A single large batch of P815-X2 was prepared in DBA/2J mice and preserved at low temperature in 10 percent dimethyl sulfoxide and P815-X2 ascites fluid by controlled rate freezing. A single vial was thawed and washed twice in RPMI 1640 medium with penicillin and streptomycin added and 5 million cells were injected into a female DBA/2J mouse. Tumor for experimental studies was harvested after 13 days. By this protocol, the variability of tumor weights of individual mice after 10 days did not differ from the variability in the means of numerous experiments.
12. Mantel-Cox logrank test was used as described by R. Peto and J. Peto [*J. R. Stat. Acad. A* **135**, 185 (1972)].
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Mutagenicity of Glutathione and Cysteine in the Ames Test

Abstract. *Postmitochondrial supernatant from rat liver and kidney homogenates transformed cysteine into a mutagen that reverted bacteria of the strain* Salmonella typhimurium *TA100 to histidine independence. Glutathione was also activated by kidney postmitochondrial supernatant but not by liver preparations. Hence, important endogenous compounds of mammals are positive in the most commonly used short-term test for carcinogenicity and mutagenicity. Glutathione is positive in the test even at concentrations found in mammalian tissues.*

Bacterial tests of mutagenicity mediated by mammalian enzymes have demonstrated that numerous compounds, including substances from plants and microorganisms, are mutagenic (*1*). We now report that even compounds that are physiologically important for mammals and present in these organisms in large concentrations, such as the tripeptide glutathione or the amino acid cysteine, can exert mutagenic effects. Biological functions of glutathione include transport of amino acids through membranes, protection of lipids against autoxidation, inactivation of electrophilic intermediates, and modulation of proteins by the formation of mixed disulfides (*2*). The sulfhydryl group of cysteine, one of the three amino acids that form glutathione, is of primary importance in most functions and dominates its biochemistry.

Mutagenicity was determined in bacteria in the presence of mammalian subcellular preparations. The Ames test (*3*) was used. In some experiments the S9 fraction of liver [the postmitochondrial supernatant fraction supplemented with a system generating reduced nicotinamide adenine dinucleotide phosphate (NADPH)] was replaced by different metabolizing systems. Postmitochondrial supernatant, microsomal, and cytosolic fractions were prepared from homogenates of kidney and liver from untreated (*4*) adult male Sprague-Dawley rats. Histidine-dependent bacteria of the strain *Salmonella typhimurium* TA100, the subcellular fraction from 100 mg of tissue with or without an NADPH-generating system, a neutralized solution of the test compound in water, and histidine-poor soft agar were mixed and added to culture plates containing minimal agar. The colonies reverting to histidine independence were counted after incubation for 2 days in the dark.

Figure 1 shows the results of experiments in which glutathione and cysteine were tested for mutagenicity in the absence of mammalian enzymes and in the presence of liver or kidney S9. In the absence of S9 neither compound increased the number of revertants. In fact, glutathione led to a small decrease, probably due to toxicity. With the addition of liver S9, cysteine increased the number of revertants. Under the same conditions glutathione did not increase the number of revertants. However, when liver S9 was replaced by kidney S9, both glutathione and cysteine increased the number of revertants severalfold above the spontaneous level. Batches of glutathione and cysteine from different manufacturers (Boehringer, Sigma, and Merck) did not show any quantitative differences, strongly suggesting that the compounds rather than impurities were responsible for the effects. Moreover, glutathione formed enzymatically in situ from glutathione disulfide also acted as a mutagen (*5*).

From Fig. 1 it can be calculated that the number of revertants was twice the control number with 6 m*M* glutathione in the top layer of agar (5 mg per plate) or with 8 m*M* cysteine (3 mg per plate). Although these concentrations are high compared to those of many other mutagens, physiological levels of glutathione in mammalian cells are also high (*2*); values from 2 to 10 m*M* are typical for rat liver and kidney. Concentrations of glutathione in *S. typhimurium* (*6*) and of cysteine in mammalian tissues (*2*) are much lower. Meijer *et al.* (*7*) reported 0.15 m*M* (150 nmole per gram wet weight) glutathione in *S. typhimurium* TA100. Cysteine is present in rat liver and kidney at concentrations of 0.1 to 0.5 m*M*. Hence, whereas glutathione was clearly mutagenic at concentrations that are physiological for mammals, cysteine

