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CONJUGATION OF ACRYLAMIDE WITH GLUTATHIONE CATALYSED BY GLUTATHIONE-*S*-TRANSFERASES OF RAT LIVER AND BRAIN

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Abstract—Acrylamide, an α,β unsaturated electrophile and a cumulative neurotoxin, was found to react with glutathione giving rise to an *S*-conjugate of acrylamide. Glutathione *S*-transferase of rat liver and brain cytosols (active on both acrylamide and 1-chloro 2,4 dinitrobenzene) emerged as a single major peak on elution from Sephadex G-75. The enzymic conjugation of acrylamide with glutathione increased with protein and was dependent on incubation time and pH of medium. Acrylamide inhibited glutathione-*S*-transferase activity towards 1-chloro 2,4-dinitro-benzene of both liver and brain cytosol, in a concentration and time dependent manner. Enzyme catalyzed conjugation of acrylamide with glutathione was induced significantly by phenobarbital and t-SO (*trans*-stilbene oxide). The enzymic conjugation of acrylamide increased two fold from neonatal to adult and then showed a decreasing pattern at subsequent ages.

Reduced glutathione forms *S*-conjugates with a wide variety of electrophiles, alkylating agents, carcinogens and/or their metabolites by a reaction catalyzed by glutathione-*S*-transferases (E.C.2.5.1.18) [1] or non-enzymically by nucleophilic substitution reactions of the SN_2 type [2]. Glutathione-*S*-transferases activity has been detected in the cytosol fractions of tissue homogenates and show broad overlapping substrate specificities. *S*-Conjugate formation by glutathione-*S*-transferases represents the first step in the synthesis of mercapturic acid [3].

Acrylamide (CH_2=$CHCONH_2$), is a widely used chemical in the polymer industry and is a potent neurotoxin [4]. Exposure of acrylamide to humans and animals leads to distal axonopathy of 'dying back type' [4]. Biotransformation of acrylamide is very poorly understood and attempts to evaluate the role of microsomal mixed function oxidase in this process have not met with much success [5].

Acrylamide is capable of interacting with vital cellular nucleophiles possessing —SH, —NH_2 or —OH as reactive groups [4]. Administration of acrylamide to rats leads to biliary excretion of glutathione conjugates accompanied by significant depletion of cellular glutathione [6]. Other than this no direct evidence has so far been presented for an enzyme catalyzed conjugation of acrylamide with glutathione. The structural similarity of acrylamide to known α,β unsaturated substrates of glutathione-*S*-transferases prompted an investigation into the possibility of enzyme catalyzed conjugation of acrylamide with glutathione. In a preliminary communication from this laboratory cytosol fractions of mammalian and avain brain homogenates have been shown to be equipped with GST† mediated conjugation of CDNB with GSH [7]. Evidence is adduced in this report to establish the enzymic nature of the conjugation reaction of acrylamide with glutathione.

MATERIALS AND METHODS

Reaction of acrylamide with glutathione was measured by following the disappearance of glutathione. Glutathione was measured colorimetrically using Ellman's reagent [8]. Activity was expressed as nmoles of glutathione disappeared (reacted) per minute. Reaction mixtures contained 1–10 mM of acrylamide in 3 ml, 0.1 M phosphate buffer pH 8.0 and glutathione (1–10 mM). Reaction was also carried out at various pH values (pH 6 to 8.0, 0.1 M phosphate buffer) using equimolar concentration of ACR and GSH.

Suitable aliquots from blank (only glutathione reduced or oxidized) and test samples were applied on silica gel thin layer chromatographic plates. Chromatograms were developed in solvent system containing *n*-butanol:acetic acid:12:3:5 and spots were detected by ninhydrin (0.2% ninhydrin in alcohol). Areas visualized on plate were eluted with 5.0 ml of 85% ethanol containing 5% $CuSO_4$ and absorbance of the solutions read at 512 nm.

