HETEROGENEOUS PHYSICO-CHEMICAL INTERACTIONS FOLLOWING ELECTRODE REACTION: INTERACTION OF FOLATES WITH THIOLS

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Dedicated to Dr Karel Mach on the occasion of his 60th birthday.

Folates do not react chemically with thiols in solution, however, they cause shifts of voltammetric reversible reduction peaks of disulfides towards positive potentials. This is explained as due to the formation of adducts between folates and thiols at the electrode surface. Analogous shifts, but towards negative potentials, were observed with the voltammetric reversible oxidation peak of a reduced form of folic acid after addition of thiols, and were ascribed to the same cause. According to this interpretation the shifts would provide a measure of free enthalpy of the weak interaction between the two partners and the electrode. It is suggested that the observed effect could serve as a simplified model of processes which occur between folates and proteins in the microheterogeneous structure of the living matter.

Key words: Folates; Thiols; Voltammetry; Weak interactions; Heterogeneous medium.

In the classical theory of kinetic currents in polarogaphy[1,2] the chemical step in the electrode process is considered as a homogeneous reaction taking place within a thin layer of solution adjacent to the electrode. It was soon established that if the values of the first-order rate constants calculated from experimental data by means of the theoretically derived formulae are higher than 10^8 s^{-1}, the conditions for applying the theory are not fulfilled[3]. In the case of the dissociation rate constants of phenylglyoxylic acid[4] and its derivatives[5], *e.g.*, the values of the order of 10^{10} s^{-1} are obviously due to the fact that the compounds are adsorbed at the electrode surface and that their dissociation and recombination occur essentially as heterogeneous reactions. The kinetics of these chemical reactions follows obviously different rules which are strongly dependent on the type, extent and potential dependence of adsorption of the compounds on the electrode and on the electrostatic field of the electrode double layer.

The effect of a chemical reaction following a reversible electron transfer is a facilitation of the redox process, *i.e.*, a shift of electroreduction towards positive and of

electrooxidation towards negative potentials. This has been established both experimentally[6–8] and theoretically[1,9–12].

In our study on interaction of folates with thiols in aqueous media by means of voltammetry with hanging mercury drop electrode (HMDE) we have observed[13,14] that the reduction peaks of disulfides shift to positive potentials when folates or their components are added to the solution, and that the oxidation peak of a reduced form of folic acid shifts to negative potentials after addition of thiols. This would indicate a chemical reaction between folates and thiols following reversible electron transfer; however, no interaction could be detected between the two types of compounds in the solution either by UV and visible spectrophotometry or by dc polarography. It appears that in our experiments the folates must have interacted with thiols only after they got adsorbed at the surface of the stationary mercury drop electrode. In the present paper we discuss the general aspects of this special kind of heterogeneous interaction.

EXPERIMENTAL

Reagents

The following folate compounds were used: folic acid (Sigma), folinic acid (Ca salt, Sigma), *N*(5)-methyl-5,6,7,8-tetrahydrofolic acid (Ca salt, Ciba Geigy), methotrexate (*pro inj.*, Lachema, Brno). Neopterin and xanthopterin were provided from the collection of pteridines synthesized by the late Prof. K. Slavik of the Faculty of Medicine, Charles University, Prague. The *p*-aminobenzoic and glutamic acids were the products of Lachema. The following sulfides and disulfides were used: dibenzyl disulfide (Lachema), 2-mercaptoethanol (Fluka), L-cysteine (Reanal, Budapest), L-cystine (BC Cleveland), glutathiones oxidized and reduced (Serva), acetyl methyl oxytocin and insulin (Leciva, Prague). Chemicals used for preparing the acetate and borate buffer solutions (acetic acid, Na acetate, boric acid, NaOH) were products of Lachema, Brno, analytical grade. The reagents were applied without further purification. Mercury for HMDE was of polarographic purity (Sluzba vyzkumu, Prague).

