Electro-oxidation of 6-mercaptopurine riboside with special emphasis on the stability of the dimer in aqueous solutions

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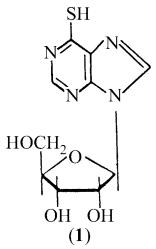
The electrochemical oxidation of 6-mercaptopurine riboside in aqueous solutions has been studied in the pH range 2.2–9.5 at a pyrolytic graphite electrode. The evidence strongly indicates that the electrochemical oxidation of 6-mercaptopurine riboside occurs in a single well-defined oxidation peak by loss of a proton and an electron to give a free radical, which instantaneously dimerises to give the corresponding disulfide. The disulfide formed was found to be unstable and decayed in a pseudo-first-order reaction with essentially similar k values in the presence and absence of an inert atmosphere. The ultimate products of oxidation in a controlled potential electrolysis have been identified as 6-sulfopurin-9-yl riboside and 6-sulfenopurin-9-yl riboside. A small amount of the starting material was also detected. A tentative mechanism for the formation of these products is suggested. A comparison of the electrochemical behaviour of 6-mercaptopurine riboside with 6-mercaptopurine is also presented.

6-Mercaptopurine (6-MP) has been considered as one of the most effective drugs used in cancer chemotherapy[1,2] and for the treatment of a number of types of leukemia and other neoplastic conditions.[3–5] It usually gets converted in the cell to ribonucleotides, which are 'fraudulent' nucleotides producing their cytotoxic actions by many different mechanisms. Mercaptopurines have several inhibitory actions on *denovo* purine synthesis and may be incorporated into DNA. The most acceptable metabolism of 6-mercaptopurine has been suggested through the formation of its nucleosides and nucleotides by Zimm and Strong.[6] The detection and characterisation of thiopurines (mainly 6-mercaptopurine) in physiological systems by various analytical, chromatographic and spectroscopic techniques have also attracted considerable attention.[7–9] As electron transfer or oxidation–reduction reactions play an important role in biological processes, attempts have also been made to study the electroreduction and electrooxidation of 6-mercaptopurine.[10,11]

The effect of 6-MP on tumour cells has been investigated and it was found that the activity of 6-MP is due to its conversion into the corresponding ribosides by the tumour cells. A good correlation between the chemotherapeutic response and the ability of tumour cells to form nucleotides has been found in animal tumours. Similarly, studies with human leukemic leukocytes incubated *in vitro* show, in general, a correlation between nucleotide formation from 6-mercaptopurine and the response of patients to treatment with this drug.[12] Thus, the importance of the riboside of 6-mercaptopurine was also realised and its electrochemical behaviour was studied by Banica *et al.*[13] using catalytic cathodic stripping voltammetry. At pH 7.0, 6-mercaptopurine riboside (6-MPR) catalysed the reduction of nickel. However, no attempt has been made to study the electro-oxidation of 6-mercaptopurine riboside (**1**). It was therefore considered desirable to study the electrochemical oxidation of 6-MPR at a pyrolytic graphite electrode over a large pH range with emphasis on the stability of the dimer formed in the presence and absence of an inert atmosphere. A comparison of the observed behaviour has also been made with 6-mercaptopurine to elucidate the effect of the ribose unit on the redox reactions of 6-MPR.



(**1**)

Experimental

6-Mercaptopurine riboside was obtained from Sigma Chemical Co. (USA) and was used as received. All other chemicals used were of analytical grade. The stock solution (1 mM) of 6-MPR and phosphate buffers ($\mu = 1.0$ M) were prepared in doubly-distilled water.

The instrumentation used for the electrochemical studies was essentially the same as that described earlier.[14] The pyrolytic graphite electrode (PGE) used for the electrochemical studies was fabricated in the laboratory by the reported method[15] and had a surface area of *ca.* 7 mm^2. The renewal of the PGE surface after each voltammogram was carried out by polishing on a 600 grit metallographic polishing disc.[16] The studies were carried out in phosphate buffers of 0.5 M ionic strength at 22 $\pm$ 1 °C. Nitrogen (Grade A) was passed through traps of pyrogallol and calcium chloride to remove the last traces of oxygen before bubbling through the solutions for 8–10 min, after which the voltammograms were recorded. All potentials are referenced to the saturated calomel electrode.

