These results taken together confirm the heterogeneity of [^{3}H]AMPH-binding sites and strongly support the hypothesis that the drug could bind to membrane-acceptor sites which are neither a receptor nor MAO-A. The effects of varying pH or salt concentrations suggest that both ionic and hydrophobic interactions are present. Our previous study [3] concerning (±)*p*-chloroamphetamine, which exhibited a greater affinity for [^{3}H]AMPH sites than did (+)amphetamine, supports the existence of hydrophobic interactions. In addition the acceptor sites for [^{3}H]AMPH can be membrane phospholipids since amphiphilic drugs possessing structural analogies with amphetamine (e.g. chlorphentermine and phentermine) exhibit binding characteristics to phospholipids [16] which are similar to those of [^{3}H]AMPH binding.

In summary, the present data demonstrate that [^{3}H] AMPH binding does not reflect an accumulation process but rather a binding phenomenon to an acceptor site which is probably a polar lipid but not MAO-A. Our results and the multiple biochemical actions of amphetamine (e.g. inhihition of dopamine and noradrenaline uptake [6] and inhibition of MAO-A [17]) lead to the conclusion that [^{3}H]AMPH is not suitable for biochemical *in vitro* study.

*Centre de Recherche Delalande
10 rue des Carrières
92500 Rueil-Malmaison, France
‡Laboratoire Fournier
Centre de Recherche
50 rue de Dijon
Fontaine-lès-Dijon
21121 Daix, France

ARIANE LESAGE*†
MARGHERITA STROLIN BENEDETTI‡
JEAN FRANÇOIS RUMIGNY*

REFERENCES

1. S. M. Paul, B. Hulihan, R. Hauger and P. Skolnick, *Eur. J. Pharmac.* **78**, 145 (1982).
2. S. M. Paul, B. Hulihan-Giblin and P. Skolnick, *Science, Wash.* **218**, 487 (1982).
3. A. Lesage, M. Strolin Benedetti and J. F. Rumigny, *Neurochem. Int.* **6**, 283 (1984).
4. D. L. Nelson, A. Herbert, Y. Petillot, L. Pichat, J. Glowinski and M. Hamon, *J. Neurochem.* **32**, 1817 (1979).
5. A. Lesage, M. Strolin Benedetti and J. F. Rumigny, in *Monoamine Oxidase and Disease—Prospects for Therapy with Reversible Inhibitors* (Eds. K. F. Tipton, P. Dostert and M. Strolin Benedetti), p. 580. Academic Press, London (1984).
6. H. H. Keller, R. Schaffner, M. O. Carruba, W. P. Burkard, M. Pieri, E. P. Bonetti, R. Scherschlicht, M. Da Prada and W. E. Heafely, in *Typical and Atypical Antidepressants: Molecular Mechanisms* (Eds. E. Costa and G. Racagni), p. 249. Raven Press, New York (1982).
7. J. Glowinski and L. L. Iversen, *J. Neurochem.* **13**, 655 (1966)
8. S. Urwyler and J. P. Von Wartburg, *Biochem. Pharmac.* **29**, 3067 (1980).
9. P. J. Munson and D. Rodbard, *Analyt. Biochem.* **107**, 220 (1980).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
11. C. J. Fowler and M. Strolin Benedetti, *J. Neurochem.* **40**, 1534 (1983).
12. H. Bönisch, *Archs Pharmac.* **327**, 267 (1984).
13. R. L. Hauger, B. Hulihan-Giblin, P. Skolnick and S. M. Paul, *Life Sci.* **34**, 771 (1984).
14. J. M. Maloteaux, A. Gossuin, C. Waterkeyn and P. M. Laduron, *Biochem. Pharmac.* **32**, 2543 (1983).
15. *The Pharmaceutical Codex* (11th Edn). The Pharmaceutical Press, London (1974).
16. H. Lüllmann and M. Wehling, *Biochem. Pharmac.* **28**, 3409 (1979).
17. T. J. Mantle, K. F. Tipton and N. J. Garrett, *Biochem. Pharmac.* **25**, 2073 (1976).

† To whom all correspondence should be addressed.

Biochemical Pharmacology, Vol. 34, No. 16, pp. 3002–3005, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00
© 1985 Pergamon Press Ltd.

