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Mutagenic activity of group VIII metal–organic complexes in the Ames test: evaluation of potential glucose biosensor components

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Five novel tris-(4,4′-substituted-2,2′-bipyridine) complexes of the group VIII metals [Fe(II), Ru(II) and Os(II)] (all having been previously examined electrochemically with respect to their ability to act as electron-transfer mediators for redox enzymes) were assayed in the Ames test to evaluate their mutagenic activity. For the bipyridine complexes, some complexes did exhibit mutagenic activity in the circumstances of this test, whereas others did not. this is comparable to the results known from the literature for ferrocene mediators. There was no specific correlation with the identities of the metal and ligand type; the possible explanations of the results are discussed in this paper. It seems probable that the penetration of the whole compound inside bacterial cells depends on the organic part of a complex, with only then the possible genotoxic activity of the complex being revealed. In view of our mutagenic test results, such complexes found to be mutagenic should not be taken into consideration as components of implanted glucose sensors in future *in vivo* experiments.

Keywords: Ames test, group VIII metals, mutagenicity, tris-bipyridine complexes

Introduction

Biosensors are devices where a biochemical/biological event is interfaced to a means of signal transduction, be it electrical, thermal or optical; upon analysis of the signal, the biological event is quantified. In particular, one of the most widely studied forms of biosensor is the glucose biosensor, due to the medical and commercial interest in controlling blood glucose levels for type I (insulin-dependent) diabetics (Reach & Wilson 1992). The most popular configuration for a glucose biosensor is that of the amperometric enzyme electrode (Hill & Sanghera 1990) based on glucose oxidase (GOD) of *Aspergillus niger* (Wilson & Turner 1992). In such a system, one option is to detect, by oxidation at an electrode, the hydrogen peroxide produced by GOD. Also, over the last 20 years, a range of 'mediated' electrodes has been developed, employing non-physiological, redox-active molecules that serve as electron transfer mediators between redox enzymes and electrodes. In the case of GOD, such a 'mediator', in its oxidized form, substitutes for molecular oxygen by accepting one or two electrons from the reduced flavin group – the prosthetic group of GOD. The mediator then passes its electron(s) on to an electrode held at an oxidizing potential, with concomitant regeneration of the oxidized mediator. Examples of such redox mediators included the much-studied ferrocenes (Cass *et al.* 1984, Cooper *et al.* 1991), organic dyes (Kulys & Cenas 1983) and 'conducting salts' (Albery *et al.* 1985), and the recently developed tris-(4,4′-substituted-2,2′-bipyridine) complexes of the group VIII metals (Zakeeruddin *et al.* 1992, Fraser *et al.* 1993). The latter compounds have been shown to be the most effective mediators to date for GOD mediation. Ferrocene mediators have been exploited commercially (The 'Exactech' sensor series from MediSense (Cambridge, MA)] in disposable electrodes *in vitro* used in conjunction with a hand-held meter for home-testing of glucose by diabetics.

One other notable area of glucose biosensing is *in vivo* monitoring (Vadgama & Desai 1991, Shaw *et al.* 1992); a biosensor giving a continuous, real-time read-out would be most desirable for monitoring blood glucose levels of diabetics. Notably, needle-type, subcutaneous sensors containing GOD have been developed. In some cases,

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mediated sensors have also been proposed (Matthews *et al.* 1988, Boutelle *et al.* 1986; Sakakida *et al.* 1993, Linke *et al.* 1994), despite the fact that it is difficult to retain low-molecular weight mediators at the electrode (Schuhmann *et al.* 1990) and in face of evidence that xenobiotic mediators such as ferrocenes can have toxic effects (Leung *et al.* 1987, Nikula *et al.* 1993). Evaluation of the mutagenic activity of redox mediators seems to be necessary for at least two reasons: (1) safety of investigators constructing new biosensors and (2) preventing patients from possible prolonged exposure in the case of *in vivo* sensors.

