Immunological studies using monoclonal antibodies against the α and β subunit of phosphorylase kinase showed also the presence of both these subunits in SR membranes [16]. The data presented in this report confirm and extend these observations. It has been shown here chemically that the α and β subunits of phosphorylase kinase are associated with SR membranes. Since phosphorylase kinase exhibits phosphatidylinositol kinase activity, it might be possible that the phosphatidylinositol kinase present in these membranes is also associated with these polypeptides identified here.

The National Hellenic Research Foundation
48 Vas. Constantinou Ave.
Athens 116 35, Greece
ZAFIROULA GEORGOUSSI
ATHANASSIOS EVANGELOPOULOS

**Ruhr-Universität Bochum*
Institut für Physiologische Chemie
Lehrstuhl I
Universitätsstraße 150
4630 Bochum, Federal Republic of Germany
LUDWIG M. G. HEILMEYER, JR.*†

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† To whom correspondence should be sent.

Biochemical Pharmacology, Vol. 35, No. 24, pp. 4573–4576, 1986.
Printed in Great Britain.
0006–2952/86 $3.00 + 0.00

Inactivation of the carcinogen, 5-hydroxymethylchrysene, by glutathione conjugation *via* a sulphate ester in hepatic cytosol

(*Received* 19 *May* 1986; *accepted* 1 *July* 1986)

5-Hydroxymethylchrysene (5-HCR), a potent carcinogen [1] and a major metabolite of 5-methylchrysene in rat liver [2], has been reported by Okuda *et al.* [3] to be activated to the highly mutagenic sulphate conjugate, 5-HCR sulphate, by rat liver sulphotransferase in the presence of a 3′-phosphoadenosine 5′-phosphosulphate (PAPS)-generating system. The mutagenicity of 5-HCR towards *Salmonella typhimurium* TA98, exerted by a dialysed soluble supernatant fraction (S105) of a liver homogenate from the untreated rat in the presence of the PAPS-generating system, was much higher than that exerted by the hepatic post-mitochondrial fraction in the presence of an NADPH-generating system [3]. This strongly suggests that, so far as examined by the mutagenicity test, sulphate conjugation plays a more important role than monooxygenation in the metabolic activation of 5-HCR in untreated rat liver. 5-HCR sulphate, isolated from the incubation mixture and identified with the synthetic specimen, has been demonstrated to have potent intrinsic mutagenicity towards TA98 and also demonstrated by a fluorophotometric study of calf thymus DNA incubated with the sulphate to bind covalently to the nucleic acid through its 5-methylene carbon with loss of a sulphate anion [3].

During the course of our investigation on the metabolic activation of 5-HCR in the presence of rat liver S105 fortified with PAPS, the authors found addition of glutathione (GSH) to the S105-PAPS system retard the mutagenicity of 5-HCR towards TA98. The present communication deals with (1) metabolic inactivation of the intrinsic mutagenicity of 5-HCR sulphate by S105 in the presence of GSH, (2) isolation and characterization of a GSH conjugate formed from 5-HCR by the rat liver S105-PAPS system fortified with GSH as well as from 5-HCR sulphate by S105 in the presence of GSH, and (3) inhibition of covalent binding of 5-HCR sulphate to calf thymus DNA by S105 in the presence of GSH.

5-HCR dissolved in dimethyl sulphoxide was incubated at 37° for 20 min with S105 of a liver homogenate from male Wistar rats, weighing 160–180 g, in 0.1 M phosphate buffer, pH 7.4, containing TA98 and PAPS in the presence and in the absence of GSH. According to the method of Ames *et al.* [4], the bacterial suspension was then diluted with soft agar and placed on hard agar plates to count the number of His^+ revertant colonies appearing after 48 hr at 37°. The mutagenicity of 5-HCR exerted by the rat liver S105-PAPS system was completely retarded in the presence of GSH (Fig. 1A).