All solutions were prepared in double distilled water, in some cases the dissolution needed acceleration by warming up. Cystine was first dissolved in 0.1 M NaOH and the solution was then neutralized by dilute $HClO_4$. The weighted amount of dibenzyl disulfide was first dissolved in a small volume of pure ethanol and then the measuring flask was filled up to the mark by water.

Apparatus

Voltammetric measurements were carried out with the PA 4 Polarograph in combination with the SMDE 1 electrode. The curves were recorded by means of the XY 4106 recorder, all products of Laboratorni pristroje, Prague. For polarographic measurements with the dropping mercury electrode, carried out in the SMDE 1 cell, a glass capillary of 54 μm inner diameter, bent by 90° and cut at 45°, was used. A separated saturated calomel electrode served as a reference; all values of potentials are referred to that electrode. A platinum flag served as an auxiliary electrode in the three-electrode system. The size of HMDE was kept at its least (given by the relay impulse of 40 ms).

Spectrophotometric measurements were done with the Diode Array Spectrophotometer HP 8452 A (Hewlett–Packard) in the wavelength span 180–400 nm using 10 mm quartz cuvets.

The pH values of the measured solutions were checked by the portable CPH 51 pH meter of Crytur, Turnov.

Procedure

The voltammetric, the polarographic and the spectrophotometric measurements of each solution were carried out in parallel. Solutions of 0.1 M acetate buffer (pH 4.7) and borate buffer (pH 8.6) served as supporting electrolytes. Prior to each polarographic and voltammetric measurement the solution was deaerated by a stream of pure nitrogen. During measurements nitrogen was passed above the solution in the cell. The experiments were carried out at the room temperature 25 ± 1 °C.

RESULTS AND DISCUSSION

Follow-Up Chemical Reactions in Voltammetry

In the simple case of a fast irreversible chemical reaction inactivating the product of a reversible electroreduction

$$\mathrm{Ox} + n\,\mathrm{e} \rightleftharpoons \mathrm{Red} \qquad (1)$$

$$\mathrm{Red} + \mathrm{A} \xrightarrow{k} \mathrm{B}\ , \qquad (2)$$

where k is the pseudo-first order rate constant of the inactivation reaction (the concentration of the agent A being in excess over that of the redox system) and B is the product, the voltammetric peak potential E_p shifts to positive values with increasing rate constant k and decreasing scanning rate v according to the theory[8,10] as follows:

$$E_\mathrm{p} = E_{1/2} - 0.78\frac{RT}{nF} + \frac{RT}{2nF}\ln\frac{RTk}{nFv}\ . \qquad (3)$$

In this equation $E_{1/2}$ is the polarographic half-wave potential pertaining to the simple reversible redox system and the other symbols have their usual significance. For a 2-electron electrode reaction (n = 2) and for the temperatures between 20 °C and 25 °C, Eq. (*3*) becomes

$$E_\mathrm{p} = E_{1/2} - 0.038 + 0.015\log(k/v)\ . \qquad (4)$$

With very fast follow-up reactions the peak can shift (at a constant scan rate) by as much as 120 mV. For the special case when all three reaction partners, Ox, Red and B,

are strongly adsorbed at the electrode, the theory[12] predicts that the coefficient of the last term in Eq. (*4*) should be 0.0296 instead of 0.015.

Unfortunately, the above theoretical expressions cannot be used for studying the interaction of folates with thiols. Above all, the concept of what is obviously a case of weak interaction precludes the idea of a fast irreversible chemical reaction. Further, the reversible reduction of disulfides on mercury takes place as a reduction of the bond between sulfur and mercury[15]. Thiols and mercury thiolates are adsorbed on mercury[16,17] and the reversible reduction of the oxidized forms of folates or the oxidation of their reduced forms occur in adsorbed state[18–20]. The components of our reaction system are hence adsorbed at the electrode, the interactions between them take place in adsorbed state and the theory derived for volume chemical reactions is therefore not applicable to them. However, our results cannot be interpreted either in terms of the simplified theory[12] which considers strong adsorption of the whole system: the adsorptivity of the interaction partners which we have tested varies widely.