The aim of this study was to evaluate the mutagenic activity of certain tris(bipyridine) complexes of the group VIII metals [Fe(II), Ru(II) and Os(II)] in the Ames test (Ames *et al.* 1975, Maron & Ames 1983) with two *Salmonella typhimurium* tester strains, TA 98 snd TA 100, in the presence and absence of promutagen-activating fraction S9. These results were then compared with those in the literature for the ferrocene family of compounds.

Materials and methods

Chemicals

Tris-bipyridine complexes of the group (VIII) metals were synthesized as described previously (Zakeeruddin *et al.* 1992, Fraser *et al.* 1993). The denotations of the complexes used in this paper are as follows:

A = $[Os(DMO\text{-}bpy)_3]Cl_2$
B = $[Ru(DA\text{-}bpy)_3]Cl_2$
C = $[Fe(DA\text{-}bpy)_3](ClO_4)_2$
D = $[Os(DA\text{-}bpy)_2TEAM\text{-}bpy](PF_6)_4$
E = $[Os(DA\text{-}bpy)_3]Cl_2$

where:

DMO-bpy = 4,4′-dimethoxy-2,2′-bipyridine
DA-bpy = 4,4′-diamino-2,2′-bipyridine
TEAM-bpy = 4,4′-(trimethylammoniummethyl)-2,2′-bipyridine

The simple inorganic salts of the tested metals were used as control substances. K_2OsCl_6 was purchased from Janssen Chemicals (Beerse, Belgium), whilst $RuCl_3xH_2O$ and $FeCl_2$ were obtained from Fluka. Standard mutagens [4-nitroquinoline-*N*-oxide (NQNO) and 2-aminofluorene (2-AF)], NADP and glucose-6-phosphate were purchased from Sigma (St Louis, MO). Oxoid nutrient (Oxoid, Bicester, UK), Difco nutrient broth and Difco agar (Difco, Detroit, MI) were applied for bacterial growth. Other chemicals used for buffers and media preparation were obtained from POCH (Poland).

Mutagenicity

The mutagenic activity of Os(II), Ru(II) and Fe(II) complexes with organic ligands and simple salts of these metals was tested in accordance with the Ames test (Maron & Ames 1983) with *S. typhimurium* TA 98 and TA 100 strains (kindly supplied by Dr B. N. Ames) with and without an activation system (S9 fraction). The tested compounds were dissolved in water and assayed in doses ranging from 8 to 400 μmol per plate, depending on their toxicity to bacterial cells. The activating S9 fraction was prepared following Ames (Ames *et al.* 1975, Maron & Ames 1983), i.e. from the liver of Aroclor-1254-treated male Wistar rats. The S9 fraction was used at a volume of 50 μl (containing 1.6 mg protein) per plate. The compounds were tested in three independent experiments and, within each of these, each dose was tested twice. In all experiments, control assays of bacterial strains were performed to check the efficiency of histidine prototrophy reversion. The standard mutagens used in these control experiments were NQNO (at a dose of 0.5 μg per plate) without the S9 fraction and 2-AF (5 μg per plate) with the S9 fraction. The spontaneous revertant numbers were on average 26 rev plate^{-1} without S9 and 35 rev plate^{-1} with S9 for YA 98 strains, and 145 and 159 rev plate^{-1} for TA 100 strain, respectively.

Statistical analysis

Regression equations and correlation coefficients were calculated in accordance with routine statistical methods (e.g. Bourke *et al.* 1985).

Results

Mutagenic activities of the group VIII metal complexes are presented in Figure 1(A–E). Each panel depicts the activity of a particular complex in four mutagenic activity testing systems (TA 98 and TA 100 strains, +S9 and −S9 fraction). Regression equations, as well as linear correlation coefficients of the dose–response direct-line relation, are also included in Figure 1. As may be noticed in Figure 1, in some cases the lines of the dose–response relation fit the direct-line relation with a high correlation coefficient and in other cases such a correlation is low. The differences were particularly striking in the case of compound **C**—in (Figure 1C) the lines prove that in experiments with TA 100 strain, the dose–response relation was strong both in the presence and in the absence of S9 fraction, whereas in the case of experments with TA 98, the dose–response relation was very weak.

