Glutathione S-Transferase in Human Lymphoid Cell Lines and Fractionated Peripheral Leucocytes

By NEIL KAPLOWITZ,* CELSA SPINA, MELISSA GRAHAM and JOHN KUHLENKAMP

Medical and Research Services, Veterans Administration Wadsworth Hospital Center and the Department of Microbiology and Immunology, University of California (Los Angeles) School of Medicine, Los Angeles, CA 90073, U.S.A.

(Received 20 June 1977)

Glutathione S-transferase activity was identified in cytosol from human lymphoid-cell lines and peripheral leucocytes (polymorphonuclear-leucocyte/monocyte and small-lymphocyte fractions) and compared with human liver enzyme. The findings of closely similar elution volume in gel filtration, substrate (1-chloro-2,4-dinitrobenzene) and inhibitory (probenecid) kinetics indicate that the liver, leucocyte and lymphoid-cell transferases are closely related. The interaction of reduced glutathione and 1-chloro-2,4-dinitrobenzene was shown to occur in intact-lymphoid-cell culture, to be linear with time and quantity of cells and to have kinetics similar to those of the enzyme reaction catalysed by cytosol.

Both mitogen-stimulated human lymphocytes (Busbee *et al.*, 1972; Whitlock *et al.*, 1972; Gurtoo *et al.*, 1975) and peripheral blood monocytes (Bast *et al.*, 1976) have been shown to contain aryl hydrocarbon hydroxylase, which is induced by the addition of polycyclic aromatic hydrocarbons to these cells in culture (Busbee *et al.*, 1972; Whitlock *et al.*, 1972; Gurtoo *et al.*, 1975; Bast *et al.*, 1976). Aryl hydrocarbon hydroxylase is a component of the microsomal mixed-function oxidase system that metabolizes polycyclic aromatic hydrocarbons, a critical step in determining the carcinogenicity of these compounds (Conney, 1967). Much work is being done to evaluate the usefulness of these cell models as a means of studying the genetics of aryl hydrocarbon hydroxylase induction *in vitro* and its relation to cancer risk in man (Kellerman *et al.*, 1973; Atlas *et al.*, 1976).

The probable active carcinogenic metabolites of polycyclic aromatic hydrocarbons, arene oxides, produced by aryl hydrocarbon hydroxylase (Jerina & Daly, 1974), may be detoxified by two different enzymic reactions: microsomal epoxide hydratase (EC 4.2.1.63) and cytosolic glutathione S-transferase (EC 2.5.1.18). The former converts arene oxides into dihydrodiols and appears to be present particularly in the liver (Lu *et al.*, 1975), but is very low in activity or absent in other organs having aryl hydrocarbon hydroxylase (Oesch *et al.*, 1973; Stoming & Bresnick, 1973), including human lymphocytes (Selkirk *et al.*, 1975). Glutathione S-transferase catalyses the nucleophilic attack by reduced glutathione on a variety of electrophilic compounds (Habig *et al.*,

1974), including arene oxides (Nemoto *et al.*, 1975), and parallels aryl hydrocarbon hydroxylase in occurrence in organs where both have been sought, including liver (Habig *et al.*, 1974; Kaplowitz *et al.*, 1975b), kidney (Clifton *et al.*, 1975) and small intestine (Clifton & Kaplowitz, 1977). Because of our interest in the role of glutathione S-transferase in detoxifying the products of aryl hydrocarbon hydroxylase, the former was sought and identified in both human lymphoid-cell lines and peripheral leucocyte fractions and compared with human liver enzyme.

Materials and Methods

Preparation of cells

The B-lymphoid-cell lines, LA-85, LA-96 and LA-237, were originally established in culture from peripheral blood samples of normal donors by a method routinely used in our laboratory (Chang *et al.*, 1971). The T-cell line, Molt-4F, was kindly supplied by Dr. Jan Minowada, Roswell Park Memorial Institute, Buffalo, NY, U.S.A. The cell lines were maintained in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY, U.S.A.) supplemented with 20% (v/v) foetal calf serum (Grand Island Biological Co.) and gentamicin (50 mg/ml; Microbiological Associates, Bethesda, MD, U.S.A.). To harvest large cell pools, the lymphoid lines were grown in plastic, tissue-culture flasks (150 cm² surface area; Corning Scientific Products, Corning, NY, U.S.A.) at a concentration of 1×10^6 – 2×10^6 cells/ml in a total volume of 100 ml, and maintained at 37°C in an atmosphere of CO₂/air

* To whom reprint requests should be addressed.