UV-Vis spectral studies and the kinetic studies for the decomposition of the UV-absorbing intermediate were carried out employing a Beckmann DU-6 spectrophotometer. IR spectra were recorded on a Perkin–Elmer 1600 FT-IR spectrophotometer using silver chloride optics.

Product isolation and characterisation

For the identification of products, nearly 20 mg of 6-MPR was electrolysed by applying a potential 100 mV more positive than oxidation peak I(a) (see below) at a large PGE (*ca.* 6 cm^2) at pH 3.25. With the progress of the electrolysis, the

colourless solution of 6-MPR turned yellow and after about 8 h of electrolysis a yellow–brown precipitate appeared in the H-cell. When peak Ia had almost disappeared (≈12 h), the electrolysis was stopped and the electrolysed solution was removed from the cell and filtered using Whatman filter paper 42. While the yellow–brown coloured residue left on the filter paper was washed with doubly-distilled water, dried and collected for analysis, the filtrate was lyophilised using Herysun lyophiliser. The freeze-dried material obtained was dissolved in the minimum amount of water (2–3 ml) and passed through a glass column packed with Sephadex G-10 (Pharmacia, NJ). Doubly-distilled water was used as eluent and fractions of 10 ml each were collected. The flow rate of the eluent was fixed at 0.8 ml min^{-1}. The first peak P_1 (100–240 ml) that appeared during gel permeation chromatography was found to be due to phosphate, as identified by qualitative testing of the yellow precipitate with $(NH_4)_2MoO_4$. The remaining three phosphate-free fractions were collected separately and again lyophilised. The dried materials were characterised using ^1H NMR and mass spectra, which were recorded using Bruker AC 300 F NMR and Jeol JMS D 300 mass spectrometers.

The colourless material obtained on lyophilisation of the volume collected under peak P_2 (250–330 ml) has m.p. 160 °C (decomp.) and exhibited a single spot when subjected to TLC (R_f *ca.* 0.36). The ^1H NMR spectrum of this material exhibits signals at δ 8.31 (s,1H), 8.05 (s,1H), 5.86 (d,1H), 5.44 (d,1H), 5.18 (s,1H), 5.04 (s,1H), 4.47 (s,1H), 4.11 (s,1H) and 3.93 (d,1H), which indicates that the product is purine 6-sulfinic acid, having a ribose unit attached at position 9. The mass spectrum of this material exhibits a clear molecular ion peak at $m/z = 316$ (7.1%), identifying the product as 6-sulfinopurine-9-yl riboside (**5**). The other high mass peaks observed in the fragmentation pattern are at 298 (8.6); 258 (3.6); 218 (10.3), 198 (18.4); 164 (18.2); 140 (36.6) and 135 (18.7%).

The volume collected under peak P_3 (410–550 ml) during gel permeation chromatography, on lyophilisation, gave a light yellow material having m.p. 180 °C (decomp.). The material exhibits ^1H NMR signals at δ 8.34 (s,1H), 8.04 (s,1H), 5.84 (d,2H), 5.05 (s,1H), 4.90 (s,1H), 4.70 (d,1H), 4.46 (s,1H) and 4.11 (s,1H). The resonances for the 3-OH ribose protons of the molecule were not clearly observed and appeared to merge with water peaks in the range $\delta = 3.2$–3.8. The ^1H NMR data indicate that the material is 6-sulfopurin-9-yl riboside. The mass spectrum of the material also exhibits a clear molecular ion peak at $m/z = 332$ (10.0%). The other high mass peaks observed in the fragmentation pattern are at 278 (9.9), 218 (18.7); 198 (27.8); 166 (48.4), 148 (78.4) and 130 (16.8%), which further confirm the identity of the material as 6-sulfopurin-9-yl riboside.