Animals and preparation of cytosols. Male albino rats (160–180 g) derived from Industrial Toxicology Research Centre breeding colony and raised on commercial pellets (Hindustan Lever, Bombay) were used. Liver and brain of rats obtained after exsanguination of rats were removed, blotted free of blood and transferred to containers, kept on an ice basket. Liver and brain pooled from 2–4 rats were homogenized using 4 vol. chilled 0.25 M sucrose, homogenates were centrifuged at 9000 *g* and 14,000 *g*

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† Abbreviations used: Acrylamide: ACR; glutathione: GSH; glutathione-*S*-transferase: GST; 1-chloro 2,4-dinitrobenzene: CDNB; 1-2,-dichloro 4-nitrobenzene: DCNB; phenobarbital: PB; *trans*-Stilbene oxide: t-SO; tri-chloroacetic acid: TCA.

for 20 min to recover post mitochondrial fractions respectively. These were subsequently contrifuged at 104,000 *g* for an hour to obtain organelle free cytosol fractions.

Gel filtration. A 9 ml portion of rat liver and brain cytosol was applied in columns (36 × 2.5 cm) containing Sephadex G-75 equilibrated with 10 mM Tris-buffer pH 8.2, which also served as a mobile. The flow rate was fixed at 30 ml/hr and 5 and 10 ml fractions were collected respectively for liver and brain cytosols.

Induction experiments. Phenobarbital (80 mg/kg in 0.5 ml of 0.15 M NaCl) or *trans*-stilbene oxide (400 mg/kg in 0.5 ml peanut oil) was given intraperitoneally, daily for three days. Control animals received identical volumes of 0.15 M NaCl or peanut oil.

Enzyme assay. Glutathione-*S*-transferase activity towards CDNB or DCNB was assayed essentially as described by Habig *et al.* [1]. The cuvettes in a final volume of 3 ml contained 0.1 M phosphate buffer (pH 6.5 for CDNB and pH 7.8 for DCNB), 1 mM glutathione and 1 mM CDNB or DCNB and suitable aliquots of enzyme source. Change in absorbance at 340 nm (for CDNB) or 344 nm (DCNB) was measured at room temperature (25–35°) against blanks containing all the reagents excepting the enzyme. Specific activity was expressed as nmoles conjugate formed/min/mg protein.

GST activity with acrylamide as substrate was assayed by following the disappearance of glutathione as described for conjugation of unsaturated compounds by Boyland and Chasseaud [15]. Specific activity was expressed as nmoles GSH disappeared/min/mg protein. Assay mixture in 3 ml contained 5 mM ACR, 5 mM GSH, 0.1 M phosphate buffer pH 7.4 and suitable aliquots of enzyme source. For non-enzymic reactions assays were conducted under the above conditions except that of denatured enzyme (prepared by heating the enzyme at 100° for 10 min) was used in place of enzyme source. Reaction was stopped by adding 1 ml of 5% TCA. The residual glutathione was determined in supernatants free of TCA precipitable materials.

Enzyme catalyzed conjugation of ACR with GSH was studied as a function of protein concentration and incubation time. Effect of pH on enzymic conjugation could not be accurately assayed because of a high non-enzymic rate of conjugation at higher pH values i.e. above pH 7.4 and 8.0. Activity of the enzyme catalysed conjugation of ACR with GSH was calculated by substracting the activity of non-enzymic reaction from total activity of conjugation (nonenzymic + enzymic) reaction.

GST activity of liver and brain cytosols obtained by gel filtration was assayed over a wide range of substrate concentrations of CDNB. K_m was derived from abscissa intercepts of the Lineweaver–Burk plot [10].

GST activity towards CDNB was investigated over a wide range of concentration of acrylamide. Inhibitor constant was derived from least square regressions equation using the Dixon plot [11].

Protein was measured by the method of Lowry *et al.* [12] using bovine serum albumin as reference standard.