(1:19). The lymphoid suspensions were fed and split 1:1 every 3–4 days until approx. 0.5 ml of packed cells were accumulated. Viable counts were done by the dye-exclusion technique (1% Trypan Blue); most samples had at least 80–90% viable cells. Lymphoid-cell pools were concentrated by centrifugation (400g for 15 mins), and cell pellets were placed on ice until homogenized approx. 30 min later.

For the purpose of harvesting circulating leucocytes, approx. 90 ml of peripheral blood from four volunteers (referred to in the Tables as NK, JK, BK and JS) was collected by venepuncture into tubes containing sodium heparin (286 units/tube). Separation of the leucocyte population into a lymphocyte-enriched fraction and a polymorphonuclear-leucocyte-enriched fraction was accomplished by a modification of the density-gradient technique described by Boyum (1968); 10 ml of a 33% (w/v) Hypaque/Ficoll solution (10 parts 33% Hypaque, 24 parts 9% Ficoll) was layered over 10 ml of a 50% Hypaque/Ficoll solution (10 parts 50% Hypaque, 24 parts 9% Ficoll) in 50 ml conical glass centrifuge tubes, and 15 ml of a 1:1 mixture of blood and 0.9% NaCl was then layered on the gradient and centrifuged at 400g for 30 min. The two cell bands were collected by aspiration, treated briefly with NH_4Cl solution (0.75%, w/v), and washed three times with serum-free medium. Differential leucocyte counts revealed that the polymorphonuclear-leucocyte-enriched fraction contained only polymorphonuclear leucocytes and large monocytes and the lymphocyte-enriched fraction contained small lymphocytes with no more than a 10% contamination by polymorphonuclear leucocytes and monocytes.

Human livers were obtained from five autopsied individuals (referred to in the Tables as A223-76, A227-76, A228-76, A229-76, A236-76) dying of sudden pulmonary embolus and had no gross or histological evidence of liver disease. Samples were obtained within 12 h of death, during which time cadavers were kept at 4°C.

Preparation of cytosol and enzyme assay

Homogenates (20%, w/v) of packed leucocytes, lymphoid cells and liver were prepared in 0.25 M-sucrose/0.01 M-sodium phosphate buffer, pH 7.4, by using a glass tissue homogenizer. Homogenates were centrifuged at 5000g for 10 min and 100000g for 60 min in a Beckman L3-50 ultracentrifuge. The supernatant fraction (cytosol) was removed by pipette and kept at -15°C for up to 2 weeks without loss of enzymic activity. Glutathione *S*-transferase activity was measured spectrophotometrically at 340 nm by using a Beckman ACTA MVI spectrophotometer (0–0.1 absorbance scale). Reaction mixtures contained 1-chloro-2,4-dinitrobenzene (0.1 mM) and glutathione (1.0 mM) as co-substrates, and 0.01 M-sodium phosphate buffer, pH 6.5 (Habig

et al., 1974). Reactions were initiated by the addition of 5–50 μl of cytosol. Spontaneous interaction of substrates was negligible under these assay conditions. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Enzyme specific activity is expressed as $\mu\text{mol/min}$ per mg of cytosol protein.

Gel filtration of cytosol (3.0 ml) from liver, lymphoid cells and leucocytes was performed sequentially in an upward-flow system by using a column (38 cm $\times$ 2.5 cm) of Sephadex G-100 (Pharmacia, Uppsala, Sweden) with 0.01 M-sodium phosphate buffer, pH 7.4, as mobile phase at a flow rate of 22 ml/h. Glutathione *S*-transferase activity for 1-chloro-2,4-dinitrobenzene was assayed in each column fraction. The column was standardized with a mixture of proteins of known molecular weight (Pharmacia Protein Kit) which was loaded on the column dissolved in 3.0 ml of buffer (10 mg of each protein).