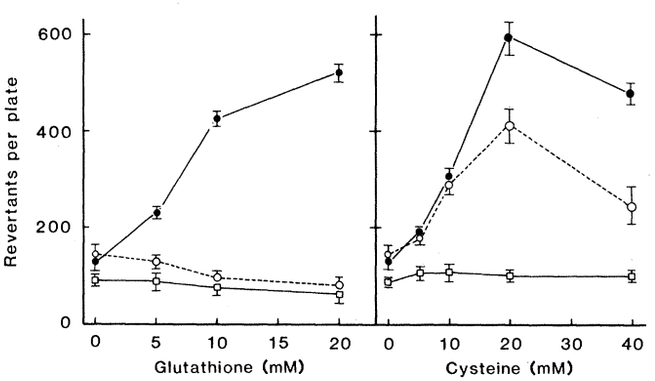

Fig. 1. Effect of glutathione and cysteine on the reversion of histidine-dependent *S. typhimurium* TA100 directly (□) and in the presence of liver (○) or kidney (●) postmitochondrial supernatant. Kidneys and livers from adult male Sprague-Dawley rats were homogenized in three volumes of 150 m*M* KCl with 10 m*M* sodium phosphate buffer (*p*H 7.4). The homogenate was centrifuged for 10 minutes at 9000*g* to yield the postmitochondrial supernatant fraction. For each incubation the following were mixed in a test tube and poured onto plates containing minimal agar: 0.97×10^8 bacteria; 330 μl of postmitochondrial supernatant fraction from liver (containing 8.2 mg of protein) or kidney (5.6 mg of protein); an NADPH-generating system (2 μmole of $NADP^+$ and 2.5 μmole of glucose-6-phosphate) in 330 μl of a solution containing 50 m*M* KCl, 12 m*M* $MgSO_4$, and 75 m*M* sodium phosphate buffer (*p*H 7.4); the test compound in 400 μl of neutralized aqueous solution; and 2 ml soft agar (0.55 percent NaCl, 0.55 percent agar, 50 μ*M* biotin, 50 μ*M* histidine, and 25 m*M* sodium phosphate buffer at *p*H 7.4 and 45°C). We incubated the cultures for 2 days in the dark at 37°C and then counted the colonies that had reverted to histidine independence. Values are means ± standard deviations for three replicate incubations.

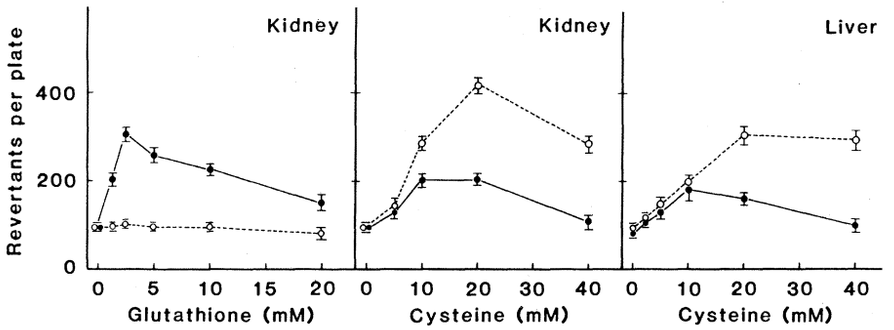

Fig. 2. Comparison of the ability of microsomal (●) and cytosolic (○) fractions to activate glutathione and cysteine. A portion of the supernatant obtained by centrifugation at 9000*g* was centrifuged for 1 hour at 100,000*g*. The resulting cytosolic supernatant was dialyzed for 3 hours against homogenization buffer. The microsomal pellet was resuspended in homogenization buffer and recentrifuged. The mutagenicity assay was performed as described in the legend to Fig. 1, except that the NADPH-generating system was omitted and the postmitochondrial fraction was replaced by the microsomal and cytosolic fractions from an equivalent amount of tissue. Protein content of kidney cytosol, kidney microsomes, liver cytosol, and liver microsomes was 4.3, 1.2, 4.8, and 1.5 mg per incubation, respectively. Values are means ± standard deviations for three replicate incubations. The experiment was also performed with an NADPH-generating system. The results were nearly the same and therefore are not presented.

was mutagenic only at higher concentrations. When environmental chemicals are screened for genotoxic hazards with the Ames test, it is recommended that they be studied at concentrations up to 5 mg per plate (*8*), if possible.

Conceivably, the increased number of mutants in the presence of relatively high concentrations of physiological compounds could be the result not of mutagenicity but of nutritional interactions between these compounds and histidine. This is, however, unlikely in our experiments, because the histidine-dependent strains TA1537, TA1538, TA98, and TA1535, which differ from TA100 in the mutation leading to histidine dependence or in the DNA repair system, showed either no revertants or very low numbers of revertants in response to glutathione and cysteine compared with the number induced in strain TA100. Moreover, TA100 colonies induced on histidine-poor plates by glutathione (in the presence of kidney S9) continued to grow after being transferred to glutathione- and histidine-free plates, showing that heritable reversions had occurred.