After incubation of 5-HCR under these conditions without the bacterial suspension, methanol was added to the incubation mixture (1 ml) in order to remove cytosolic proteins and most of inorganic salts. The aqueous methanolic solution was subjected to high pressure liquid chromatography (HPLC) carried out on an octadecylsilica column (Nucleosil $7C_{18}$, 7 μm in particle size, 4 × 300 mm)

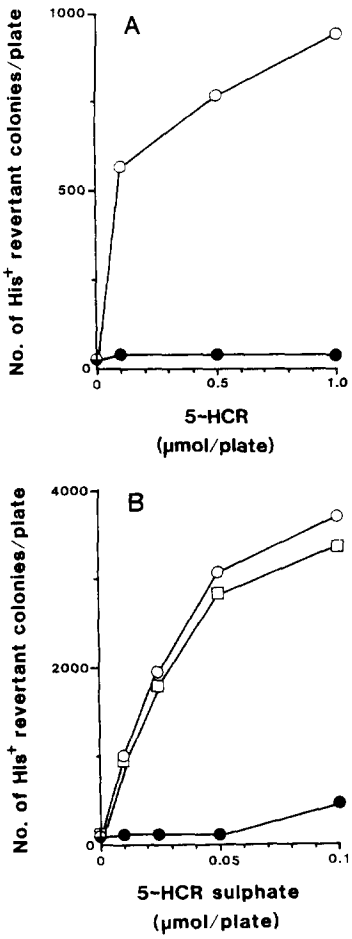

Fig. 1. Effect of GSH on mutagenicity towards *Salmonella typhimurium* TA98 of 5-HCR and its active metabolite 5-HCR sulphate in rat liver cytosol. (A) Open circles: each mixture, consisting of S105 (2.8 mg of protein, equivalent to 50 mg of rat liver), 5-HCR dissolved in dimethyl sulphoxide (0.1 ml), and PAPS (0.6 μmol), was incubated with an overnight culture (0.1 ml) of TA98 (10^8 cells) at 37° for 20 min in a final volume of 1 ml of 0.1 M KH_2PO_4–Na_2HPO_4 buffer, pH 7.4, diluted with soft agar and poured onto hard agar plates to count His^+ revertant colonies after incubation at 37° for 48 hr. Closed circles: S105 was mixed with GSH (4 μmol) and then with the aforementioned ingredients. (B) Open circles: 5-HCR sulphate (Na) dissolved in dimethyl sulphoxide (0.1 ml) was incubated with TA98 (10^8 cells) in the absence of GSH and S105 under the aforementioned conditions. Open squares: the sulphate was incubated with TA98 (10^8 cells) in the presence of GSH (4 μmol). Closed circles: the sulphate was incubated with TA98 (10^8 cells) in the presence of GSH (4 μmol) and S105 (2.8 mg of protein).

in methanol–water (4:1, 0.8 ml/min) containing tetra-*n*-butylammonium bromide (TBA, 2 mM). As previously reported [3], 5-HCR sulphate formed from 5-HCR under these conditions in the absence of GSH appeared as an intense peak at a retention time of 11.5 min in the HPLC-chromatogram obtained by monitoring at 269 nm. In the presence of GSH, however, an intensely u.v.-absorbing metabolite with higher polarity than 5-HCR sulphate was found at a retention time of 10 min, but no detectable peak corresponding to the sulphate appeared in the chromatogram. The more polar metabolite, eluted from the HPLC column, showed a ninhydrin-positive and u.v.-absorbing spot at R_f 0.51 on a silica thin-layer plate (Merck F_{254}) developed with *n*-butanol–acetic acid–water (4:1:2) and u.v.-absorption and fluorescence emission spectra characteristic of 5-alkyl-substituted chrysene. The HPLC study also indicated that neither 5-HCR sulphate nor the more polar metabolite was formed when PAPS was omitted from the incubation mixture or boiled S105 was used.