The colourless material obtained under peak P_4 (640–890 ml) has m.p. 221–223 °C (decomp.). The IR spectrum shows important bands at 3944, 3820, 3596, 3419, 3259, 3022, 2100, 1736, 1580, 1438, 1165, 1075, 734 and 469 cm^{-1}, indicating that the product is 6-mercaptopurine riboside (the starting material). The amount of starting material obtained under this chromatographic peak was very small. It appears that the 6-mercaptopurine riboside observed under this peak is due to the formation of the starting material during the hydrolysis of disulfide, as shown in the mechanism. One more possibility for the observation of compound **1** in the product is its incomplete oxidation during CPE. However, this possibility was ruled out by repetitive cleaning of the working electrode during the controlled potential electrolysis (CPE) and allowing sufficient electrolysis time to ensure that all the starting material was consumed.

Attempts were also made to analyse the product precipitated during CPE (see above). This material exhibits a m.p. of 180 °C (decomp.) and its NMR and mass spectral data are essentially the same as those of the material obtained under chromatographic peak P_3. It appears that the solubility of this compound at pH 3.25 is relatively low and hence a part of it precipitated during CPE.

Results and discussion

Voltammetric behaviour

Some of the typical cyclic voltammograms of 6-MPR are presented in Fig. 1. At a sweep rate of 100 mV s^{-1}, 6-MPR exhibits a well-defined anodic peak (Ia) in the entire pH range 2.2–9.5. In the reverse sweep, cathodic peak IIc is observed. The peak potential of peak Ia is dependent on pH and shifts to less positive potential with increasing pH. The peak potential of peak IIc is also dependent on pH and shifts to more negative potential with increase in pH. The dependence of E_p on pH for peak IIc can be expressed by the linear relation $E_p = [-195.74 - 45.408\ pH]$ mV *vs.* SCE, having a correlation coefficient of 0.986.

The peak current of oxidation peak Ia is found to increase with increasing concentration of 6-MPR. The plot of i_p *vs.* concentration is practically linear up to 0.6 mM and at higher concentrations (>0.6 mM) the peak current has a tendency to limit (Fig. 2). This behaviour suggests the involvement of adsorption phenomena in the electrode reaction,[17] which is further confirmed by the increase in peak current function ($i_p/\sqrt{v}$) with increasing sweep rate[18] in the range 50–4000 mV s^{-1} (Fig. 3). It is also observed that the peak potential of peak Ia is dependent on sweep rate and shifts to a more positive potential with increasing sweep rate. The dependence of E_p on log v can be expressed by the linear relation $E_p = 107.88 \log v + 2.0647$ having a correlation coefficient of 0.986 (Fig. 4).

Cyclic voltammograms were also recorded with the initial sweep in the negative direction to confirm whether peak IIc is due to the reduction of the species generated in peak Ia or is due to the independent reduction of 6-MPR. Peak IIc is not observed in the voltammograms, if the sweep is initiated in the negative direction. This observation confirms that the species responsible for reduction peak IIc is a result of the oxidation of 6-MPR in the peak Ia reaction.

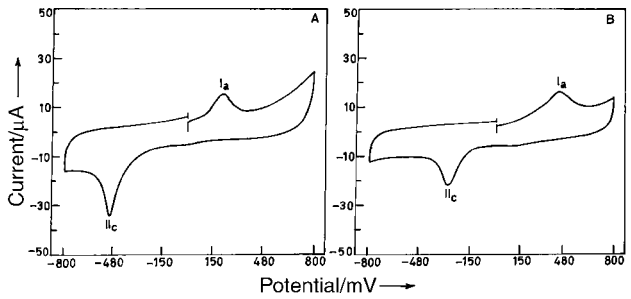


Fig. 1 Typical cyclic voltammograms observed for 0.5 mM 6-mercaptopurine riboside in phosphate buffers of pH (A) 2.69 and (B) 6.85 at a sweep rate of 100 mV s^{-1}.