On the other hand, an added adsorbable substance acts usually as an inhibitor of a reversible electrode reaction and shifts reduction processes to negative and oxidation processes to positive potentials[21]. The fact that in spite of the inhibiting tendencies the adsorbed folates shift the reduction of disulfides in the positive direction and the adsorbed mercury thiolates shift the oxidation of reduced folates to negative potentials only corroborates the conclusion that the mutual interaction of folates with thiols in adsorbed state has qualitatively the same effect on the electrochemical reaction what would have a homogenenous follow-up chemical reaction between them.

Without theoretical background for any specific quantitative measurements we proceeded in our research by comparing the shifts of voltammetric peak potentials caused by additions of the interaction partners to the electrolyzed solution.

Electroreduction of Disulfides

The essential condition for observing the effect of follow-up reactions in polarography or voltammetry is the reversible character of the electrode reaction. Hence for studying the reactions of thiols we have to use as their source the electroreduction of those disulfides which are reduced reversibly. A simple reduction of the covalent –S–S– bond is an irreversible process. If the disulfide molecule in its solvated form in a given solution can approach the electrode in such a way that the sulfur atoms come into direct contact with the mercury atoms of the electrode surface and if this contact occurs when the mercury electrode is under a favorable potential, the –S–S– bond spontaneously breaks and two Hg–S– bonds are formed in its place. When the electrode is only slightly negatively or not too positively charged and the sum of all interfacial molecular forces results in a weak interaction, the process of bond breaking and bond formation is reversible.

$$\mathrm{R{-}S{-}S{-}R' + HgHgHgHg} \rightleftharpoons \mathrm{R{-}S{-}S{-}R'} \rightleftharpoons \begin{matrix}\mathrm{R{-}S\ \ S{-}R'}\\ \mathrm{\ \ |\ \ \ |}\\ \mathrm{HgHgHgHg\ \ \ HgHgHgHg}\end{matrix} \qquad (5)$$

(Here R–S–S–R′ symbolizes the disulfide and HgHgHgHg the mercury surface.) Otherwise, with a strongly negative electrode the disulfides do not interact at all, and at a strongly positively charged electrode the disulfides after splitting become irreversibly chemisorbed.

In our experiments we tried the effects of folates upon the reduction peaks of dibenzyl disulfide, cystine, oxidized glutathione, acetyl methyl oxytocin and insulin.

Dibenzyl disulfide. The reduction of this compound in aqueous or mixed aqueous-alcoholic media is reportedly irreversible[22]. On cyclic voltammograms we observed at higher scan rates a small drawn-out anodic response to the cathodic peak, displaced in the direction of positive potentials. This is presumably because in presence of water in the solution the two benzene rings in the molecule are preferentially adsorbed at the electrode in planar orientation and in that way for steric reasons the –S–S– bridge cannot come into contact with mercury. Consequently, the electroreduction of the dibenzyl disufide occurs irreversibly and hence the reduction peak did not shift to positive potentials upon addition of any of the four folates or the two pteridines.

Cystine. The electroreduction of cystine on mercury electrodes occurs primarily *via* reversible interaction with mercury[15] and the shifts of the reduction peak potential in positive direction can thus serve as an indication of interactions of the reduction product – cysteine – with the solution components.

In Fig. 1 is plotted the potential of the voltammetric peak due to the reduction of cystine as a function of concentration of various compounds added to the solutions of cystine in acetate buffer (1a) and borate buffer (1b), respectively. We can see that all the folates that had been tried cause a measurable shift of the peak potential in both media. Of the folate components the pteridines are as active as folates, the *p*-aminobenzoic acid produces a small shift at a higher concentration and the glutamic acid is practically inactive.

Oxidized glutathione. The electroreduction of this compound[23] follows to a large extent the mechanism of reduction of cystine[15], *i.e.*, it starts by interaction with mercury and the primary electron transfer is reversible. That shows also on the shifts of the voltammetric peak potential after additions of folates to the solution, as demonstrated in Figs 2 and 5. It can be seen that the potential shifts are comparable or even greater than in the case of cystine.