We calculated the reversion coefficients for direct comparison of the mutagenic potency of the complexes and the results are presented in Figure 2(1–4). The coefficients were calculated on the basis of the mutagenicity data obtained at a dose of 200 μM of the complexes. The reversion coefficients were calculated as follows:

$$\text{reversion coefficient} = \frac{\text{revertant number per tested plate}}{\text{revertant number per control plate (spontaneous)}}$$

According to Ames (Ames *et al.* 1975, Maron & Ames 1983), the compound should be estimated as mutagenic if the reversion coefficient exceeds 2.0. According to these criteria, compound **A** can be said to be mutagenic in the

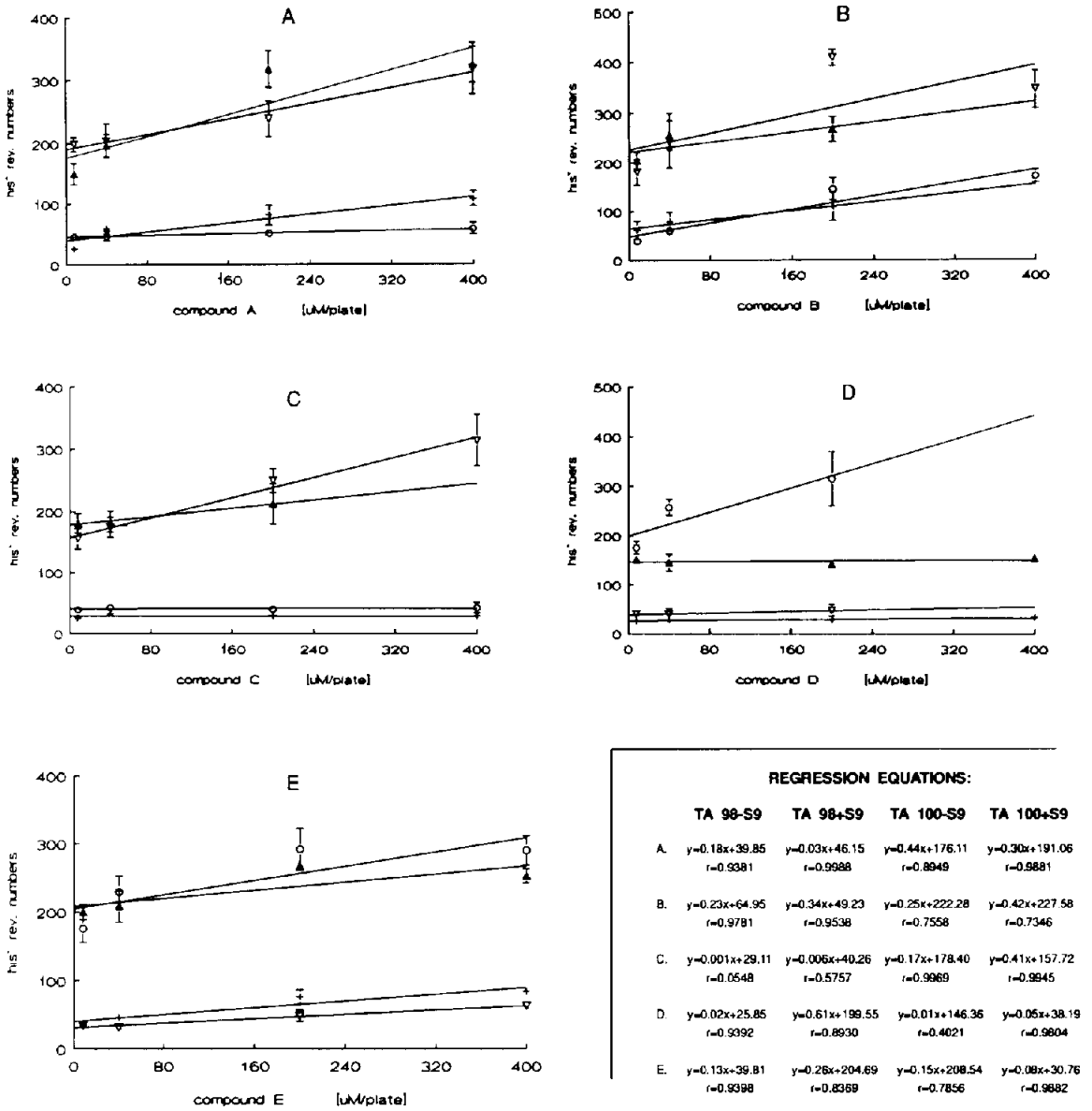

Figure 1. Regression lines and regression equations describing dose–response relationship of the mutagenic activity of tested compounds. The results of each compound are presented separately (**A–E**), corresponding to compounds **A–E**. +, TA 98 (−S9); ○, TA 98 (+S9); ▲, TA 100 (−S9); ▽, TA 100 (+S9).

experiments with TA 100 − S9 and TA 98 − S9, while compound **B** is mutagenic in the experiments with TA 100 + S9, TA 98 + S9 and TA 98 − S9. In the experiments with TA 98 − S9, **E** also exceeded 2.0 and can be said to be mutagenic. However, compounds **C** and **D** were found not to be mutagenic in all strains and under all conditions.

Combining the data presented in Figures 1 and 2, we can consider that compounds **A**, **B** and **E** are mutagenic (compound **B** exhibiting the highest mutagenic potency, while compounds **A** and **E** showed a slightly lower but noticeable activity), whereas compounds **C** and **D** are not.

We found that the simple inorganic salts of the tested metals did not exhibit mutagenic activity in the Ames test, since the revertant numbers were at the level of spontaneous revertant numbers (the reversion coefficients were 1.0).

Discussion

We estimated the mutagenic activity of five tris-bipyridine complexes of group VIII metals in the Ames test with two of the *S. typhimurium* tester strains: TA 98 and TA 100. The tester strains used in our experiments detect two different types of mutational DNA change, i.e. frame shift (TA 98) and base substitutions (TA 100) (Maron & Ames 1983). For the bipyridine complexes, we observed that complexes **A**, **B**