The carcinogen, 7-hydroxymethyl-12-methylbenz[*a*]anthracene, is activated and covalently binds to DNA via a sulphate ester

(*Received* 7 *January* 1985; *accepted* 2 *April* 1985)

7-Hydroxymethyl-12-methylbenz[*a*]anthracene(7-HMBA), a potently carcinogenic major metabolite of 7,12-dimethylbenz[*a*]-anthracene (DMBA) in rat liver [1–3], has recently been demonstrated to induce His$^+$ reverse mutation in *Salmonella typhimurium* TA 98 to a remarkable extent in the presence of liver cytosol fortified with 3′-phosphoadenosine 5′-phosphosulphate (PAPS) and to a slight extent in the presence of liver microsomes or a 9000 *g* supernatant fraction fortified with NADPH [4]. From the cytosol-PAPS system, a highly reactive 7-hydroxymethyl sulphate ester of 7-HMBA has been isolated and identified as a directly acting mutagenic metabolite [4]. The sulphate ester conjugate (7-HMBA sulphate) is yielded at a significant rate by cytosolic sulphotransferase and inactivated by cytosolic glutathione *S*-transferase in the presence of glutathione (GSH) to form an unreactive and non-mutagenic GSH *S*-conjugate [5]. In addition, biologically formed 7-HMBA sulphate bound covalently through its 7-methylene carbon with loss of a sulphate anion to nucleophilic residues of amino acids consisting in hepatic cytosolic proteins; *S*-cysteine, ε-*N*-lysine, and *S*-methionine adducts of the carcinogen have been isolated from the digested proteins and identified with the corresponding synthetic specimens [6].

These facts might provide us an important clue to solve the long-arising question about the significant increase in carcinogenicity of the weak carcinogen, benz[*a*]anthracene (BA), up to the highest level among polynuclear aromatic hydrocarbons by methylation at the "L-region" (7- and 12-positions) of BA as seen in DMBA, 7-methyl-BA and 12-methyl-BA [7].

Sulphate esters have long been putative, biologically reactive intermediates since Miller and his co-workers proposed them for the metabolic activation of the proximate carcinogens such as *N*-OH-FAA, *N*-OH-MAB and 1′-hydroxysafrole [8–12]. However, they have not yet succeeded in detecting the sulphates from biological systems. *N*-Hydroxyl derivatives of carcinogenic aromatic amines have also been demonstrated to be activated at weakly acidic pH, probably possible in urinary bladder [13], through their *O*-acetates [14], *O*- [15, 16] and *N*-glucuronides [13], aminoacyl esters [17] or nitrosyl radicals

[18, 19] in hepatic *in vitro* systems. An approach has been made by Cavalieri and his co-workers to confirm whether a sulphate ester is a directly acting carcinogen or not by using synthetic 6-hydroxymethylbenzo[*a*]pyrene (6-HMBP) sulphate [7]. They have demonstrated that the synthetic sulphate is a more potent carcinogen than 6-HMBP but have not yet confirmed whether or not it is a true metabolite in a biological system. The present paper provides the first direct evidence for covalent binding to DNA of a biologically formed reactive sulphate which can be isolated from the biological system.

7-HMBA (0.1 mM) dissolved in dimethyl sulphoxide (10% v/v) was incubated at 37° and pH 7.4 (0.1 M KH_2PO_4-Na_2HPO_4) for 20 min with a calf-thymus DNA preparation (2 mg/ml, Sigma Type I), in the presence of a dialysed preparation of male Wistar rat liver cytosol (3.5 mg protein/ml) fortified with disodium ATP (5 mM), sodium sulphate (5 mM), magnesium chloride (3 mM) and EDTA (0.1 mM). The DNA was isolated from the mixture (10 ml) by the previously reported method [20], washed 3 times with ethanol and then with acetone (10 ml each), dried *in vacuo* and dissolved in water (2 mg/5 ml). The DNA solution showed intense fluorescence emission and excitation spectra which closely resembled the ones of 7-HMBA but shifted to longer wavelength regions (Fig. 1). No fluorescence was emitted from an aqueous solution of the isolated and washed DNA either when the hepatic preparation was boiled or when any one of the components for the PAPS-generating system, ATP, sodium sulphate or the dialysed cytosolic fraction, was omitted from the mixture.