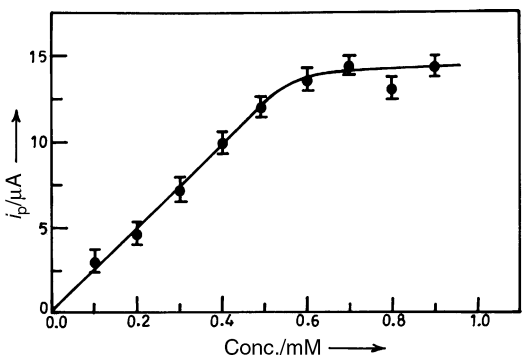


Fig. 2 Dependence of peak current (i_p) of peak Ia on concentration of 6-mercaptopurine riboside at pH 6.85, sweep rate 100 mV s^{-1}.

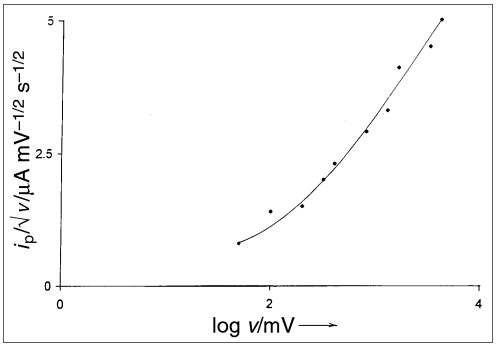


Fig. 3 Dependence of $i_p/\sqrt{v}$ on log v observed for peak Ia of 0.5 mM 6-mercaptopurine riboside at pH 6.85.

Linear sweep voltammetry of 6-mercaptopurine riboside at a sweep rate of 20 mV s^{-1} exhibits one well-defined oxidation peak Ia in the pH range 2.2–9.5. However, if the sweep is initiated in the negative direction, no reduction peak is noticed which suggests that 6-MPR only undergoes oxidation at the PGE. The peak potential of the oxidation peak Ia is dependent on pH and shifts to less positive potentials with an increase in pH. The plot of E_p *vs.* pH for peak Ia is linear (Fig. 5) and the dependence can be expressed by the relation E_p (2.0–9.5) = [607.06 − 53.883 pH] mV *vs.* SCE, with a correlation coefficient 0.984.

The progress of electrolysis during CPE of 6-MPR was monitored by recording cyclic voltammograms at different times. The complete oxidation of a 0.5 mM solution of 6-MPR required about 4 h. The PGE surface had to be cleaned several times during electrolysis due to adsorption complications. Well-defined oxidation and reduction peaks Ia and IIc were seen before the electrolysis began. As the electrolysis progressed, peak Ia systematically decreased and the peak current for peak IIc also decreased. At the end of the electrolysis, peak Ia had completely vanished; however, peak IIc was still observed and in the second positive direction sweep, peak Ia was again present. This behaviour clearly indicated that the species responsible for peak IIc exists in the exhaustively electrolysed solution. The reduction product generated in peak IIc on oxidation again gives starting compound 6-MPR, which oxidises in the peak Ia reaction in the second positive direction sweep. This would suggest that

$$2\,RSH \rightleftharpoons RSSR + 2\,H^+ + 2\,e \qquad (1)$$

It was found that during electrolysis, the peak current of peak Ia showed a systematic decrease and the plot of log i_p *vs.* time was linear for the first 15–20 min of electrolysis, after which a positive deviation from the straight line was observed. This behaviour indicates that the electrolysis follows a simple reaction path only for the first 15–20 min and thereafter secondary chemical reactions play a significant role. The n values determined for 0.2 mM 6-MPR in the entire pH range were 4.3 ± 0.1.

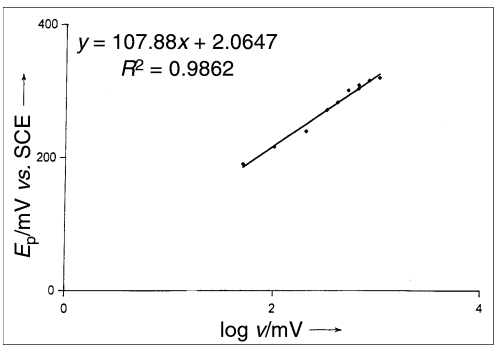


Fig. 4 Dependence of peak potential (E_p) on log v for peak Ia of 0.5 mM 6-mercaptopurine riboside at pH 6.85.