Cysteine and glutathione are cofactors or substrates for many enzymes and are essential in various types of biochemical reactions (*2*). To study the mode of activation, we attempted to determine whether cofactors are required, whether the oxidized forms of glutathione and cysteine (glutathione disulfide and cystine) exert mutagenic effects, and whether the microsomal or cytosolic fraction can perform the activation. Omission of the NADPH-generating system in S9 did not noticeably affect the mutagenicity of glutathione or cysteine. This implies that the activation is not dependent on cytochrome P-450–dependent monooxygenases, unlike the case for numerous xenobiotics.

Cytosolic and microsomal enzymes were separated by centrifugation of the postmitochondrial fraction for 1 hour at 100,000*g*. The pellet was resuspended in the homogenization buffer and the procedure was repeated to remove cytosolic contamination. The cytosol was dialyzed for 3 hours against homogenization buffer to remove components of low molecular weight. These preparations were then used in mutagenicity tests (Fig. 2). Glutathione was efficiently activated by the microsomal fraction of the kidney. Low concentrations of glutathione were substantially more mutagenic when the microsomal fraction was used instead of postmitochondrial supernatant. The maximum achievable effect was weaker, however, because of increased toxicity. Cytosol did not activate glutathione. In contrast, cysteine was more mutagenic in the presence of cytosol than microsomes, but the latter subcellular fraction also led to a weak activation. The dependence of the mutagenicity of glutathione on kidney microsomal enzymes might indicate the involvement of γ-glutamyl transpeptidase, a membrane-bound enzyme found in especially high concentrations in the kidney (*2*). It can hydrolyze glutathione to cysteinyl glycine, which could be split by another kidney enzyme, cysteinyl-glycine dipeptidase, into its amino acid components (*2*). However, because glutathione was more strongly mutagenic than cysteine, activation via cysteine only is unlikely. We propose a chemically analogous activation that is either differently catalyzed or preceded by a reaction mediated by microsomal kidney enzymes (for example, hydrolysis to cysteinyl glycine).

Thiols such as cysteine and, to a lesser extent, glutathione, are oxidized in the presence of air, yielding hydrogen peroxide, superoxide, and disulfides. This oxidation can occur nonenzymatically and is catalyzed by transition metals (*2*). Due to the lack of mutagenicity in the absence of mammalian tissue preparations, oxidation can account for the mutagenicity of cysteine and glutathione only if it is substantially catalyzed by microsomes or cytosol or if an additional enzyme-mediated reaction is involved in the activation. Hydrogen peroxide was not mutagenic at concentrations up to the limits of toxicity, either when tested alone or in the presence of kidney S9. Addition of catalase (up to 750 μg or 50,000 U per incubation) or superoxide dismutase (up to 200 μg or 500 U) did not affect kidney S9–mediated mutagenicity of glutathione. Hence, hydrogen peroxide and superoxide were not the ultimate mutagen, nor were they involved in a rate-limiting step in its formation. Glutathione disulfide and cystine cannot account for the mutagenic effect either, as they were not mutagenic in the absence of reducing cofactors. Thiol-free radicals, which are probable intermediates in the oxidation process (*9*), are good candidates for the active species, but further experiments are required.

Our study shows that at least two naturally occurring compounds in mammals, cysteine and glutathione, react positively in the Ames test. Future studies should include an investigation of the biochemical mechanism of this phenomenon and, in particular, the mutagenicity or carcinogenicity of these compounds in animal models.

HANSRUEDI GLATT
MIROSLAVA PROTIĆ-SABLJIĆ*
FRANZ OESCH
Institute of Pharmacology,
University of Mainz, D-6500
Mainz, Federal Republic of Germany

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* Present address: Laboratory of Molecular Carcinogenesis, National Cancer Institute, Bethesda, Md. 20205; permanent address: Laboratory for Marine Molecular Biology, Institute Rudjer Bošković Zagreb, Jugoslavia.

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Human Platelet–Derived Growth Factor (PDGF): Amino-Terminal Amino Acid Sequence

Abstract. *Human platelet-derived growth factor (PDGF) obtained from outdated human platelets was subjected to amino-terminal amino acid sequence analysis by automated Edman degradation. Despite the apparent presence of limited proteolytic degradation of the protein derived from this method, the sequence analysis reveals two primary peptide sequences and suggests that active PDGF is composed of two, possibly homologous, peptides linked by a disulfide bond or bonds.*

Platelet-derived growth factor (PDGF) is a heat-stable (100°C), cationic (isoelectric point, *p*I, 9.8) polypeptide (*1*) that circulates in blood stored in the α granules of platelets (*2*). It is released into serum during blood clotting, and it represents the major polypeptide growth factor of human serum. It is a potent mitogen for cultured fibroblasts, smooth muscle cells, and glial cells (*3–5*). Platelet-derived growth factor was isolated originally from whole human serum (*6*) and subsequently from clinically outdated human platelets (*1, 5, 7–9*) and from human platelet-rich plasma (*10*).