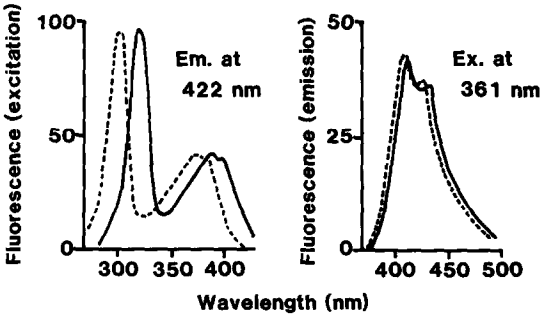

Fig. 1. Fluorescence excitation and emission spectra of DNA-bound and free 7-HMBA. Fluorescence intensity is expressed in arbitrary units. Solid and broken lines represent the spectra of DNA-bound and free 7-HMBA, respectively. The aqueous solution of the isolated DNA (0.4 mg/ml) showed the same spectral intensities as those of an aqueous solution of 7-HMBA (6.7 μM) dissolved in ethanol (0.1%, v/v) at the wavelengths for their peak maxima.

A fluorophotometric study demonstrated that under the aforementioned incubation conditions, 7-HMBA bound to DNA in the ratio 1.4 nmoles/mg DNA and to cytosolic proteins 1.25 nmoles/mg protein, the latter of which was determined according to the previously reported method [6]. Free 7-HMBA sulphate was isolated [4] and determined [6] from the incubation mixture as previously reported; it existed in the incubation medium in the ratio 0.4 nmoles/mg cytosolic protein. The fluorophotometric study also indicated that in the absence of the subcellular preparation and the fortifying agents, synthetic 7-HMBA sulphate [4] (Na salt, 0.1 mM) bound to calf-thymus DNA in the ratio 17 nmoles/mg DNA under the same incubation conditions as mentioned above. The fluorescence emission and excitation spectra recorded were identical with those of the DNA isolated from the incubation mixture consisting of 7-HMBA, hepatic cytosol and the PAPS-generating system.

The DNA (20 mg) treated with 7-HMBA and the hepatic sulphotransferase system or directly with 7-HMBA sulphate as mentioned above was dissolved in water (10 ml) and denatured by heating at 100° for 10 min and rapid cooling to 0° after being isolated and thoroughly washed with the organic solvents. The mixture was diluted with 1/35 M veronal-acetate buffer, pH 5.3 (10 ml), containing zinc chloride (0.2 mM) and nuclease P_1 (2 mg, Yamasa Shoyu Co. Ltd., Choushi, Japan) and incubated at 50° for 2 hr. The DNA digest was adjusted to pH 2.8 with 0.05 N HCl and heated at 80° for 1 hr to hydrolyse the base-deoxyribosyl bonds. The acid hydrolysate was made alkaline with concentrated ammonia and extracted with ethyl acetate (5 volumes) containing 12-hydroxymethyl-7-methyl-BA as an internal standard. High performance liquid chromatography (HPLC) on an octadecylsilicone column (Nucleosil $7C_{18}$, 7 μm in particle size, 4 mm × 25 cm) showed the extract to contain three intense fluorescence materials which were eluted with methanol-water (8:2, 1 ml/min) as well-separated peaks at 5.8 (I), 8.2 (II) and 18 (III) min in the peak area ratio 2:1:2. Under these conditions, the internal standard was eluted at 6.5 min. The ethyl acetate extract contained 76% of total fluorescence emitted by the isolated DNA.

The peak I material isolated was found to be a condensation product of guanine and 7-HMBA with loss of H_2O, which showed molecular and fragment ion peaks in the mass spectrum (MS) at m/z (relative intensity, %): 405 (M^+, 5), 389 (3), 256 (100), 255 (85), 252 (93), 251 (59), 241 (45), 240 (38), 238 (78), 226 (19) and 151 (45). The guanine adduct was acid-labile and slowly hydrolysed at pH 2.8 on heating at 80° to yield the peak II material which was isolated by HPLC and identified as 7-HMBA by MS, u.v.-absorption spectroscopy, and silica gel TLC. Thus, the peak II material in the acid-treated digest proved to be an artifact from the guanine adduct since the free carcinogen, if present, had been confirmed by fluorophotometry to be completely removed from the isolated DNA through the washing procedures used. The peak III material, highly stable to the acidic conditions used, was found to be a condensation product of adenine and 7-HMBA with loss of H_2O, which showed molecular and fragment ion peaks in MS at m/z (relative intensity, %): 390 (M^+, +1, 20), 389 (M^+, 62), 374 (8), 297 (2), 257 (24), 255 (100), 254 (22), 253 (21), 252 (16), 240 (19), 238 (35) and 228 (9). Fluorometry following HPLC indicated that the ratio of the adenine and guanine adducts, estimating the latter from peaks I and II, was 2:3 with each digest of DNA which was incubated with 7-HMBA sulphate alone as well as with 7-HMBA in the presence of hepatic cytosol fortified with the PAPS-generating system.