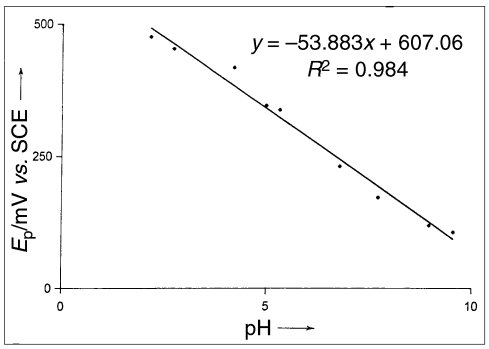


Fig. 5 Dependence of peak potential (E_p) on pH for the voltammetric peak Ia for 0.5 mM 6-mercaptopurine riboside, sweep rate 20 mV s^{-1}.

Spectral studies

The UV spectra of 0.1 mM solutions of 6-mercaptopurine riboside were recorded at different pH values in the range 2.0–9.5. In the entire pH range 6-MPR exhibited two well-defined λ_{max}. In the pH range 2.0–6.8, λ_{max} at 320 and 206 nm and a shoulder at *ca.* 225 nm were observed. The maximum at 206 nm does not appear to be real, as oxygen strongly absorbs below 210 nm and hence reference to this maximum is not made. At pH > 6.8, the maximum at 320 nm shifted to 310 nm and the shoulder became to a well-defined maximum with λ_{max} 232 nm. The progress of electrolysis was monitored by recording UV-Vis spectra in the region 200–350 nm during CPE in the presence (by bubbling nitrogen gas) and absence of an inert atmosphere. 6-MPR exhibited well-defined absorption bands at 320 nm and a shoulder at 225 nm in phosphate buffer of pH 7.0 (Fig. 6). When a potential of 0.4 V was applied, the absorbance at λ_{max} 320 nm systematically decreased with the progress of electrolysis (curves 2 to 8). The shoulder at 226 nm also disappeared with time. The absorbance in the regions 205–225 and 240–295 nm showed an increase with the progress of electrolysis whereas absorbance in the region 225–240 nm systematically decreased. Three clear isosbestic points at 225, 240 and 295 nm were seen. Curve 8, recorded after 70 min of electrolysis, exhibited a shoulder at around 295 nm. The spectral changes observed in an inert atmosphere were basically similar to the ones observed in the presence of air.

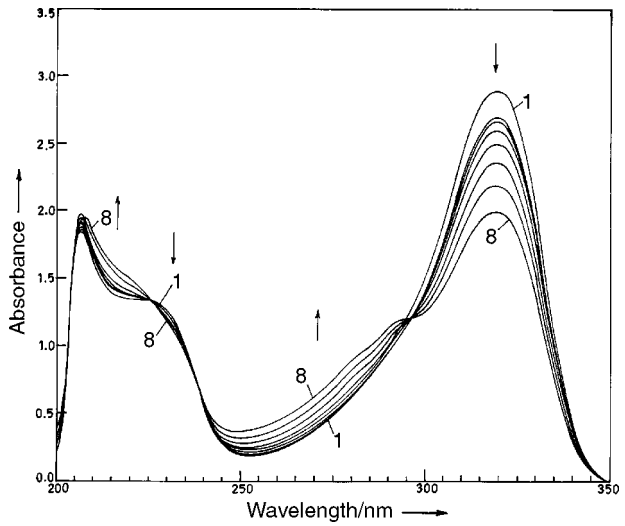


Fig. 6 Observed spectral changes during oxidation of 0.1 mM 6-mercaptopurine riboside at pH 6.8, potential 0.4 V *vs.* SCE. Curves recorded at intervals of 5 min (curves 1–5) and 10 min (curves 6–8).