Acetyl methyl oxytocin. Oxytocin is a nonapeptide containing one cystine unit which is the reason of its faradaic activity. The derivatives of oxytocin display various degrees of irreversibility in their electroreduction[24], presumably because of steric factors inter-

fering in their interaction with mercury. Also our derivative is reduced irreversibly, and no folate could cause any shift of its voltammetric peak to positive potentials.

Insulin. This two-chain polypeptide of molecular weight about 6 000 contains two –S–S– links which hold the chains together and one –S–S– group localized in the shorter chain. Two of the three disulfide groups are accessible to electroreduction[25]. On mercury electrodes the reduction involves a reversible exchange of 2 electrons. The effect of addition of folates upon the potential of the reduction peak of insulin is illustrated by Fig. 3.

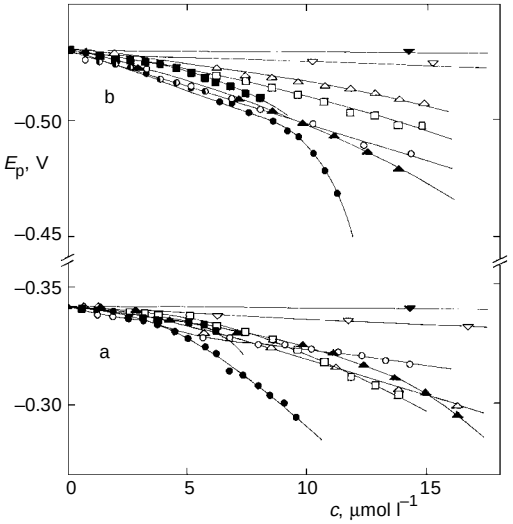

FIG. 1
Potential dependence of voltammetric peak of reduction of l-cystine, on concentration of added various forms of folates and their components, in solutions of a acetate buffer; b borate buffer. Individual points pertain to: ❍ folic acid; ● Ca folinate; Δ Ca salt of *N*(5)-methyl-5,6,7,8-tetrahydrofolic acid; ▲ methotrexate; ❒ neopterin; ■ xanthopterin; ∇ *p*-aminobenzoic acid; ▼ glutamic acid. Concentration of cystine between 3.3 and 2.8 . 10^{-5} mol l^{-1}

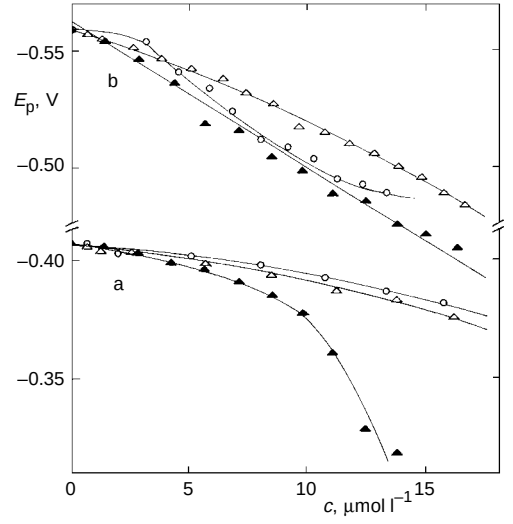

FIG. 2
Potential dependence of voltammetric peak of reduction of oxidized glutathione on concentration of added folates, in solutions of: a acetate buffer; b borate buffer. Individual points pertain to: ❍ folic acid; Δ Ca salt of *N*(5)-methyl-5,6,7,8-tetrahydrofolic acid; ▲ methotrexate. Concentration of glutathione between 3.3 and 2.8 . 10^{-5} mol l^{-1}

Interactions in Adsorbed State

The theoretical formula (Eq. (*4*)) for the volume chemical reaction following a reversible electroreduction predicts at the temperature of 25 °C a shift of the voltammetric peak to negative potentials by 15 mV at a tenfold increase of the scan rate. For the case of all reaction partners strongly adsorbed the theory predicts the shift by 30 mV. In Fig. 4 we have shown the experimental values of the peak potential of cystine measured in presence of methotrexate at different rates of potential scan in two buffers. The peak shifts to negative potentials with increasing scanning rate as expected; however, the dependence of E_p on log v can be approximated in both solutions by a straight line with the slope of 50 mV per log unit which cannot be reconciled with the requirements of the theory either for a volume reaction or for strong adsorption. Obviously, the interaction between folates and thiols is a more complex case.