Structural evidence for the purine-base adducts was obtained by a synthetic method. 7-Aminomethyl-12-methyl-BA (7-AMBA) which was synthesized from the known compound, 7-bromomethyl-12-methyl-BA [22], via *N*-(12-methyl-BA-7-yl)methylphthalimide became a key compound for synthesizing both adducts. Namely, 7-bromomethyl-12-methyl-BA (0.3 M) was agitated at 70° for 1 hr with excess potassium phthalimide (0.36 M) in dimethyl formamide. *N*-(12-Methyl-BA-7-yl)methyl-phthalimide (m.p. 203–205°) obtained was heated for 2 hr with excess hydrazine hydrate in methanol under the refluxing condition. 7-AMBA, unstable in free form in air, was thus obtained in almost quantitative yield from the bromide; MS m/z: 271 (M^+); NMR $\delta_{TMS}^{CDCl_3}$ ppm: 3.35 (*s*, 3 H, 12-CH_3), 4.77 (broad *s*, 2 H, 7-CH_2-) and 7.33–8.13 (*m*, 10 H, aromatic H).

Heating (80°) 2-chloro-6-hydroxypurine and 7-AMBA (40 mM each) for 18 hr in dimethyl sulphoxide and nitrogen as a gaseous phase gave the guanine adduct in 40% yield after silica gel chromatography in acetone–benzene (5:3) and recrystallizations from acetic acid–methanol; u.v. λ_{max}^{EtOH} nm (log ε): 395 (3.10), 376 (3.68), 359 (3.79), 343

(3.64), 328 (3.50), 294 (4.43), 282 (4.43), 273 (4.37), 263 (3.31), 255 (4.20), 233 (4.09) and 220 (4.24); i.r. ν_{max}^{KBr} cm^{-1}: 3321, 3120, 1690, 1670, 1479, 1464, 1418 and 1375. Chromatographic data, mass, u.v.-absorption, fluorescence emission and excitation spectra of the synthetic adduct were all identical with those of the guanine adduct isolated from the DNA. Thus the structure of the guanine adduct was identified as N^2-(12-methyl-BA-7-yl)methylguanine. Heating 6-chloropurine and 7-AMBA (40 mM each) under the same conditions gave the adenine adduct in 65% yield after silica gel chromatography in acetone–benzene (5:1) and recrystallizations from acetone–methanol; u.v. λ_{max}^{EtOH} nm (log ε): 395 (3.10), 376 (3.76), 359 (3.79), 343 (3.63), 328 (3.50), 294 (4.43), 283 (4.41), 273 (4.35), 262 (3.30), 255 (4.20), 233 (4.0) and 220 (4.20); i.r. ν_{max}^{EtOH} cm^{-1}: 3295, 3120, 1675, 1605, 1452, 1420 and 1370. The synthetic adduct was chromatographically and spectroscopically identical with the adenine adduct from the DNA. Thus, the structure of the adenine adduct was identified as N^6-(12-methyl-BA-7-yl)methyladenine.

Therefore, metabolic activation of 7-HMBA and its covalent binding to DNA via the sulphate ester conjugate can be illustrated as summarized in Fig. 2. The sulphate ester moiety plays a role as a leaving group in the formation of the 7-methylene carbonium ion which acts as an alkylating agent on DNA and proteins. In fact, so far as examined by a bacterial mutagenicity test, synthetic sulphates of arylmethanols such as benzyl alcohol, 1- and 2-hydroxymethylnaphthalenes, 1-hydroxymethylpyrene [4] and 7-hydroxymethyl-BA [7] were all mutagenic towards *Salmonella*, of which 7-hydroxymethyl-BA sulphate has recently proved to be a metabolite of the carcinogen, 7-hydroxymethyl-BA, in rat liver [22].