In a second set of experiments, the circuit was disconnected (open circuit relaxation) when the absorbance at the longer wavelength maximum was reduced to 50%. Spectral changes at different times were then monitored to detect the wavelength region in which the UV-Vis absorbing intermediate is generated. Below pH 6.8, a systematic increase in absorbance at around 320 nm, and decrease in absorbance around 220 nm, was observed. At pH > 6.8, the decrease in absorbance after turning off the potential was centred at around 260 nm and an increase in absorbance at around 310 nm was seen. The kinetics for the decomposition of the UV-Vis absorbing intermediate generated was monitored by recording changes in absorbance with time at selected wavelengths. The resulting absorbance *vs.* time plots were found to be exponential in nature (Fig. 7). The values of the pseudo-first-order rate constant were determined at different pH, using linear log $(A - A_\infty)$ *vs.* time plots (insert, Fig. 7). The values of k calculated at different pH are presented in Table 1. The kinetics of decomposition of the UV-absorbing intermediate in an inert atmosphere was also monitored at different pH values; the values of k were practically the same as those presented in Table 1.

Identical changes in spectral behaviour were observed at pH 3.25 and 4.86. But at pH 9.54, the UV-Vis spectrum of 6-MPR exhibited three well-defined absorption bands at 310, 232 and 202 nm. When a potential of about 0.3 V was applied, it was observed that the absorbance at λ_{max} 310 and 232 nm decreased whereas absorbance at 202 nm increased systematically with time.

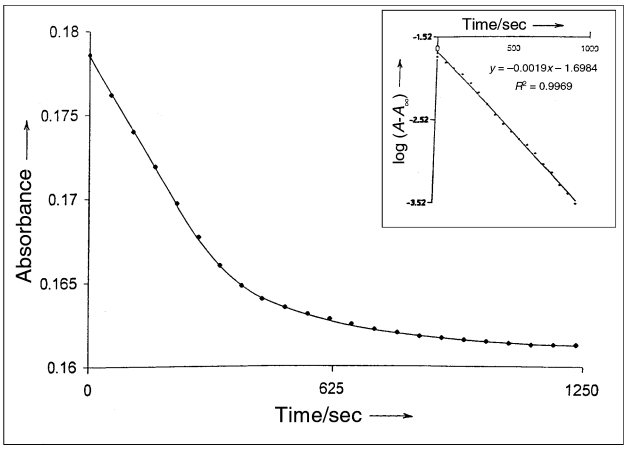


Fig. 7 Observed changes in absorbance with time and plot of log $(A - A_\infty)$ *vs.* time [inset] at pH 6.8 for the UV-absorbing intermediate generated at 260 nm for 6-mercatopurine riboside.

Table 1 Rate constants observed for the decay of the UV-absorbing intermediate of 6-MPR at different pH on PGE

		$k/10^{-3}$ s^{-1} [a]	
pH	λ/nm	N_2	Air
2.31	320	2.5	3.2
	220	1.8	2.1
3.25	320	2.5	2.1
	220	1.8	1.6
4.86	320	1.9	1.8
	220	1.8	1.6
6.84	320	2.0	1.2
	260	4.5	4.6
9.06	260	1.8	1.7
9.54	310	2.9	2.8
	260	2.0	2.1

[a] Average of at least three replicate determinations.