The intrinsic mutagenicity of 5-HCR sulphate towards TA98 was also inhibited by preincubation of the sulphate with GSH potently in the presence of S105, but to a small extent in the absence of S105 (Fig. 1B). No appreciable reduction of mutagenicity was observed when the sulphate was preincubated with S105 alone. From the incubation mixture containing 5-HCR sulphate, S105, and GSH, was isolated the more polar ninhydrin-positive metabolite that was chromatographically and spectroscopically identical with that formed from 5-HCR.

The more polar, ninhydrin-positive metabolite was isolated by using an Amberlite XAD-2 resin column (2 × 20 cm) for the structural assignment from a large scale incubation mixture (50 ml), consisting of 5-HCR sulphate (0.1 mM), S105 (equivalent to 2.5 g of liver), and GSH (4 mM) as previously reported [5]. Under these incubation conditions, 5-HCR sulphate used completely disappeared until 3 hr after incubation at 37° and pH 7.4 with almost quantitative formation of the more polar metabolite and with concomitant formation of a little of the non-enzymic hydrolysis product 5-HCR (≤2%). The more polar metabolite obtained by eluting the resin column with methanol was purified by HPLC carried out on the octadecylsilica column in methanol–water (1:1, 0.8 ml/min) free from TBA for subsequent mass spectrometry; the metabolite, eluted as a u.v.-absorbing peak at 14 min from the HPLC column under these conditions, showed a base ion peak at *m/z* 548 ($M^+ + 1$) and a fragment ion peak at *m/z* 308 ($GSH^+ + 1$) with relative intensity of 75% in the field desorption mass spectrum recorded at an accelerating voltage of 4 kV and an emitter current of 20 mA.

For further structural assignment, the more polar metabolite (1 mg) was treated in 0.1 M Tris–HCl buffer, pH 8.5 (5 ml) at 37° for 3 hr with a γ-glutamyltranspeptidase preparation (5 units, Sigma Chemical Co., St. Louis, MO) which also had a potent peptidase activity. A single, less polar product was formed in almost quantitative yield and isolated from the reaction mixture on the XAD-2 resin column. The product, eluted from the resin column with methanol, showed a ninhydrin-positive and u.v.-absorbing spot at R_f 0.65 in the thin-layer chromatogram obtained under the aforementioned conditions and u.v.-absorption and fluorescence emission spectra almost superimposable on those of the mother metabolite. A field desorption mass spectrum of the less polar product, recorded under the aforementioned conditions, showed a base ion peak at *m/z* 362 ($M^+ + 1$) after it was purified by HPLC; it was eluted at 10 min from the aforementioned column with methanol–water (4:1, 0.8 ml/min) without TBA. An acetyl-methyl derivative, obtained by sequential treatments of the less polar product with acetic anhydride in aqueous sodium hydroxide and an ethereal solution of diazomethane, followed by purification by TLC, showed a molecular ion peak at *m/z* 417 ($C_{25}H_{23}NO_3S^+ = M^+$) and fragment ion peaks at *m/z* 374 ($M^+ - COCH_3$), 241 ($M^+ - SCH_2CH(NHAc)COOCH_3$), and 226 ($M^+ - CH_2SCH_2CH(NHAc)COOCH_3 - H$) with relative intensities of 19, 7.5, 100, and 11%, respectively, in the electron-impact mass spectrum, indicating the structure of the product to be *S*-(chrysen-5-yl)methylcysteine.

The more polar metabolite, thus assigned as *S*-(chrysen-5-yl)methylglutathione, showed no intrinsic mutagenicity towards TA98. The non-enzymic formation of the GSH conjugate from 5-HCR sulphate was found by the HPLC

study not to occur to any detectable extent merely by the incubation with GSH in the absence of S105. The apparent rate for the enzymic GSH conjugation of the sulphate was 5.6 nmol/50 mg liver/min, which was 190 times higher than that for sulphation of 5-HCR in the S105-PAPS system, strongly suggesting the reason of why the bacterial mutagenicity of 5-HCR was completely retarded by the addition of GSH to the S105-PAPS system. The well known inhibitor for GSH *S*-transferase, bromosulphophthalene (0.5 mM) added to the S105-GSH system, potently inhibited (86%) the formation of the GSH conjugate from 5-HCR sulphate (0.1 mM) used as a substrate.