When folates are added to reversibly reduced disulfidic compounds the positive shift of the voltammetric reduction peak is invariably accompanied by a decrease of the

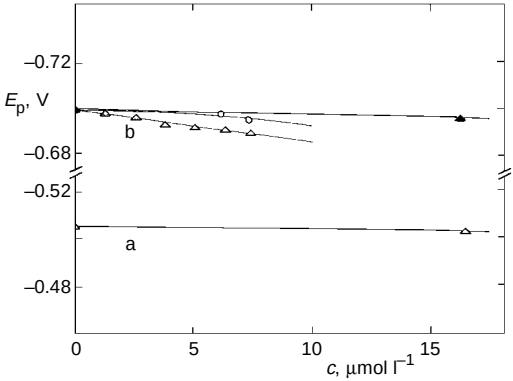

FIG. 3
Potential dependence of voltammetric peak of reduction of insulin on concentration of added folates, in solutions of: a acetate buffer; b borate buffer. Individual points pertain to: ❍ folic acid; Δ Ca salt of *N*(5)-methyl-5,6,7,8-tetrahydrofolic acid; ▲ methotrexate. Concentration of insulin between 3.3 and 3.1 . 10^{-5} mol l^{-1}

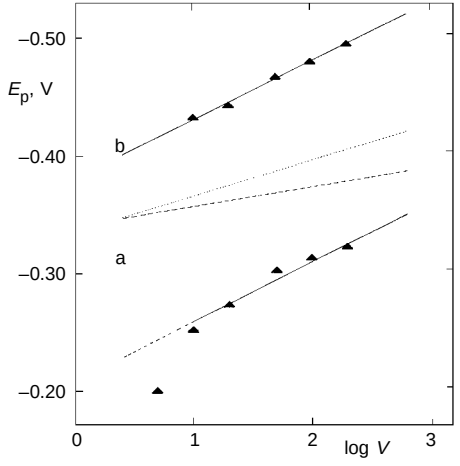

FIG. 4
Potential dependence of voltammetric peak of electroreduction of 3.1 . 10^{-5} mol l^{-1} cystine in presence of 1.3 . 10^{-5} mol l^{-1} methotrexate on the logarithm of the scan rate v (in mV s^{-1}) in solution of: a acetate buffer; b borate buffer. Dashed line: straight line with the slope of 15 mV/log unit; dotted line: straight line with the slope of 30 mV/log unit

peak, as can be seen in Fig. 5. A comparison of the real peak height and of that expected in the theoretical case of a mere dilution effect, caused by additions of small volumes of folate solutions, is made in Fig. 6. The folates, adsorbed at mercury electrodes, apart from their surface interaction with the mercury thiolates generated in the electrode reaction, exert an inhibitive effect upon the faradaic reaction itself displacing the disulfides from the electrode surface when added in gradually increasing concentration.