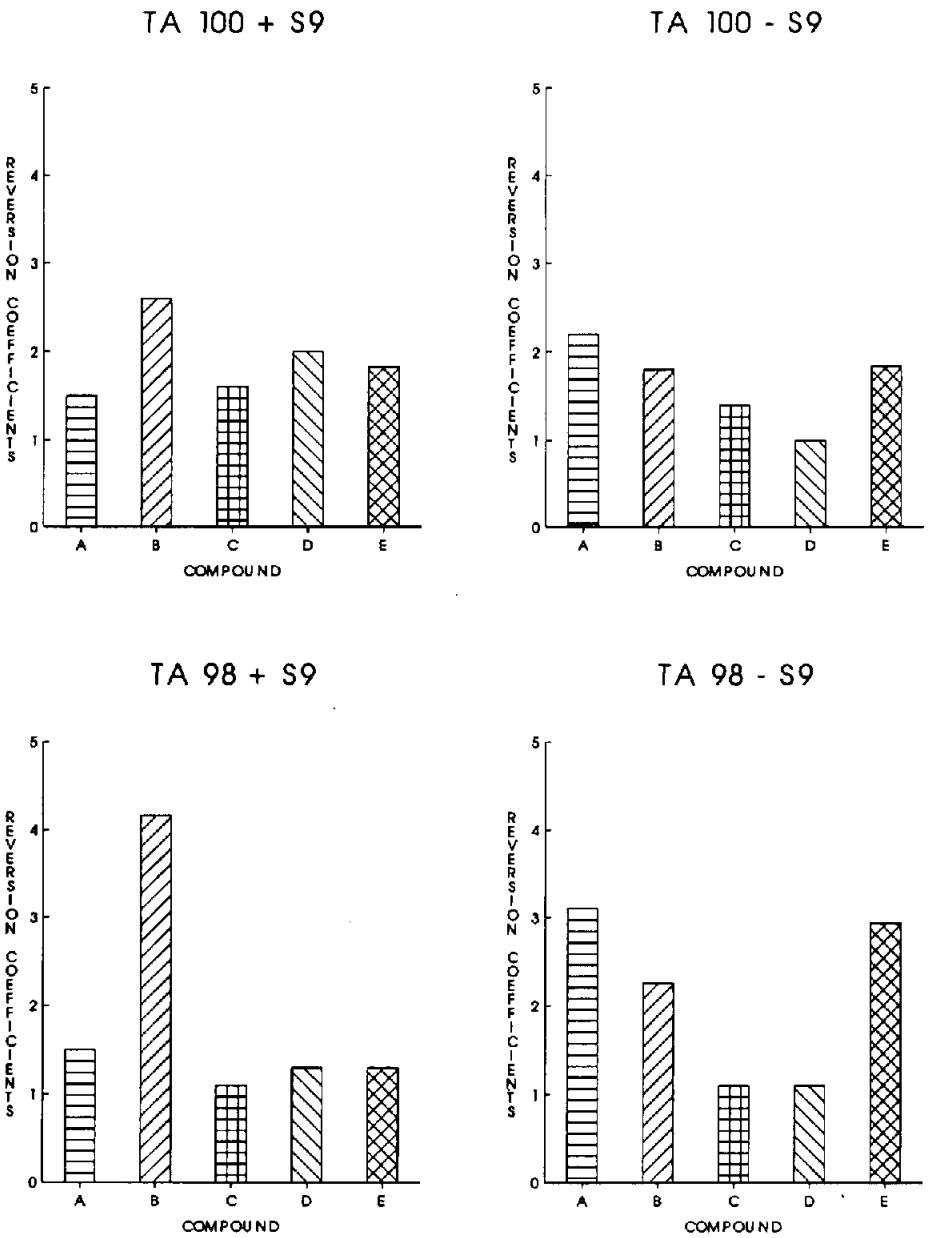

Figure 2. Reversion coefficients calculated on the basis of mutagenicity data obtained at a dose of 200 μM of the compounds evaluated in four mutagenic activity testing systems.

and **E** were mutagenic, and complexes **A** and **B** were able to generate both types of mutational DNA changes (the reversion coefficient exceeded 2.0 in both TA 98 and TA 100 experiments), whereas in the case of complex **E** this level of the reversion coefficient was exceeded only in TA 98 – S9 experiments.

The organic metal complexes were often activated in their mutagenic activity by microsomal enzymes, routinely obtained from livers of polyphenol-treated Wistar rats (Maron & Ames 1983). Among the tested compounds, it was true especially in the case of compound **B**, where in the experiments with the S9 fraction the reversion coefficients were remarkably higher than in the experiments without S9 fraction. However, in the TA 98 – S9 experiments, compound **B** was also estimated as mutagenic. It should be concluded that compound **B** revealed a direct mutagenic activity of the frameshift type. The chemical modification of this compound by the S9 fraction enzymes considerably enhanced its frame shift type mutagenic activity (TA 98 + S9) and it also revealed a significant level of the second type of mutations, i.e. base substitution (TA 100 + S9). The enzymes capable of activating a diverse number of mutagens are omnipresent in different mammalian cell types *in vivo* (Guengerich 1992, Goldstein & Faletto 1993). Therefore, it should be pointed out that such activation processes of compound **B** would also take place in humans.