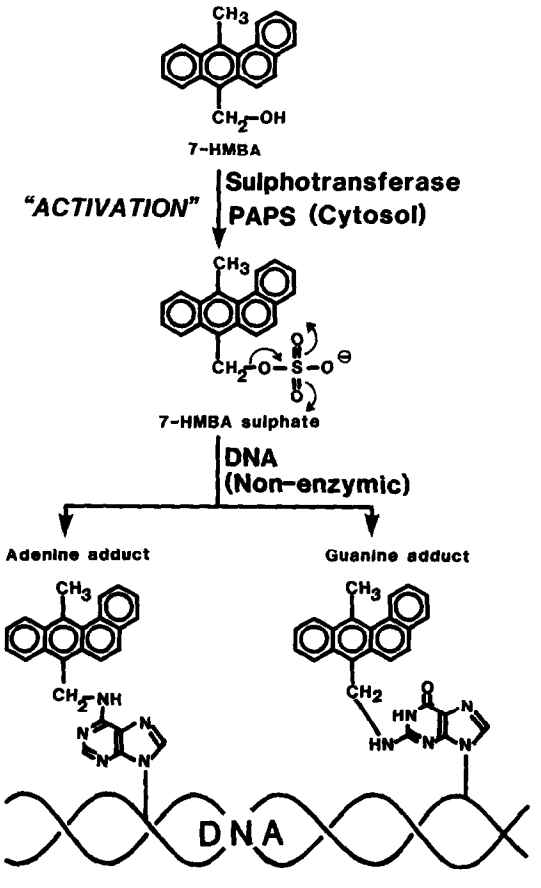

Fig. 2. Covalent binding of the carcinogen, 7-HMBA, to DNA via reactive 7-HMBA sulphate formed by sulphotransferase in rat liver cytosol.

Although bay or non-bay region epoxides have been strongly suggested to be ultimate forms of DMBA or 7-HMBA [23–30], they do not give any answer as to why 7-HMBA may be directly correlated to the activation of DMBA since the carcinogenic alcohol is a major hepatic metabolite [30–32]. The present investigation provides the first direct evidence not only for the participation of the sulphate ester in the metabolic activation of the carcinogen, but also for the presence of another type of metabolic activation mechanism in addition to the well-known epoxide formation in polynuclear aromatic hydrocarbons.

Laboratory of Drug Metabolism and Toxicology
Department of Hygienic Chemistry
Tokyo College of Pharmacy
1432-1 Horinouchi
Hachioji-shi
Tokyo 192-03
Japan