Substrate and inhibitory kinetics

The kinetics of the enzyme activity for 1-chloro-2,4-dinitrobenzene in cytosol were determined by the method of Lineweaver & Burk (1934). The apparent K_m and V_{max} were calculated from least-squares regression analysis (Batson, 1956). Comparison of kinetic parameters for the different cell types was performed by the unpaired *t* test (Batson, 1956). The kinetics of the inhibition of glutathione *S*-transferase by probenecid {4-[(dipropylamino)sulphonyl]benzoic acid} (Sigma Chemical Co., St. Louis, MO, U.S.A.) were determined by the method of Dixon (1953) by using least-squares regression analysis (Batson, 1956) to calculate the inhibition constants (K_i), which correspond to the value on the abscissa for the intersection of lines for two substrate concentrations. Comparison of the inhibitory kinetics for different cell types was performed by the unpaired *t* test (Batson, 1956).

Glutathione conjugation in intact cells

Glutathione (non-protein thiol) concentration was determined in cell pellets from lymphoid culture centrifuged at 400g for 15 min by the method of Owens & Belcher (1965), and was 0.11 ± 0.01 mM. Therefore, to ensure an excess of intracellular glutathione similar to the conditions for the cell-free assay, substrate concentrations in the whole culture in the range 0.005–0.020 mM were used.

Incubation (37°C) of 1-chloro-2,4-dinitrobenzene (0.5 mM) and reduced glutathione (10 mM) at pH 8.0 in 0.01 M-sodium phosphate buffer for 2 h resulted in complete conversion of the former into glutathione conjugate. The λ_{max} of this product (345 nm) corresponds exactly to that published for the glutathione conjugate (Habig *et al.*, 1974). Complete conversion was confirmed by the addition after 2 h of reduced

glutathione (10mM) with no change in A_{345} after another 1h incubation. This incubation mixture, which served as a source of reaction product, was added to lymphoid-cell culture in the range 0.005–0.020mM for the construction of a standard curve. Identical recovery of the added reaction product was obtained by spectrophotometric assay both of whole culture or of culture supernatant (see below).

Lymphoid-cell cultures (1×10^6 – 2×10^6 cells/ml) were mixed in a shaker bath at 37°C for 60 min. The substrate 1-chloro-2,4-dinitrobenzene was then added from stock solution (0.5mM) to cultures to give the desired concentration and incubations were carried out as above for 0–60 min. To assay the conversion of substrate into glutathione conjugate, 2.0ml samples of the culture were removed in duplicate and either placed in a cuvette for immediate reading of A_{345} by using the light-scattering mode of a Beckman ACTA M VI spectrophotometer, or immediately centrifuged at 400g (4°C) for 5 min followed by similar assay of the culture supernatant. No difference was found between these two assay methods. For each determination, cell culture without added substrate served as a control blank.

In all experiments, supernatant from centrifuged cultures contained no detectable glutathione S-transferase activity or glutathione. This was done as a check for cell lysis.

Results

Cytosol from lymphoid cells and the two fractions of peripheral blood leucocytes were found to have glutathione S-transferase activity. The enzyme activity was identified in both B- and T-cell lines. When expressed as specific activity per mg of cytosol protein, values significantly lower than that of liver were identified only for the polymorphonuclear-leucocyte/monocyte fraction of peripheral leucocytes. The mean specific activities $\pm$ s.e.m. are as follows: liver 93 ± 9 , lymphoid-cell lines 74 ± 13 , peripheral small lymphocytes 43 ± 4 , and peripheral polymorphonuclear leucocytes/monocytes 27 ± 3 μ mol/min per mg ($P < 0.02$ versus values for liver and lymphoid cell).

Gel filtration of cytosol samples demonstrated an identical elution volume for glutathione S-transferase activity from liver, lymphoid cells and peripheral leucocytes. An example of these results is shown in Fig. 1. The identical elution volume as for hepatic and lymphocytic glutathione S-transferase was found for ovalbumin (mol.wt. 45000).

Table 1 gives the results of the kinetic parameters for the glutathione S-transferase activity with 1-chloro-2,4-dinitrobenzene from liver, lymphoid cells and peripheral leucocyte fractions. Comparison of the data for each group showed no significant difference ($P > 0.1$) between liver and lymphoid cells

Fig. 1. Elution of glutathione S-transferase in gel filtration of cytosol from human liver (●) and peripheral small lymphocytes (○)

Enzyme activity in each fraction is expressed relative to peak activity. The two cytosol samples were run on two successive days under the same conditions. The elution volumes of hepatic and lymphocytic glutathione S-transferases were identical (82 ml).