Table 1. Non-enzymic conjugation of ACR with GSH was carried out at various concentrations and ratio of the substrates, i.e. ACR and GSH as described below at pH 8.0 (0.1 M phosphate buffer) and as described in text. Activity of reaction was expressed as nmoles GSH disappeared/min

Ratio of ACR and GSH (mM) in incubation mixture	Activity (nmoles GSH disappeared/min)
ACR:GSH::1:1	0.040
ACR:GSH::1:0.5	0.030
ACR:GSH::1:0.2	0.015
ACR:GSH::1:0.1	0.010
GSH:ACR::1:0.5	0.030
GSH:ACR::1:0.2	0.020

Data represent mean of three values.

RESULTS

Data presented in Table 1 show that acrylamide reacts with glutathione and the activity was dependent on concentration of both the reactants i.e. acrylamide and glutathione. It is also apparent that maximum reaction occurred when both the reactants were present in equimolar proportion. Table 2 shows the effect of pH on reaction of acrylamide with glutathione. A significant rate of conjugation reaction was evident at higher pH values (pH 7.5 and pH 8.0). Glutathione adduct of acrylamide, separated on silica gel thin layer chromatographic plate, exhibited a R_f value of 0.5 against R_f values of 0.4 and 0.32 for reduced and oxidized glutathione respectively.

Elution profile of glutathione-S-transferase. Elution profile of glutathione-*S*-transferases of rat liver and brain cytosol towards CDNB or DCNB and ACR as substrates is depicted by Fig. 1(a) and 1(b) respectively. It would be seen that enzyme conjugating acrylamide with glutathione was eluted in a single major peak along with GST activity on CDNB or DCNB. A similar elution profile of GST of rat liver from Sephadex G-75 towards CDNB has also been reported earlier [16].

Characterization of liver and brain enzyme catalyzed conjugation of acrylamide. Enzyme catalyzed reaction of ACR with GSH was studied using GST rich fraction peak of rat liver and brain cytosol.

Table 2. Effect of pH on the activity of non-enzymic conjugation of ACR with GSH: Reaction in 3 ml incubation mixture contained 1 mM ACR and 1 mM GSH in 0.1 M phosphate buffer of various pH values as described below. Reaction was carried out and activity was measured essentially as described in text

pH	Activity (nmoles GSH disappeared/min)
6.0	N.D.
6.5	0.03
7.0	0.04
7.5	0.09
8.0	0.23

Data represent mean of three values.
N.D.—Not detectable.