TADASHI WATABE*
TOSHIKO FUJIEDA
AKIRA HIRATSUKA
TSUNEO ISHIZUKA
YOSHIKI HAKAMATA
KENICHIRO OGURA

REFERENCES

1. E. Boyland, P. Sims and C. Huggins, *Nature, Lond.* **207**, 816 (1965).
2. C. Huggins and S. Morii, *J. exp. Med.* **114**, 741 (1961).
3. J. W. Flesher and K. L. Sydnor, *Cancer Res.* **31**, 1951 (1971).
4. T. Watabe, T. Ishizuka, M. Isobe and N. Ozawa, *Science* **215**, 403 (1982).
5. T. Watabe, T. Ishizuka, N. Ozawa and M. Isobe, *Biochem. Pharmac.* **31**, 2542 (1982).
6. T. Watabe, T. Ishizuka, Y. Hakamata, T. Aizawa and M. Isobe, *Biochem. Pharmac.* **32**, 2120 (1983).
7. E. Cavalieri, R. Roth and E. Rogan, in *Polynuclear Aromatic Hydrocarbons, Third International Symposium on Chemistry and Biology, Carcinogenesis and Mutagenesis* (Eds. P. W. Jones and P. Leber), pp. 517–529. Ann Arbor Science, Ann Arbor, Michigan (1979).
8. J. R. DeBaun, J. Y. R. Smith, E. C. Miller and J. A. Miller, *Science* **167**, 184 (1970).
9. F. F. Kadlubar, J. A. Miller and E. C. Miller, *Cancer Res.* **36**, 2350 (1976).
10. P. G. Wislocki, P. Borchert, J. A. Miller and E. C. Miller, *Cancer Res.* **36**, 1686 (1976).
11. J. R. DeBaun, E. C. Miller and J. A. Miller, *Cancer Res.* **30**, 577 (1970).
12. E. W. Boberg, E. C. Miller, J. A. Miller, A. Poland and A. Liem, *Cancer Res.* **43**, 5163 (1983).
13. F. F. Kadlubar, J. A. Miller and E. C. Miller, *Cancer Res.* **37**, 805 (1977).
14. C. M. King and I. B. Glowinski, *Envir. Hlth Perspect.* **49**, 43 (1983).
15. C. C. Irving, *Xenobiotica* **1**, 387 (1971).
16. E. C. Miller, P. D. Lotlikar, J. A. Miller, B. W. Butler, C. C. Irving and J. T. Hill, *Molec. Pharmac.* **4**, 147 (1968).
17. Y. Yamazoe, M. Shimada, T. Kamataki and R. Kato, *Biochem. biophys. Res. Commun.* **107**, 165 (1982).
18. T. Kimura, M. Kodama and C. Nagata, *Gann* **71**, 417 (1979).
19. T. Kimura, M. Kodama and C. Nagata, *Biochem. Pharmac.* **28**, 557 (1979).
20. C. C. Irving and R. A. Veazey, *Biochim. biophys. Acta* **166**, 246 (1968).
21. A. Dipple and T. A. Slade, *Eur. J. Cancer* **6**, 417 (1970).
22. Y. Hakamata and T. Watabe, *Proceedings of the 55th*

* To whom all correspondence should be addressed.

Annual Meeting for the Japanese Society of Biochemistry, p. 889. Osaka, Japan (1982).
23. E. Huberman, L. Aspiras, C. Heidelberger, P. L. Grover and P. Sims, *Proc. natn. Acad. Sci. U.S.A.* **68**, 3195 (1971).
24. H. Marquardt, J. E. Sodergren, P. Sims and P. L. Grover, *Int. J. Cancer* **13**, 304 (1974).
25. D. M. Jerina, H. Yagi, R. E. Lehr, D. R. Thakker, M. Schaefer-Ridder, J. M. Karle, W. Levin, A. W. Wood, R. L. Chang and A. H. Conney, in *Polycyclic Hydrocarbons and Cancer*, Vol. 1 (Eds. H. V. Gelboin and P. O. P. Ts'O), pp. 173–188. Academic Press, New York (1978).
26. C. Malaveille, H. Bartsch, B. Tierney, P. L. Grover and P. Sims, *Biochem. biophys. Res. Commun.* **83**, 1468 (1978).
27. P. G. Wislocki, K. M. Gadek, M. W. Chou, S. K. Yang and A. Y. H. Lu, *Cancer Res.* **40**, 3661 (1980).
28. J. T. Sawicki, R. C. Moschel and A. Dipple, *Cancer Res.* **43**, 3212 (1983).
29. A. Dipple, M. Pigott, R. C. Moschel and N. Costantino, *Cancer Res.* **43**, 4132 (1983).
30. E. Boyland and P. Sims, *Biochem. J.* **95**, 780 (1965).
31. J. DiGiovanni and M. R. Juchau, *Drug Metab. Rev.* **11**, 61 (1980).
32. P. H. Jellinck and B. Goudy, *Science* **152**, 1375 (1966).

Biochemical Pharmacology, Vol. 34, No. 16, pp. 3005–3008, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00
© 1985 Pergamon Press Ltd.

Microsomal reduction of bisulfite (aqueous sulfur dioxide)—Sulfur dioxide anion free radical formation by cytochrome P-450

(*Received* 17 *October* 1984; *accepted* 1 *February* 1985)

In aqueous medium near pH 7, SO_2 exists primarily as sulfite ($SO_3^=$) and bisulfite (HSO_3^-) and, to a minor extent, as hydrated SO_2 [1, 2]. The nomenclature (bi)sulfite is used when it is not known which of these species, all of which have a sulfur atom with a formal oxidation state of plus four, is involved in a reaction.