Table 1. Comparison of kinetics of enzyme from human lymphoid cells, peripheral leucocytes and liver

Each individual value represents the mean of triplicate determinations. The mean ($\pm$ s.e.m.) value for each group is also given. See the text for details of tissue preparation and enzyme assays.

Tissue	K_m (mM)	V_{max} (μ mol/min per mg)
Liver		
A-223-76	0.45	0.79
A-227-76	0.23	0.26
A-228-76	0.36	0.60
A-229-76	0.72	0.50
A-236-76	0.35	0.20
	0.42 ± 0.08	0.43 ± 0.10
Lymphoid-cell lines		
LA-85	0.88	0.93
LA-237	0.54	0.55
LA-96	0.75	0.67
Molt-4F	0.46	0.21
	0.66 ± 0.10	0.59 ± 0.15
Peripheral polymorphonuclear leucocytes/monocytes		
NK-1A	1.32	0.46
JK-1A	0.66	0.77
BK-1A	0.32	0.10
JS-1A	1.19	0.44
	0.87 ± 0.23	0.44 ± 0.08
Peripheral lymphocytes		
NK-1B	0.68	0.33
JK-1B	0.56	0.71
BK-1B	0.82	0.35
JS-1B	0.55	0.27
	0.65 ± 0.06	0.42 ± 0.10

or leucocyte fractions. An example of these results is depicted in Fig. 2.

Probenecid, a known inhibitor of rat liver and

Fig. 2. Lineweaver-Burk plot of the kinetics of glutathione *S*-transferase from two human livers (○, ■) and lymphoid cells (T-cell line Molt-4F, ▲; B-cell line 237, ●)

Enzymic activity is represented by v and substrate concentration (1-chloro-2,4-dinitrobenzene) by $[S]$. There is close correspondence in abscissa intercept (representing $-1/K_m$) for liver, B-cell and T-cell lines. For each line, $r = 0.99$. The equations for the lines from top to bottom are: $y = 2.15x + 4.82$; $y = 0.99x + 1.79$; $y = 0.60x + 1.67$; $y = 0.56x + 1.33$.

Fig. 3. Dixon plot showing the competitive inhibition by probenecid of glutathione *S*-transferase from human liver (○) and lymphoid-cell line (●)

Concentrations given in parentheses are those of the substrate, 1-chloro-2,4-dinitrobenzene. There is close correspondence in the intersection of the lines for the two substrate concentrations with each source of enzyme. From top to bottom the equation for each line and correlation coefficient are as follows: $y = 0.74x + 16.52$, $r = 0.97$; $y = 0.42x + 7.21$, $r = 0.96$; $y = 0.38x + 6.52$, $r = 0.99$; $y = 0.11x + 4.17$, $r = 0.98$.

kidney glutathione *S*-transferases (Kaplowitz *et al.*, 1976), was found to be a competitive inhibitor of human hepatic and lymphoid-cell enzyme (Fig. 3). The K_i values listed in Table 2 were not significantly different for the two sources of enzyme.

Table 2. Competitive inhibition by probenecid of human lymphoid-cell line and hepatic glutathione *S*-transferase. Each individual value represents the mean of triplicate determinations. The mean ($\pm$ S.E.M.) value for each group is also given.

Tissue	K_i (mM)
Lymphoid-cell lines	
A-223-76	2.39
A-227-76	2.36
A-228-76	2.76
	2.50 ± 0.13
Liver	
LA-85	2.78
LA-237	1.95
LA-96	2.79
Molt-4F	3.28
	2.70 ± 0.28

Fig. 4. Linear relationship between volume of cell suspension (10^6 cells/ml) and glutathione conjugate produced. Cell line LA-85 was maintained at 37°C . Samples (0.4–2.0 ml) were removed in triplicate, diluted to 2 ml with culture medium (at 37°C) and 1-chloro-2,4-dinitrobenzene was added ($10\mu\text{M}$). After precisely 2 min, the reaction products were determined spectrophotometrically (see the text). The equation of the line is $y = 0.99x - 0.07$; $r = 0.99$.