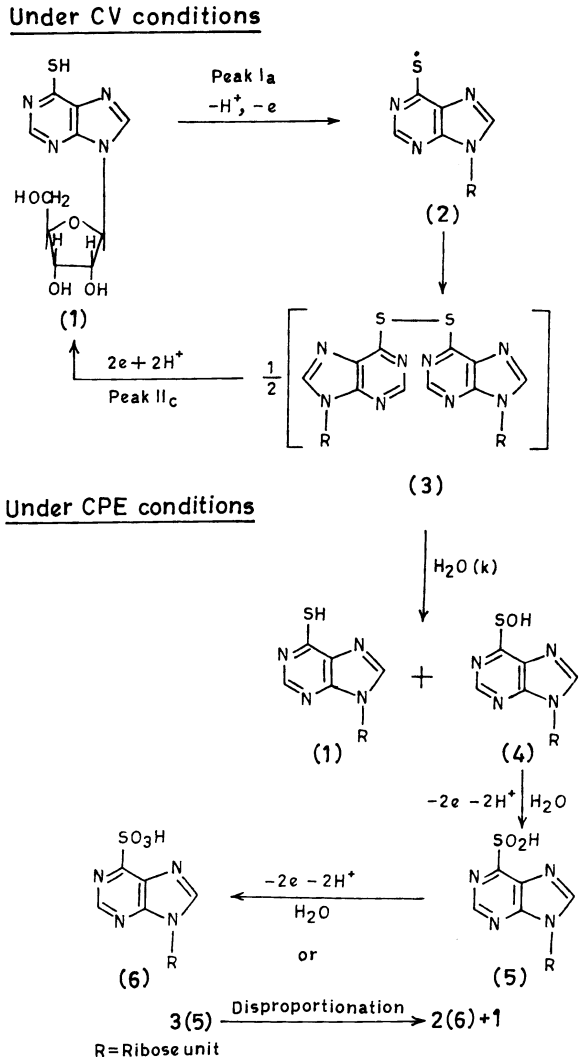
The FTIR spectra of 6-MPR during the course of electrolysis were also recorded in order to identify the intermediates and products of electrochemical oxidation. A solution of 0.25 mM 6-MPR at pH 6.8, just before the oxidation, exhibited all the important IR bands at 3918, 3855, 3496, 3444, 3317, 2099, 1646, 1490, 1080, 535 and 441 cm^{-1}. The solution of 6-MPR was then electrolysed at a potential corresponding to peak Ia and the IR spectra were then recorded at different times of electrolysis. After 10 min of electrolysis a new band at 496 cm^{-1} was observed in the spectrum. This band appeared up to about 1 h of electrolysis and then slowly disappeared. The exhaustively electrolysed solution exhibited bands at 3976, 3931, 3883, 3790, 3361, 2064, 1636, 1357, 1079 and 550 cm^{-1}. Though, the stretching vibrations for a S–S linkage normally show weak absorption and are of little value,[19] the appearance of a band at 496 cm^{-1} is assigned to the formation of the disulfide (S–S) linkage. The band at 1357 cm^{-1} appears to be due to sulfur–oxygen linkages. Thus, it seems reasonable to conclude that purinyl-6-disulfide (**3**), having a ribose moiety, and various purines having sulfur–oxygen linkages are formed. It is difficult to characterise the products of oxidation on the basis of FT-IR studies alone.

Reaction mechanism

As evidenced from the above studies, it is inferred that electrochemical oxidaton of 6-mercaptopurine riboside occurs in a single well-defined oxidation peak, Ia. Mercapto compounds have been reported to oxidise by readily losing an electron and proton to give the corresponding disulfdes.[20] Thus, it seems reasonable to conclude that 6-MPR (**1**) loses a proton and an electron in the peak Ia process to give a free radical (**2**), which rapidly dimerizes to give the corresponding disulfide (**3**) [Scheme 1]. The diagnostic criteria suggested by Andrieux *et al.*[21] for electrodimerization were applied to prove radical–radical coupling; however, adsorption complications involved in the electrode reaction did not allow this evidence to be obtained. It was found that no reverse peak corresponding to peak Ia was observed, even at a sweep rate of 800 mV s^{-1}, hence it is concluded that the free radical **2** is highly unstable and undergoes instant dimerization. The S–S linkage in the disulfide **3** can be easily reduced to give the starting compound **1**. Peak IIc in cyclic voltammetry thus represents the reduction of disulfide in a 2 e, 2 H$^+$ reaction to give compound **1**.

The disulfide linkage has been shown to be unstable in acid or basic media, in a variety of aromatic and heterocyclic compouds.[22–24] Thus, it is expected that under CPE conditions, the disulfide **3** will be readily attacked by water, causing rupture of the S–S linkage giving 6-sulfenopurin-9-yl riboside (**4**) and 6-mercaptopurine riboside (**1**), the starting material. The observed increase in absorbance centered around 320 nm in the absence of an applied potential is due to the spontaneous formation of the starting compound from the disulfide **3**, as shown in Scheme 1. The kinetics of decomposition of the disulfide was followed spectrophotometrically and the values of k for pseudo-first-order kinetics were more or less the same in an inert atmosphere and in the presence of air, clearly ruling out the possibility of air oxidation of the disulfide formed.