The metabolism of (bi)sulfite has centered upon its oxidation to sulfate. Sulfite oxidase, a mitochondrial enzyme, is thought to be primarily responsible for the oxidation of (bi)sulfite *in vivo* [2, 3]. Sulfite oxidase is considered to be a two-electron acceptor of electrons from (bi)sulfite.

$$SO_3^= \rightarrow SO_3 + 2e^- \quad (1)$$

$$SO_3 + H_2O \rightarrow SO_4^= + 2H^+ \quad (2)$$

These reactions are thought to detoxify sulfite without forming free radical intermediates [4].

The one-electron oxidation of (bi)sulfite

$$SO_3^= \rightarrow \dot{S}O_3^- + e^- \quad (3)$$

is catalyzed by peroxidases. Either horseradish peroxidase [5, 6] or the prostaglandin H synthase (hydroperoxidase) in ram seminal vesicles or guinea pig lung microsomes [7] catalyzes the formation of the sulfur trioxide anion free radical ($\dot{S}O_3^-$) as demonstrated with ESR investigations. Autoxidation of (bi)sulfite is catalyzed by transition metals, but while this reaction is thought to form SO_3^-, it is not thought to be important *in vivo* [2, 4].

Although the biological oxidation of (bi)sulfite is certainly more common than (bi)sulfite reduction [1–3, 8], under anaerobic conditions (bi)sulfite is reduced to dithionite by either reduced flavodoxins or a mixture of paraquat, H_2, and hydrogenase [9]. In the latter case, the paraquat cation free radical reduces (bi)sulfite to form the sulfur dioxide anion free radical, which is in equilibrium with dithionite [10].

$$2\dot{S}O_2^- \rightleftarrows S_2O_4^= \quad (4)$$

Rat liver microsomes contain cytochrome P-450 and its flavin-containing reductase, NADPH-cytochrome P-450 reductase. The cytochrome P-450-dependent monooxygenase system is known to donate one electron to a few xenobiotics and, thereby, to form free radicals [11]. With this fact in mind, electron spin resonance was used to search for the sulfur dioxide anion free radical [the one-electron reduction product of (bi)sulfite] in rat hepatic microsomal incubations.

Materials and methods

Hepatic microsomes, mitochondria, and cytosol were prepared from fed, untreated Sprague–Dawley rats, as described [12], and kept on ice until used. ESR spectra were recorded with a Varian E-109 spectrometer equipped with an E-238 TM_{110} cavity. The g-value of the sulfur dioxide anion radical was determined relative to the g-value (2.0037) of 2,2-diphenyl-1-picrylhydrazyl (DPPH) dispersed in KCl. A capillary tube containing this secondary g-value standard was attached to an aqueous flat cell containing the hepatic protein incubation, and the unknown g-value was calculated as described in Ref. 13.

All experiments were performed at room temperature in either 0.1 M phosphate, Tris–HCl, or borate buffers (pH = 7.5) containing 1.0 mM diethylenetriaminepentaacetic acid (DETAPAC), which was necessary to prevent the autoxidation of (bi)sulfite during sample preparation [6]. An incubation contained ~1 mg/ml microsomal, mitochondrial, or cytosolic protein, 1.0 mM or 10 mM Na_2SO_3, 5.5 mM glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*, Sigma type XXIII), and 0.37 mM $NADP^+$ or NAD^+ in a total volume of 3 ml. The reaction was initiated by the addition of the pyridine nucleotide (10 μl of a 117 mM solution) under a nitrogen atmosphere. The flat cell was purged with nitrogen gas and then filled from the bottom using pressurized nitrogen gas to force the incubation into the flat cell, which was then sealed [14].

Results

After 14 min, microsomal incubations containing sodium sulfite and an NADPH-generating system developed a weak, broad ESR spectrum. This signal continued to increase linearly for hours (Fig. 1). The spectrum in Fig. 2A was obtained after 2 hr, and was completely dependent upon the presence of active microsomal protein (Fig. 2B), the NADPH-generating system (Fig. 2C), and sulfite (Fig. 2D). The sulfur dioxide radical anion has been reported to form nonenzymatically from (bi)sulfite (under unspecified conditions) in the presence of NADPH, NADH or ascorbate [15], but under our conditions absolutely no signal was detected in the absence of microsomal protein. Heat-