Unreduced, active PDGF exhibits multiple molecular weight forms ranging in size from 28,000 to 35,000 daltons, as judged by analytical sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*1, 7–10*). Reduction of PDGF produces inactive, lower molecular size polypeptides ranging from 12,000 to 18,000 daltons (*5, 7, 8*). Whether these polypeptides represent two or more subunits held together in native PDGF by disulfide bonds, as has been suggested (*7, 8*), or whether they represent pieces of a single polypeptide produced by proteolytic fragmentation during the handling and fractionation of the outdated platelets but still held together by disulfide bonds, has not been established.

Elucidation of the primary structure of PDGF will help to provide an understanding of the molecular basis for its function and its relation to other polypeptide growth factors, serum proteins, and platelet factors. Here we report the results of amino-terminal amino acid sequence analysis of both active PDGF and the major, inactive peptides derived from it by disulfide reduction.

PDGF was purified by a modification of a previously described procedure (*1, 8*). Two major bands of PDGF activity, corresponding to protein bands of 35,000 (PDGF-I) and 32,000 (PDGF-II) daltons, were recovered from the final stage of purification (SDS-PAGE). Biologic activity during purification was assessed by the ability of PDGF to stimulate DNA synthesis in cultures of BALB/c-3T3 (clone A30) cells (*1*). The specific activity of purified PDGF is estimated at about 3000 units per microgram of protein, and that of the platelet lysate is about 0.03 unit per microgram of protein. One unit of PDGF activity is defined as the amount required to induce 50 percent of the cells to synthesize DNA (*1*). The stained protein eluted from the gels seems to retain full biologic activity.

Amino-terminal amino acid sequence analysis was performed with automated Edman degradation on a gas-phase sequenator designed and constructed at Caltech (*11, 12*). Analysis of phenylthiohydantoin amino acids released by the sequenator was accomplished by reverse-phase high-performance liquid chromatography on a cyano column (IBM Instruments) (*13*).

Sequence analysis of several preparations (80 to 400 pmole) of both PDGF-I and PDGF-II revealed the presence of three to four detectable amino acid sequences in all of them. Although the relative amounts of the different sequences varied somewhat between the PDGF-I and PDGF-II preparations, they were not sufficiently unalike to allow unambiguous determination of the individual sequences. Therefore, the purified PDGF fractions were reduced, alkylated, and fractionated by SDS-PAGE. This procedure yielded two major fractions, with apparent molecular sizes of approximately 18,000 (fraction A) and 14,000 (fraction B) daltons, and one minor fraction, with an apparent molecular size of 12,000 daltons (fraction C) (Fig. 1).

Sequence analysis of fractions A, B, and C revealed that together they exhibited all of the sequences present in the unreduced PDGF preparations. Moreover, although none of the three fractions gave a single, clean sequence, each by itself gave a simpler mixture of sequences than that present in either PDGF-I or PDGF-II. Quantitative analysis of these sequence data established the individual sequences shown in Fig.

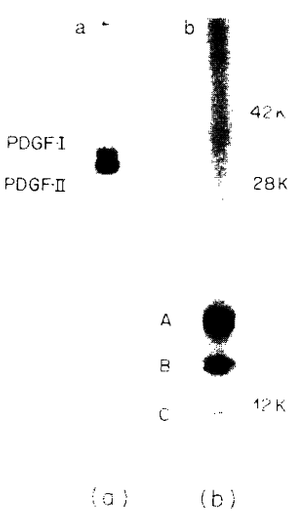

Fig. 1. SDS-PAGE of unreduced (a) and reduced or alkylated (b) PDGF. (a) The two major forms of unreduced PDGF, PDGF-I, and PDGF-II. The specific activity of PDGF-I and -II is about 3000 units per microgram of protein. (b) The sample of the reduced or alkylated PDGF demonstrates the presence of reduced fractions A, B, and C. Fractions A and B are the major reduced fractions of PDGF. Fraction C is a minor component and is present only in some reduced PDGF preparations. For reduction, purified PDGF (10 to 20 μg) was dissolved in 50 μl of 0.5*M* ammonium bicarbonate containing 20 m*M* dithiothreitol. The reduced preparation was kept for 2 hours at room temperature, and 50 μl of 40 m*M* iodoacetamide in 0.5*M* ammonium bicarbonate was added. After 5 minutes, the preparation was dialyzed against 0.02 percent SDS, lyophilized, dissolved in 25 μl of Laemmli sample buffer (*9*) containing 2 percent SDS, and subjected to SDS-PAGE (16 percent gels, 2 mA per gel). The gels were stained overnight at room temperature with 0.1 percent Coomassie brilliant blue R-250 in 10 percent acetic acid and 10 percent methanol and destained with 10 percent acetic acid and 10 percent isopropyl alcohol. The stained proteins were eluted (*8*), dialyzed at room temperature against 0.1 percent SDS, and lyophilized.