The purine-6-sulfenic acid derivative (**4**) is expected to be highly unstable and hence it will readily oxidise at the PGE surface. A large number of sulfenic acids have been obtained as key intermediates during chemical oxidation of purines[25,26] with half-lives of less than 10–15 min in the pH range 3.0–8.0. Thus, during cyclic voltammetric experiments a further oxidation of the sulfenic acid derivative **4** in a 2 e, 2 H$^+$ oxidation step will give the relatively stable 6-sulfinopurin-9-yl riboside **5**. Abraham *et al.*[27] have also suggested conversion of purine-6-sulfenic acid to purine-6-sulfinic acid during hepatic cyto-



Scheme 1 Tentative reaction scheme proposed for the electrochemical oxidation of 6-mercaptopurine riboside.

chrome P-450 oxidation of 6-thiopurine. Sulfenic acids and sulfenates are also reported to be sensitive to the presence of oxygen; however, as an inert atmosphere was maintained, conversion of **4** to **5** appears to occur by slow oxidation under CPE conditions. The conversion of compound **5** to give 6-sulfopurin-9-yl riboside (**6**) can occur either by disproportionation according to eqn. (2)

$$3\,\mathrm{PuR\text{-}SO_2H} \rightarrow 2\,\mathrm{PuR\text{-}SO_3H} + \mathrm{PuRSH} \qquad (2)$$

where PuR represents the purine riboside moiety, or by the slow oxidation of compound **5** in a 2 e, 2 H^+ process to give **6**. However, the disproportionation reaction seems to be more feasible in view of the smaller number of electrons involved in the oxidation of **1**. The formation of unstable sulfenic acid to give purine-6-sulfinate and purine-6-sulfonate during electrochemical studies of a variety of mercapto compounds has also been reported in the literature.[28]

It is interesting to note that the ribose unit at position 9 in 6-mercaptopurine does not dissociate from the molecule and remains attached in all species.

While 6-MPR exhibited a single oxidation peak (Ia) in the entire pH range of 2.2–9.5 at a sweep rate of 100 mV s^{-1}, on the other hand oxidation of 6-MP was observed in two well-defined oxidation peaks (Ia and IIa) above pH 7.0. It appears that peak IIa merges with the background due to its higher oxidation potential and hence is not observed during oxidation of 6-MPR. The k values for the decomposition of the disulfide for 6-MP and 6-MPR at pH 6.8 did not vary significantly and were 1.6×10^{-3} s^{-1} for 6-MP and 1.2×10^{-3} s^{-1} for 6-MPR. Thus, the decay of the UV-Vis absorbing intermediate generated in both compounds occurred at similar rates in the longer wavelength region. However, the UV-absorbing intermediate at around 260 nm was not noticed in the case of 6-MP. The major product of electro-oxidation of 6-MP at pH 3.0 has been characterised as purine-6-sulfinic acid, together with a small amount of starting material.[29] Dryhurst has also reported[30] initial oxidation of 6-thiopurine in a 1e, $1H^+$ process and the formation of bis(6-purine disulfide) is suggested. The disulfide was found to decompose in a series of reactions at pH 9.0 in ammonia buffer to give purin-6-sulfinic acid and purine-6-sulfonamide. No evidence for the formation of purin-6-sulfonic acid was obtained in the present studies, the products obtained being purine-6-sulfinic acid and purine-6-sulfonic acid with a ribose unit attached at position 9 in both acids. Thus, the ribose unit seems to facilitate further oxidation of **5** to **6**. A small amount of the starting material (6-MPR) was also detected.

The biological activity of mercaptopurines has been found to be related to its ease of oxidation to disulfides or sulfur oxides.[31] The results obtained provide some additional insights into the formation of disulfide and its unstable behaviour, which has been monitored by spectral changes. The formation of disulfides, sulfenic and sulfinic acids can also result from reaction of thiol radicals ($RS^{\cdot}$) with molecular oxygen. However, such a possibility in our studies was ruled out on the basis that purified nitrogen was continuously bubbled through the solutions during the course of electro-oxidation.

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