In order to obtain direct evidence for the GSH *S*-transferase-mediated reduction of the covalent binding of the active sulphate ester to DNA in the presence of GSH, 5-HCR sulphate (0.05–0.5 mM) was incubated with calf thymus DNA and GSH in the presence and in the absence of S105 in 0.1 M phosphate buffer, pH 7.4. After the DNA was isolated by the previously reported method [6], it was washed with several solvents to remove excess 5-HCR sulphate and subjected to fluorospectrophotometry to determine the 5-methylene-chrysene chromophor binding to DNA bases as previously reported [3]. The fluorophotometric data indicated that GSH also strongly inhibited the binding of 5-HCR sulphate to the DNA in the presence of S105 and also that the binding was little retarded by GSH in the absence of S105 (Fig. 2). The data were in good agreement with those on the aforementioned reduction of the intrinsic mutagenicity of 5-HCR sulphate by S105 and GSH, especially at lower concentrations of the sulphate up to 0.1 mM.

Recently, Watabe *et al.* demonstrated that highly mutagenic sulphate esters of the carcinogens such as 7-hydroxymethyl-12-methyl-benz[*a*]anthracene (-BA), 7-methyl-12-hydroxymethyl-BA, 7-hydroxymethyl-BA, and 7,12-dihydroxymethyl-BA, were inactivated to non-mutagenic GSH conjugates in rat liver S105 [5, 7–9]. The present investigation also indicates that GSH readily scavenges the reactive sulphate ester formed from 5-HCR and inhibits its binding to DNA in the presence of hepatic GSH *S*-transferase, so that the carcinogenic 5-HCR could not exert any mutagenicity in the presence of GSH. Thus, the metabolic activation and inactivation of 5-HCR in hepatic cytosol are summarized as illustrated in Fig. 3.

