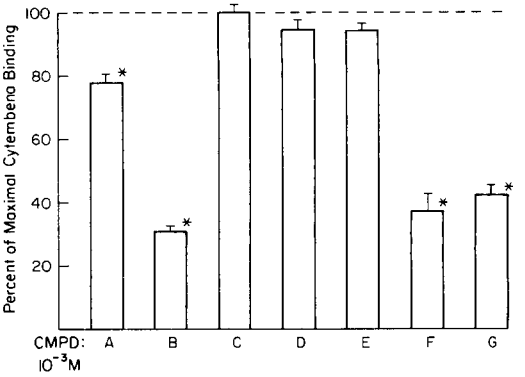

Fig. 4. Comparative effects of cytembena and related analogs on the inhibition of [^{14}C]cytembena binding to liver microsomal protein. Key: (A) cytembena; (B) 4-methoxybenzoylacrylic acid; (C) 4-methoxybenzoylpropionic acid; (D) 5-(4-methoxyphenyl)dihydro-2(3*H*)furanone; (E) 4-hydroxy-4-(4-methoxyphenyl)butyric acid; (F) β-benzoylacrylic acid; and (G) ethacrynic acid. The concentration of each compound was 10^{-3} M, and the values represent the means ± S.E.M. of N = 3. An asterisk indicates a significant difference ($P < 0.05$) between means in the presence or absence of the compound.

DISCUSSION

Our experiments have defined major characteristics of the binding process associated with CYT. These data have shown that the *in vitro* binding processes for CYT are nonenzymatic and irreversible. This proposal was supported by experiments that failed to show a difference in ^{14}C-binding after prior heat denaturation and that indicated an independence from pyridine nucleotides (NADPH, NADH) or SKF-525A. Thus, the binding of CYT to microsomal protein in our experiment is not attributable to a conversion to a more reactive metabolite.

By these studies, it is clear that thiol groups contained in liver microsomal protein are implicated as sites of CYT binding *in vitro*. Such a view has been suggested previously by Jackson *et al.* [11] with studies of CYT in the L1210 leukemia-induced mouse model. Our experiments support this view with three different lines of evidence. First, cysteine and dithiothreitol were found to significantly ($P < 0.05$) inhibit the extent of [^{14}C]CYT binding to microsomal protein in a concentration-dependent fashion while lysine was a weak inhibitor of the ^{14}C-binding process. Second, CYT was also found to bind only to the sulfur-containing amino acid, cysteine, in the absence of protein ($P < 0.05$) but not to a significant degree in the presence of glycine or lysine. Third, ethacrynic acid was found to be a potent inhibitor of CYT binding to liver microsomal proteins. Ethacrynic acid is often used as a selective sulfhydryl reagent [13, 14] and is proposed to react with thiol groups in proteins and thiol-containing amino acids *in vivo* and *in vitro* [13–16]. In addition, the profile of the interaction of ethacrynic acid [14] and CYT (Table 2) with amino acids containing various nucleophilic groups is qualitatively similar. This latter finding further indicates that CYT is not a thiol-specific reactant, as has also been suggested for ethacrynic acid [14].

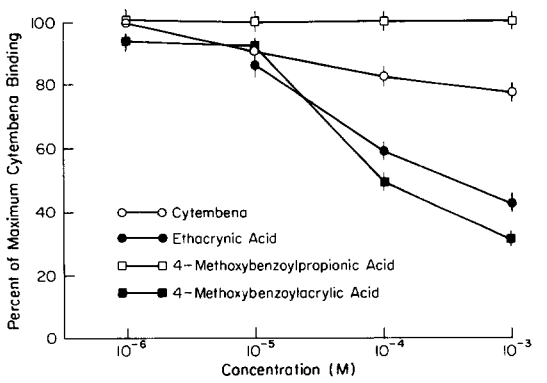

Fig. 3. Dose-dependent effects of cytembena, ethacrynic acid, 4-methoxybenzoylpropionic acid, and 4-methoxybenzoylacrylic acid on the inhibition of [^{14}C]cytembena binding to liver microsomal proteins. Plotted values represent the means ± S.E.M. as indicated by the vertical bars. Time of incubation was 40 min.

Structural requirements of CYT for the binding process were indirectly assessed by the measurement of the relative ability of selected analogues to inhibit [^{14}C]CYT binding to microsomal protein. The most effective inhibitor of ^{14}C-binding was 4-methoxybenzoylacrylic acid whereas 4-methoxybenzoypropionic acid was not an inhibitor. Moreover, ethacrynic acid and 4-methoxybenzoyacrylic acid were more effective inhibitors of [^{14}C]CYT binding than the parent drug. These data emphasize the importance of the carbon-carbon double bond on the side chain and indirectly imply that the presence of the bromine atom was not required for an efficient interaction with tissue binding sites and competition of the binding of [^{14}C]CYT to microsomal proteins.

4-Methoxybenzoylacrylic acid, 4-methoxybenzoylpropionic acid and 5-(4-methoxyphenyl)dihydro-2(3*H*)furanone have been identified as metabolites of CYT, *in vivo* and *in vitro* [17, 18]. In a recent paper Mitoma *et al.* [18] postulated that CYT formed a CYT-glutathione conjugate in liver homogenates which, in turn, was converted to 4-methoxybenzoylpropionic acid and 5(4-methoxyphenyl)dihydro-2(3*H*)furanone. Of these metabolites, we have shown that only 4-methoxybenzoylacrylic acid is a potent inhibitor of [^{14}C]CYT binding to liver microsomal proteins, *in vitro*. Thus, 4-methoxybenzoylacrylic acid may represent a reactive metabolite of CYT which forms the postulated glutathione conjugate reported initially by Mitoma *et al.* [18]. In addition, Mitoma *et al.* [18] proposed that CYT may interact directly with glutathione *S*-transferase(s) to form this thiol conjugate. Our studies do not rule out either of these possibilities. It is clear that further studies will be required to characterize the chemical nature of the protein adducts formed with CYT, *in vitro* and *in vivo*.

CYT is an antineoplastic agent that is capable of producing clear-cut nephrotoxicity while having virtually no hematologic toxicity. Further characterization of the nephrotoxicity suggests that this agent may have an effect on the proximal tubule under therapeutic conditions [9]. Suggestions have been made that some form of irreversible binding to nonspecific tissue sites in the kidney may occur with this compound [9] since high kidney concentrations have been reported both *in vivo* [1] and *in vitro* [9]. In a parallel fashion, ethacrynic acid has been shown to be actively concentrated by renal tissue [19] and is known to complex with cellular thiol groups both *in vivo* [16] and *in vitro* [15]. The mode of action of this drug is believed to clearly relate to an interaction with sulfhydryl groups [13, 15, 20].

The ability of CYT to bind to liver microsomes and the preferential interference with this drug-binding process by compounds possessing thiol groups or structural analogs possessing an acrylic acid side chain have been demonstrated in the present study. The mechanism of the irreversible binding of CYT to liver microsomes occurred by a nonenzymatic process and was correlated with the presence of thiol-containing nucleophilic groups. The relevancy of the nonenzymatic binding of CYT and/or potentially reactive metabolites (e.g. 4-methoxybenzoylacrylic acid) to the observed nephrotoxicity is unclear at the present time. However, based upon the interaction between CYT and ethacrynic acid for tissue nucleophiles and the ability of CYT to concentrate in the kidney *in vivo*, one may suggest that these two phenomena are interrelated.

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