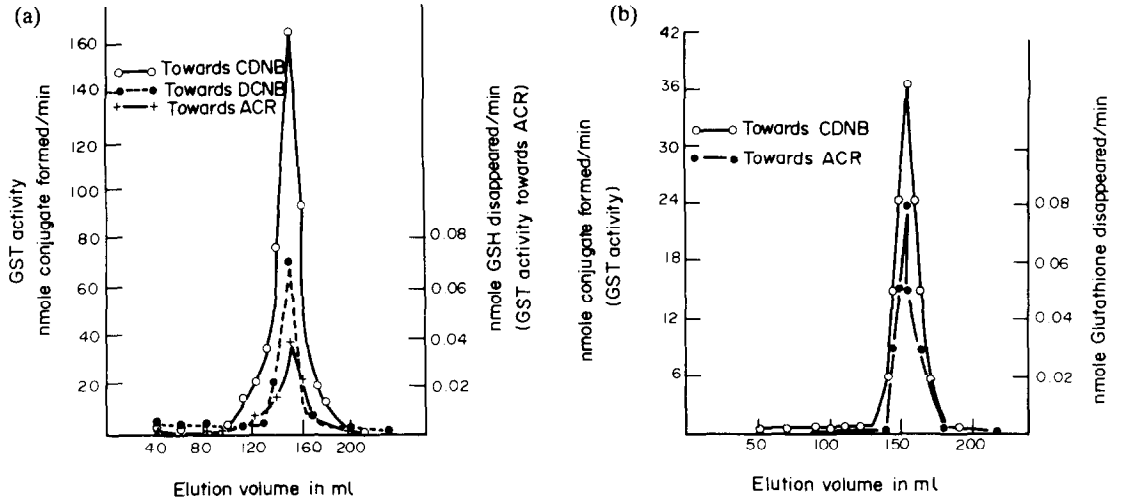

Fig. 1. Elution profile of liver and brain GST from Sephadex G-75. (a) Liver: Rat liver cytosol (9 ml) was applied in a column of Sephadex G-75 equilibrated with 10 mM tris-buffer pH 8.2. Same buffer was used as mobile phase. Fractions were collected in 10 ml volume with a flow rate of 30 ml/hr. For assay of GST activity towards CDNB and DCNB or ACR as substrates, 50–120 μg protein and 0.10–0.24 mg protein, respectively was used and respective activities were assayed as described in text. Values of enzyme activities were corrected for non-enzymic contributions. (b) Brain: Rat brain cytosol (9 ml) was applied in column of Sephadex G-75 equilibrated with 10 mM tris-buffer pH 8.2. Same buffer was also used as mobile phase. Fractions were collected in 5 ml volume. For assay of GST activity towards CDNB as substrate 40 μg protein and towards ACR 1.2 mg protein was used. Values for enzyme activities in each case were corrected for non-enzymic contribution.

Figure 2(a) and 2(b) depict the dependence of enzymic activity on protein concentration. Addition of enzyme protein significantly increased the marginal conjugation of ACR with GSH in the absence of enzyme. Enzyme activity was linear up to 30 mins. (Fig. 3(a) and 3(b) for liver and brain enzyme respectively). Enzymic activity increased upto pH 7.4, the reaction could not be studied beyond this pH value because of a interference by non-enzymic conjugation.

It may be noted that both liver and brain GST mediate the conjugation reaction (Table 3). Glutathione-*S*-transferase activity of rat liver towards CDNB was 5 times higher and towards acrylamide was 3 times more as compared to that of brain (Table 3). Further Michaelis constants (K_m) of liver and brain GST using CDNB as substrate (as derived from Fig. 4(a) and 4(b) were 1.6 mM and 2.5 mM respectively.

Inhibition of GST activity towards CDNB as substrate by acrylamide. Acrylamide elicited a concentration dependent inhibition of GST activity of liver towards CDNB (Fig. 5) No significant effect of acrylamide on non-enzymic conjugation was noticed. Inhibition of GST activity was also dependent on pre-incubation time of enzyme with ACR (Fig. 6). Inhibition of GST activity of liver and brain on CDNB as substrate was also evaluated using wide range of substrate concentrations. Figure 5(a) and 5(b) represent Dixon plot for inhibition of GST by acrylamide of rat liver and brain inhibition constants derived from the plots were 4 mM and 3.3 mM

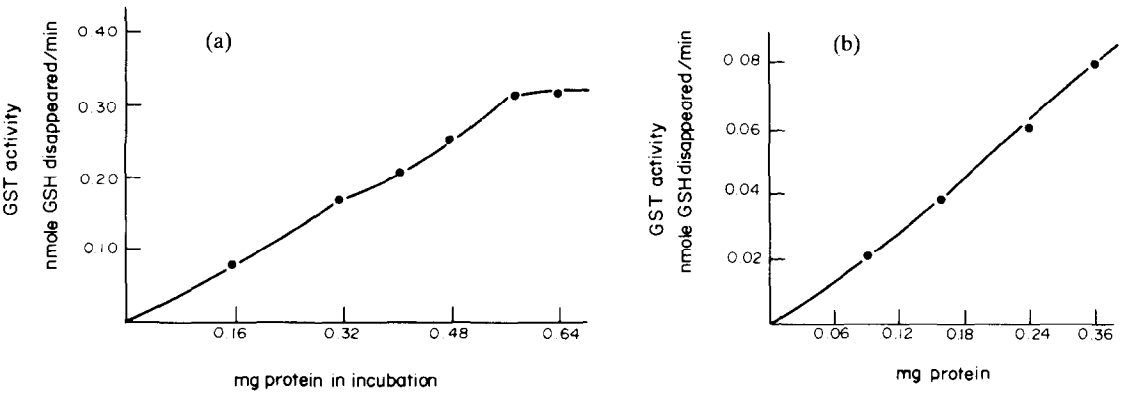

Fig. 2. Dependence of GST activity on protein concentration. (a) Liver: Assay system for GST activity towards ACR is given in text. Aliquots of enzyme (peak GST fraction) containing 0.16 mg to 0.80 mg protein were taken and unit activity vs protein concentration at pH 7.4 was determined. (b) Brain: Aliquots of enzyme (peak fraction of rat brain cytosol) containing 0.08 mg to 0.40 mg protein were added in assay mixture and unit activity vs protein concentration at pH 7.4 was determined.