In the case of **A** and **E** compounds, however, the mutagenic activity in the presence of the S9 fraction was remarkably lower than in experiments without the S9 fraction. We interpret these results as indicating that compounds **A** and **E** probably bind to some of the S9 proteins, thus causing an artificial lowering of the concentration of soluble complex in the bacterial microenvironment in our experimental system. Such a decrease of mutagenic activity after incubation with the S9 fraction is not rare and was observed by ourelves (Gasiorowski *et al.* 1993) and by other authors (Athanasiou & Kyrtopoulos 1983) after incubation of the S9 fraction with the organic extract of chlorinated drinking water, which usually contained composite metallo-organic compounds. The presented results of reduced mutagenic activity of compounds **A** and **E** after incubation with the S9 fraction may indicate that these two compounds contained the chemical assemblies able to interact with proteins *in vitro*.

The simple salts of group VIII metals were not mutagenic in our experimental conditions, probably due to their low penetration into bacterial cells. The organic part of the complex probably made some of them able to penetrate bacterial cells and the mutagenic activity could be revealed, as was discussed, for example, in the case of Cr(III)–organic complexes (Warren *et al.* 1981, Szyba *et al.* 1992, Gulanowski *et al.* 1994). In the case of our complexes, the role of the Cl^- anion seems to be crucial, since only Cl^--containing complexes were found to be mutagenic. It shows that the explanation of our mutagenicity test results is very complicated and needs further research.

Once inside the cell, such complexes may cause mutations, as their ability to bind to DNA by electrostatic and intercalative mechanisms has been well characterized *in vitro* (Pyle *et al.* 1989, Friedman *et al.* 1991, Murphy & Barton 1993). In fact, interactions of the complexes with calf thymus DNA during *in vitro* incubation have been observed previously for related Rh(I) and Ru(II) complexes, leading to the proposition of such compounds as possible anti-cancer drugs (Monti-Bragadin *et al.* 1987).

These results may be compared with those known from the literature on another series of compounds widely used in electrochemistry in general and commercial mediated biosensors in particular, i.e. the ferrocenes. A mixture of positive and negative results is reported from various sources and from various tests. In the *S. typhimurium* test system used here, the parent compound, unsubstituted ferrocene, was reported positive by one lab but not by another using the same protocol (Haworth *et al.* 1983). The latter result could have been due to the poor aqueous solubility of the compound. Ferrocene was also found positive in the *Drosophila* mutagenicity system when injected into adults (Zimmering *et al.* 1985). However, ferrocene carbamate tested negative in the *S. typhimurium* system and in the bone marrow of mice (micronucleated polychromatic erythrocyte count; Marchner *et al.* 1988). Nevertheless, in Chinese hamster ovary cells, ferrocene tested positive in the sister chromatid exchange test but negative in the chromosome aberration test (Galloway *et al.* 1985). In general, the group VIII metal complexes reported here seem comparable to the commercially-exploited ferrocenes with respect to their mutagenic activity.

In summary, regardless of the specific mutagenicity mechanism, we can point out that compounds **A**, **B** and **E** are mutagenic in the Ames test, and, as with all xenobiotics, should therefore be used with care; complex **B** possesses especially strong mutagenic activity. We also suggest that evaluation of mutagenic activity should form an integral part of any research program studying new metal–organic complexes as candidates for mediators in biosensors. Such practices would favor non-mutagenic compounds at an early stage of the research. From this point of view, complexes **C** and **D** can still be recommended as excellent electrochemical mediators for *in vitro* biosensors, whereas complexes **A**, **B** and **E** should not be given prime consideration. Certainly, the use of any mediator in *in vivo* sensor experiments on human subjects should be avoided.

As may be concluded both from the data presented in the literature and from our own results presented above, the explanation of the mutagenicity test results would be very complicated in the case of such complexes and the prediction of such an activity on the basis of their chemical structure may be impossible. Therefore it seems necessary to examine the mutagenic activity of the complexes designed for biosensors in short-term mutagenicity tests to eliminate genotoxic compounds from future contact with patients.

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