The addition of 1-chloro-2,4-dinitrobenzene ($10\mu\text{M}$) to cell culture was followed sequentially for the production of glutathione conjugate. The ensuing reaction was linear with time for the initial 5 min. Therefore, subsequent incubations were carried out for 2 min. In addition, a linear relationship was found between the quantity of cell suspension and the product of the reaction (Fig. 4). The kinetics of interaction of 1-chloro-2,4-dinitrobenzene (7.4 – $19\mu\text{M}$) and intracellular glutathione were determined

Fig. 5. Kinetics of the interaction of glutathione and 1-chloro-2,4-dinitrobenzene in intact cells

Each data-point represents the mean of triplicate determinations. The three different symbols represent assays on three separate occasions. Lymphoid-cell line LA-85 was used in this experiment. Cell protein was determined on the contents of each cuvette, and enzyme activities are expressed as μmol of product/min per mg of cell protein. For details see the text. The equations for the three lines from top to bottom are: $y = 4.4x + 20.85$, $r = 0.98$; $y = 3.43x + 18.11$, $r = 0.97$; $y = 3.40x + 19.46$, $r = 0.97$.

by using 2.0 ml portions of a well-mixed culture and 2.0 min reaction times (Fig. 5), giving an apparent K_m of 0.19 ± 0.1 mM.

Discussion

In the rat the glutathione *S*-transferases (AA, A, B, C, E, M) are a complex group of enzymes, most of which differ with respect to antigenicity, amino acid analysis, substrate specificity and kinetic parameters (Habig *et al.*, 1974), which leads to great difficulty in using activity for any given substrate as a means of measuring the amount of enzyme. In contrast, the human hepatic glutathione *S*-transferases (α , β , γ , δ , ϵ) each react with a monospecific antibody and have the same amino acid analysis, an identical spectrum of substrates, and close correspondence in enzyme kinetics (Kamisaka *et al.*, 1975). The apparent K_m values for the purified human liver enzymes, which are believed to be charge isomers, range from 0.40 to 0.63 mM with 1-chloro-2,4-dinitrobenzene as substrate (Kamisaka *et al.*, 1975). The values found with lymphoid cell, fractionated peripheral leucocyte, and liver cytosol (Table 1) in the present paper agree closely with the former results with purified enzymes. In addition to the kinetic findings, the similarity between human hepatic, lymphoid-cell and leucocytic enzyme is further supported by the identical elution volume for each in gel filtration (Fig. 1). In addition, the close correspondence in elution of ovalbumin (mol.wt. 45 000) and glutathione *S*-transferase activity

is consistent with the 48 500 mol.wt. reported for the purified liver enzymes (Kamisaka *et al.*, 1975).

Glutathione *S*-transferases are novel enzymes in having a possible physiological role other than their catalytic detoxifying function, which is to promote the interaction of nucleophilic glutathione with electrophilic toxic and carcinogenic compounds (Habig *et al.*, 1974). These enzymes bind a variety of organic anions and non-polar compounds that are not substrates (Kamisaka *et al.*, 1975). This property may be important for a cytoplasmic receptor or transport role for the glutathione *S*-transferases in the hepatocyte and proximal tubular cell of the kidney (Kaplowitz *et al.*, 1975a, 1976). Probenecid is an example of a compound that is bound and competitively inhibits the transferases without itself being altered (Kaplowitz *et al.*, 1976). The finding of competitive inhibition of the human hepatic and lymphoid-cell glutathione *S*-transferase activities by probenecid (Fig. 3) and the close agreement in K_i values for liver and lymphoid cells (Table 2) further support the validity of the identification of the transferase in lymphoid cells and its close similarity to the hepatic enzyme.

We have shown that the enzyme activity for 1-chloro-2,4-dinitrobenzene can be assayed in cell culture. The addition of this substrate to cell culture is associated with the appearance of the glutathione conjugate in the culture medium. This reaction was initially linear with respect to time and volume of cells (Fig. 4). The kinetics of this reaction were similar to that with cytosol as a source of enzyme (Fig. 5). The K_m for the reaction in cells and cytosol are of the same order of magnitude, despite inherent differences in the two assays. This finding supports the notion that the reaction with intact cells also is enzyme-mediated. This observation is of particular importance for two reasons: (1) assays using intact cultures may provide an easy means for assaying large numbers of samples; (2) in studying the products of aryl hydrocarbon hydroxylase generated by microsomal fractions from lymphocytes, the influence of lymphocytic cytosol (containing glutathione *S*-transferases and glutathione) should be considered.