Table 3. Comparison of glutathione-*S*-transferase of rat liver and brain (for details see text)

Parameter	Liver	Brain	Ratio
Glutathione-*S*-transferase* towards CDNB (peak fraction)	4836	937	5.16
Glutathione-*S*-transferase† towards acrylamide (peak fraction)	0.243	0.081	3.00
Michealis constant (K_m) of glutathione-*S*-transferase CDNB)	1.6 mM	2.5 mM	0.64
Inhibitor constant (K_i)	4.0 mM	3.0 mM	1.33

Data from a typical experiment.
* Nanomoles conjugate formed/min/mg protein (calculated from Fig. 1(a) and 1(b)).
† Nanomoles glutathione disappeared/min/mg protein (from Fig. 1(a) and 1(b)).

against K_m values of 1.6 and 2.5 M for liver and brain GST.

Effect of phenobarbital and trans*-stilbene oxide on enzyme catalyzed conjugation of ACR with GSH.* Results given in Table 4 show that phenobarbital caused 140 per cent acceleration of GST activity towards ACR and 65 per cent towards CDNB. A 50 per cent increase in enzymic conjugation of ACR and 60 per cent in conjugation of CDNB was evident in stimulation with *trans*-stilbene oxide in rats.

Influence of age on enzyme catalyzed conjugation of ACR with GHS. The enzymic conjugation of ACR with GSH was low in young rats and gradually increased with the age (two-fold from neonatal to adult). The enzymic activity of this reaction showed a decreasing pattern at subsequent ages i.e. up to 8 months (Table 5).

DISCUSSION

Present studies demonstrate that acrylamide reacts with glutathione both non-enzymically and enzymically. Non-enzymic reaction was dependent on substrate concentration and pH suggesting a direct reaction of acrylamide with glutathione resulting in acrylamide–glutathione adduct. Formation of glutathione-*S*-conjugates has long been shown to be an initial step in the biotransformation of electrophiles into mercapuric acids [13, 14]. Reaction of α,β unsaturated compounds involves the addition of nucleophilic GS^- to the β carbon atom of the double bond polarized by conjugation with strongly electron withdrawing group [15]. It is likely that acrylamide, like many other α, β unsaturated electrophiles, reacts with glutathione in a similar manner. From the elution profile of enzyme conjugating ACR with GSH, it would be evident that this enzyme is identical with the GST, which catalyze conjugation of CDNB or DCNB (the conventional substrates). The function of GST catalyzed conjugation of acrylamide with glutathione would be to accelerate the formation of *S*-conjugate and stimulate the disposal of toxic chemical. Inhibition of GST activity towards CDNB by acrylamide may possibly be due to the interaction of acrylamide at a site near to the binding site of CDNB on enzyme or may be due to a competition between CDNB and ACR for GSH. However it is difficult at present to evaluate the mechanism of this inhibition.