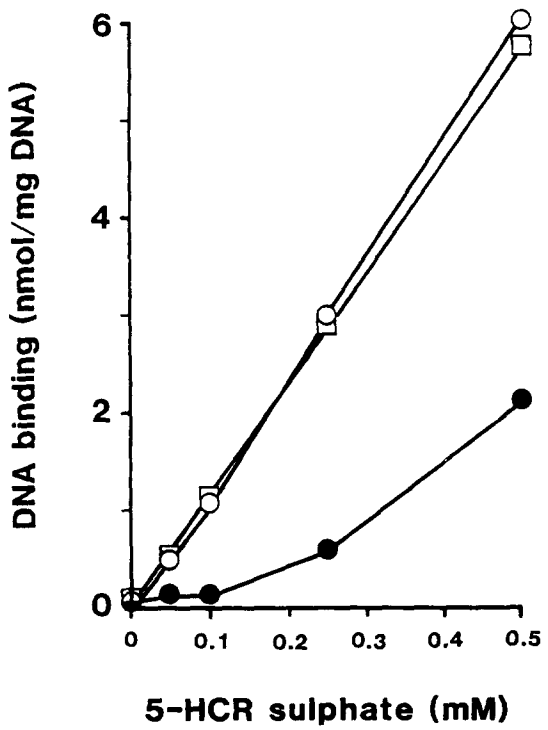

Fig. 2. Effect of GSH on covalent binding of 5-HCR sulphate to calf thymus DNA. Open circles: 5-HCR sulphate (Na) dissolved in methanol (0.05 ml) was incubated with calf thymus DNA (2 mg) at 37° for 60 min in a final volume of 1 ml of 0.1 M KH_2PO_4–Na_2HPO_4 buffer, pH 7.4. Open squares: the sulphate was incubated with DNA (2 mg) in the presence of GSH (4 μmol) under the aforementioned conditions. Closed circles: the sulphate was incubated with DNA (2 mg) in the presence of GSH (4 μmol) and S105 (2.8 mg of protein). The DNA was isolated from the mixture by the previously reported method [6], washed three times with ethanol, acetone and ether (2 ml each), dried *in vacuo*, and dissolved in water (0.04 mg/ml) for recording the fluorescence emission spectrum at an excitation wavelength of 271 nm according to the previously reported method [3]. Degree of binding of the 5-methylene-chrysene chromophor to DNA was estimated from the intensity of the fluorescence emission at 396 nm.

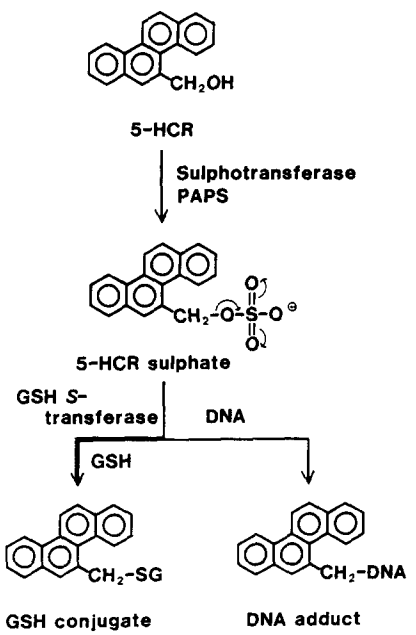

Fig. 3. Metabolic activation by sulphotransferase and inactivation by GSH *S*-transferase of 5-HCR in rat liver cytosol.

* Author to whom correspondence should be addressed.

Laboratory of Drug Metabolism & Toxicology
Department of Hygienic Chemistry
Tokyo College of Pharmacy
Hachioji, Tokyo 192-03, Japan

HARUHIRO OKUDA
KIYOSHI MIWA
HIROSHI NOJIMA
TADASHI WATABE*

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Biochemical Pharmacology, Vol. 35, No. 24, pp. 4576–4579, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

Uptake of opioid drugs by rat cerebrocortical brain slices

(*Received* 24 *January* 1986; *accepted* 1 *July* 1986)

It is widely accepted that opioid drugs elicit analgesia by binding to specific cell-surface receptors in the CNS [1, 2]. The uptake of morphine into brain slices has been reported by previous workers [3, 4], but the extent to which these drugs can penetrate into the intracellular compartment of neural cells has been little investigated. It is known that the endogenous opioid peptides may be internalized by neurons as a result of receptor recycling [5, 6], and it is possible that opioid drugs may also be translocated across neuronal membranes in this fashion. In some cases their action may involve an intracellular receptor. The present study was designed to establish the extent to which opioid drugs are able to penetrate the intracellular compartment of brain cerebrocortical slices. For this purpose radiolabelled morphine, etorphine (μ-receptor agonists) and ethylketocyclazocine (EKC*; K-agonist/μ-antagonist) were employed as the opioid drugs.