From the present findings, peripheral leucocytes (both the small lymphocyte and polymorphonuclear-leucocyte/monocyte fractions) and B- and T-lymphoid-cell lines can be added to the list of cell types in which glutathione *S*-transferases and aryl hydrocarbon hydroxylase co-exist. Therefore, an important aspect of the enzymic function of the glutathione *S*-transferases may be the modulation or counteraction of the biological effects of the toxic and carcinogenic products of aryl hydrocarbon hydroxylase. In this regard, to evaluate cancer risk, consideration must be given not only to the activation of carcinogens by aryl hydrocarbon hydroxylase but also to the inactivation or protection by glutathione

S-transferases. The peripheral leucocytes offer a readily available source of cells to measure glutathione *S*-transferase concentrations in a cell population having aryl hydrocarbon hydroxylase, which may provide an opportunity to evaluate the genetic and environmental influence of this important detoxification mechanism in cancer risk in man.

This work was supported by Veterans Administration Medical Research Funds and United States Public Health Service Grant F-32-CA05295.

References

- Atlas, S. A., Vesell, E. S. & Nebert, D. W. (1976) *Cancer Res.* **36**, 4619-4630
- Bast, R. C., Okuda, T., Plotkin, E., Tarone, R., Rapp, H. J. & Gelboin, H. V. (1976) *Cancer Res.* **36**, 1967-1974
- Batson, H. D. (1956) *An Introduction to Statistics in the Medical Sciences*, Chicago Medical Book Co., Chicago
- Boyum, A. (1968) *Scand. J. Clin. Lab. Invest.* **21**, 1-109
- Busbee, D., Shaw, C. R. & Cantrell, E. (1972) *Science* **178**, 315-316
- Chang, R. S., Hsieh, M. W. & Blankenship, W. (1971) *J. Natl. Cancer Inst.* **47**, 469-477
- Clifton, G. & Kaplowitz, N. (1977) *Cancer Res.* **37**, 788-791
- Clifton, G., Kaplowitz, N., Wallin, J. D. & Kuhlenkamp, J. (1975) *Biochem. J.* **150**, 259-262
- Conney, A. H. (1967) *Pharmacol. Rev.* **19**, 317-366
- Dixon, M. (1953) *Biochem. J.* **55**, 170-171
- Gurtoo, H. L., Bejba, N. & Minowada, J. (1975) *Cancer Res.* **35**, 1235-1243
- Habig, W. H., Pabst, M. J. & Jakoby, W. B. (1974) *J. Biol. Chem.* **249**, 7130-7139
- Jerina, D. M., Daly, J. W. (1974) *Science* **185**, 573-582
- Kamisaka, K., Habig, W. H., Ketley, J. M., Arias, I. M. & Jakoby, W. B. (1975) *Eur. J. Biochem.* **60**, 153-161
- Kaplowitz, N., Kuhlenkamp, J. & Clifton, G. (1975a) *Proc. Soc. Exp. Biol. Med.* **149**, 234-237
- Kaplowitz, N., Kuhlenkamp, J. & Clifton, G. (1975b) *Biochem. J.* **146**, 351-356
- Kaplowitz, N., Clifton, G., Kuhlenkamp, J. & Wallin, J. D. (1976) *Biochem. J.* **158**, 243-248
- Kellerman, G., Shaw, C. R., Layten, M. & Kellerman, (1973) *N. Engl. J. Med.* **289**, 934-937
- Lineweaver, H. & Burk, P. (1934) *J. Am. Chem. Soc.* **56**, 658-666
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Lu, A. Y., Ryan, D., Jerina, D. M., Daly, J. W. & Levin, W. (1975) *J. Biol. Chem.* **250**, 8283-8288
- Nemoto, N., Gelboin, H. V., Habig, W. H., Ketley, J. N. & Jakoby, W. B. (1975) *Nature (London) New Biol.* **255**, 512
- Oesch, F., Jerina, D. M., Daly, J. W. & Rice, J. M. (1973) *Chem.-Biol. Interact.* **6**, 189-202
- Owens, C. W. & Belcher, R. V. (1965) *Biochem. J.* **94**, 705-711
- Selkirk, J. K., Gray, R. G., Whitlock, J. P. & Gelboin, H. V. (1975) *Cancer Res.* **35**, 3651-3655
- Stomung, T. A. & Bresnick, E. (1973) *Science* **181**, 951-952
- Whitlock, J. P., Cooper, H. L. & Gelboin, H. V. (1972) *Science* **177**, 618-619