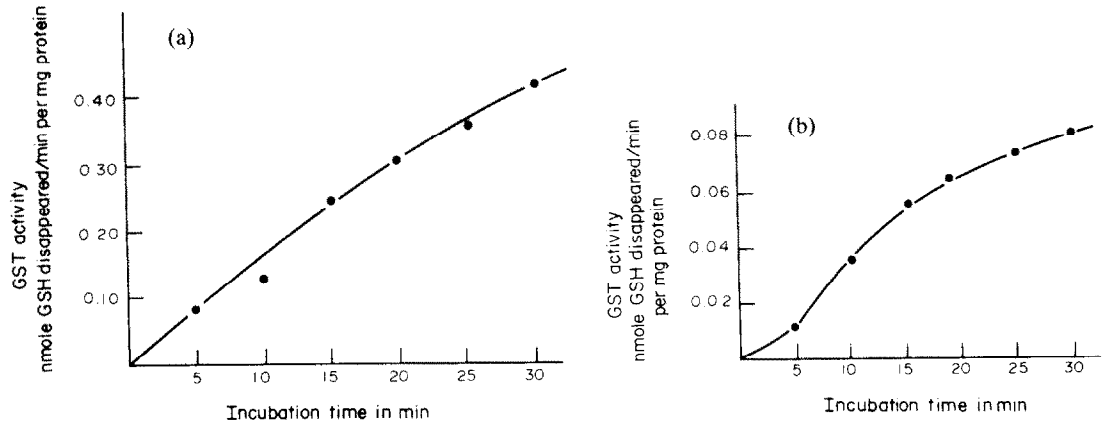

Fig. 3. Effect of incubation time on enzyme activity. (a) Liver enzyme: GST activity towards ACR using desired incubation time was carried out at pH 7.4 using 0.25 mg enzyme protein in 0.1 M phosphate buffer. A plot of GST activity vs time is presented. (b) Brain enzyme: Brain GST activity towards ACR was assayed at pH 7.4 (0.1 M phosphate buffer) using 0.4 mg enzyme protein as a function of incubation time.

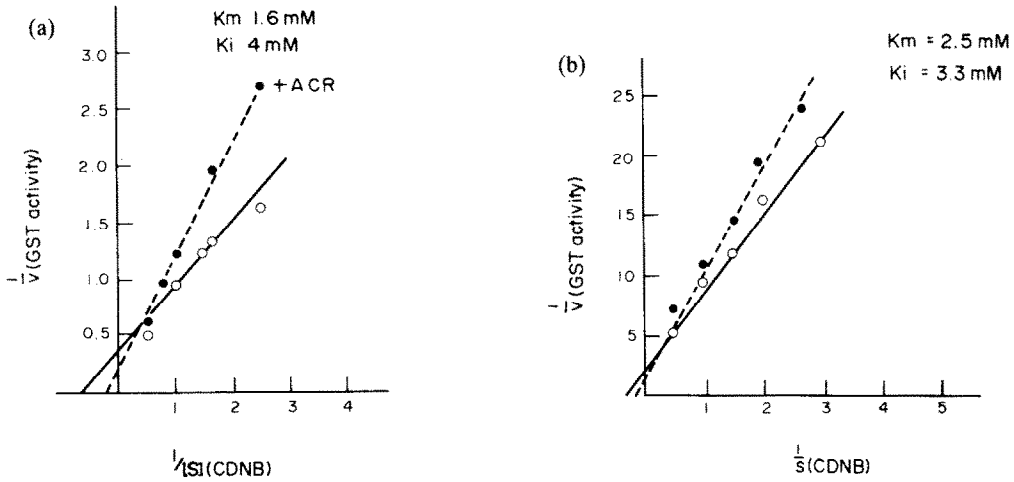

Fig. 4. Dixon plot for determination of K_i for hepatic and brain GST. (a) Liver: Activity of GST over wide range of CDNB concentration was assayed in presence of 10 mM ACR. Data are presented in the form of Dixon plot. Respective K_m and K_i were determined. GST activity is defined as nmole conjugate formed/min/mg protein. (b) Brain: procedure is same except brain GST was used in place of liver enzyme. GST activity is expressed as nmoles conjugate formed/min/mg protein.

PB and t-SO are conventional inducers of hepatic glutathione-*S*-transferase activity with CDNB and other conventional substrates (17–20). Inducibility of enzyme (GST) catalyzed conjugation of ACR with GSH by PB and t-SO provides additional evidence in favour of the occurrence of GST catalyzed conjugation of ACR. Further, postnatal developmental profile of enzyme conjugating ACR with GSH was also compatible to that of GST, catalyzing conjugation of CDNB or other substrates [21].

Although the activity for conjugation of ACR in brain was less than in liver, the presence of the enzyme provides a mechanism of detoxication particularly of neurotoxin such as acrylamide.