Materials and methods

Brain slices (approx. 1.0 × 0.5 cm and 0.3 mm thick), were prepared from the cerebral cortex of adult Sprague–Dawley rats using a blade and recessed guide [7]. Individual slices were preincubated in 4.1 ml of Krebs–Tris medium of composition (mM); NaCl 138, KCl 5.0, $MgSO_4$ 1.0, $CaCl_2$ 1.2, NaH_2PO_4 1.2, Tris–HCl 20, glucose 10, pH 7.4. The incubation was carried out at 37° in 5 ml glass beakers. Each beaker carried a gassing line which delivered pure oxygen into the medium. After 30 min preincubation, 200 μl [^{3}H]opioid compound was added to each beaker (final concentration see results, final sp. act. 0.9 Ci/mol). Also, 200 μl [^{14}C]inulin, dissolved in Krebs–Tris medium (final concentration 0.05 mg/ml, final sp. act. 10.9 Ci/mol) was added 10 sec later. The period of incubation was terminated by the removal of the slices from the incubation beakers. After draining well the final wet weight of tissue was measured. Each slice was either dissolved in 1 ml of 1 M NaOH and an aliquot taken for radioactivity counting to determine total uptake, or homogenized by hand in 1 ml of 0.32 M sucrose, centrifuged at 10,000 *g* in a microcentrifuge for 2 min, and an aliquot of the supernatant taken for radioactivity counting. In the latter case, the pellet obtained was resuspended in 500 μl trichloroacetic acid (TCA) (10%) and recentrifuged. An aliquot of this supernatant was also taken for radioactivity measurement. The radioactivity in an aliquot of the incubation medium from each beaker was also determined by liquid scintillation counting.

The Inulin Space (I.S.) (μl/100 mg final wet weight) was calculated as follows:

$$\text{I.S.} = \frac{\text{dpm [}^{14}\text{C]inulin/100 mg final wet weight}}{\text{dpm [}^{14}\text{C]inulin/}\mu\text{l incubation medium}}$$

The total volume of 100 mg final wet weight slice was taken as 80 ± 1 [8]. The "non-inulin" space was the difference between the total volume of the tissue and the I.S.

The tissue/medium (T/M) ratio for the drug was taken as the ratio:

$$\frac{\text{dpm in "non-inulin" space}}{\text{dpm in same volume of incubation medium}}$$

Using the specific radioactivity of the drug in the medium it was possible to estimate the concentration of the drug in the intracellular space.

Ethylketocyclazocine methane sulphonate and etorphine HCl were the kind gifts of Sterling-Winthrop and C-Vet Ltd. respectively. Morphine sulphate was purchased from Macfarlan Smith Ltd. The radioactive compounds were all obtained from Amersham International.

Results and discussion

As shown in previous detailed studies, the equilibration of inulin between the incubation medium and the I.S. (inulin space) occurred within 15 min [8, 9]. The half-time for inulin uptake was close to 5 min, which is in good accordance with another report [9]. The mean value for the I.S. was calculated from all measurements after equilibration of the inulin within the slices. The I.S. was 46.3 ± 1.2 μl/100 mg final wet weight (mean ± SEM, N = 30). The "non-inulin" space was calculated to be 33.7 μl/100 mg final wet weight, assuming that 80% of the slice weight was water and 20% solids [8]. This was identical to the value for the non-inulin space calculated using the total volume of tissue determined by employing ^3H_2O (0.4 μCi/incubation), following the same experimental procedure.

The accumulation of EKC (10 μM) was maximal after 45 min, when the T/M for the drug was 38. The maximal uptake was 12.9 nmol/100 mg final wet weight of slice. Morphine uptake at 10 μM, was maximal after 20 to 30 min, at a final T/M ratio of 2.9. Etorphine accumulation at 10 μM followed a similar time scale to that of morphine uptake but the final T/M was 15. The maximal uptakes of morphine and etorphine were 1.0 and 5.1 nmol/100 mg final wet weight, respectively (Fig. 1). In experiments where cerebrocortical slices were incubated under similar conditions and exposed to 10 μM of these opioid drugs for 15 min, inhibitory effects were observed on Ca^{2+} uptake and amino acid neurotransmitter release [10]. The intracellular concentration of morphine, etorphine and EKC would be 21, 122 and 228 μM respectively in these conditions. This suggests that sufficient drug would be able to translocate the cell membrane in order to have an effect at putative intracellular site(s) of action.

The time course of the accumulation of these opioid drugs is incompatible with the characteristics of binding to high-affinity sites on the plasma membrane surface. It is likely that entry of EKC, morphine and etorphine to the

*Abbreviations used: EKC, ethylketocyclazocine; I.S., inulin space; TCA, trichloroacetic acid.