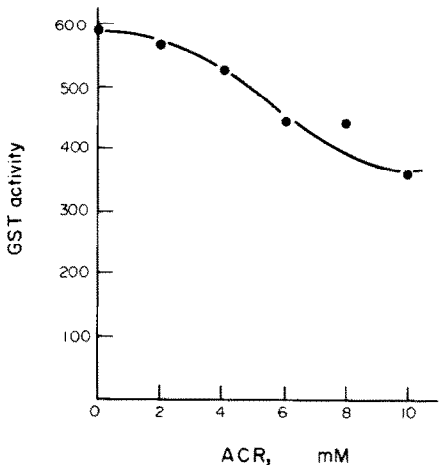

Fig. 5. Inhibition of hepatic GST (towards CDNB) by acrylamide. Acrylamide, 2 to 10 mM was added in a reaction mixture containing aliquots of enzyme source (120 μg of cytosolic protein) buffer and GSH and pre-incubated for ten minutes. Reaction was started by adding CDNB. GST activity vs acrylamide concentration is presented. GST assay towards CDNB is described in methods. GST activity is expressed as nmoles conjugate formed/min/mg protein.

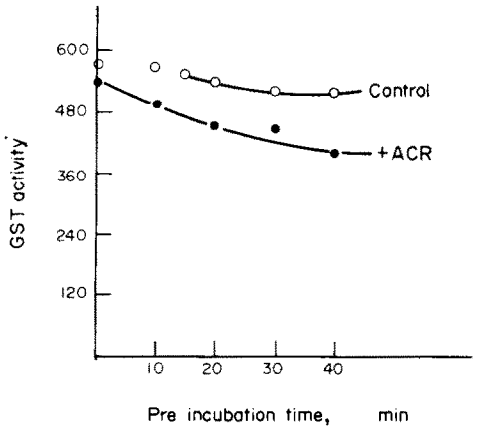

Fig. 6. Dependence of inhibition of GST on pre-incubation time. ACR (10 mM) was added in a reaction mixture and pre-incubated for different time, rest of the details are same as described in Fig. 5. GST activity is expressed as nmoles conjugate formed/min/mg protein.

Table 4. Effect of phenobarbital and *trans*-stilbene oxide administration to rats on hepatic glutathione-*S*-transferase activities towards acrylamide (for details see text)

Treatments	Glutathione-*S*-transferase activity towards CDNB*	Glutathione-*S*-transferase towards ACR†
Saline	452 ± 11	0.08 ± 0.003
Phenobarbital	737 ± 10	0.19 ± 0.03
Peanut oil	389 ± 22	0.10 ± 0.02
trans-Stilbene oxide	649 ± 26	0.15 ± 0.02

Data present mean ± S.E. of 3 experiments.
* Nanomoles conjugate formed/min/mg protein.
† Nanomoles glutathione disappeared/min/mg protein.
Male rats were injected (i.p.) for 3 consecutive days with either phenobarbital (80 mg/kg) in 0.5 ml 0.15 M NaCl or *trans*-stilbene oxide (400 mg/kg) in 0.5 ml peanut oil. Control animals received an identical volume of vehicle alone. Animals were killed 24 hr after last treatment.

Table 5. Glutathione-*S*-transferase activity towards acrylamide in liver and kidney cytosols of neonatal, adult and old rats. For experimental details see the text. Data represent the mean ± S.E. of three experiments

Age	Glutathione-*S*-transferase towards ACR*	
	Liver	Kidney
7 days	0.07 ± 0.002	0.040 ± 0.001
21 days	0.09 ± 0.002	0.082 ± 0.002
2 months	0.12 ± 0.012	0.125 ± 0.003
4 months	0.14 ± 0.014	0.140 ± 0.010
6 months	0.13 ± 0.01	0.108 ± 0.02
8 months	0.10 ± 0.014	0.090 ± 0.001

* Nanomoles glutathione disappeared/min/mg protein. For estimation of GST activity of 7 and 21 days old rats, 2–3 livers or kidneys were pooled and processed as described in text.

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