We can see that adsorption, which is the condition for interaction of the two partners, becomes a hindrance when it is too strong. Insulin, *e.g.*, a strongly adsorbed big molecule, can enter into interaction only with the relatively most strongly adsorbable folate, the methotrexate (Fig. 3), which has a chance to get adsorbed at the electrode. On the other hand, the *p*-aminobenzoic acid, less adsorptive than the folates or pteridines,

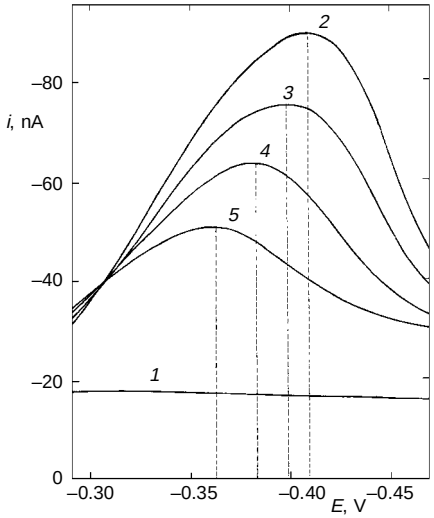

FIG. 5
Effect of folic acid upon the voltammetric reduction peak of oxidized glutathion. *1* acetate buffer; *2* + oxidized glutathion 3 . 10^{-5} mol l^{-1}; + folic acid: *3* 8 . 10^{-6} mol l^{-1}; *4* 1.6 . 10^{-5} mol l^{-1}; *5* 3 . 10^{-5} mol l^{-1}. Scan rate 100 mV s^{-1}

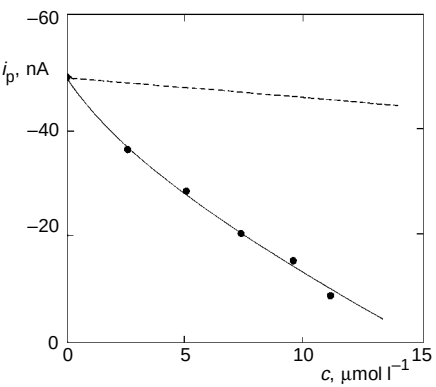

FIG. 6
Dependence of height of the voltammetric reduction peak of cystine on concentration of added Ca folinate. Concentration of cystine 3.3–3.0 . 10^{-5} mol l^{-1} in borate buffer. Dashed line: concentration dependence of hypothetical peak height due to mere dilution by additions of folinate

shows a weaker interaction than those reagents with the electrogenerated cysteine, and the least adsorbed glutamic acid does not practically interact at all.

The mechanism of the process of interaction between folates and thiols in the adsorbed state is a complex one, as indicated in Fig. 7. When methotrexate is added to the solution of cystine, the reduction peak of the latter decreases as shown, and shifts to positive potentials. If the electrode potential is kept constant at the initial value for 20 s before the scan is applied, the peak becomes still more positive. During 20 s period some mutual orientation of the folate with respect to cystine molecule at the electrode surface takes place. Apparently, it is favourable for the interaction occurring after the electron transfer. Further waiting at the initial potential prior to scan leads to an increase of the peak height, *i.e.*, more folate molecules are accumulated in the favorable orientation and included in the interaction. The peak potential, however, shifts slowly

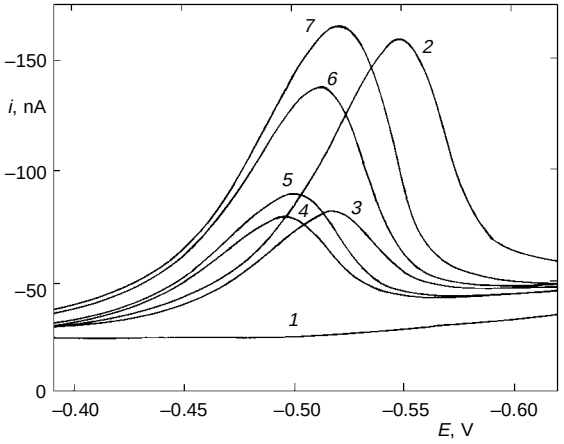

FIG. 7
Effect of accumulation of interaction partners on the surface of HMDE on the voltammetric reduction peak of cystine in presence of methotrexate. *1* borate buffer; *2* + cystine 3 . 10^{-5} mol l^{-1}; *3* + methotrexate 5.7 . 10^{-6} mol l^{-1}, scanned immediately after closing circuit; *4*–*7*: after closing circuit waited with HMDE at initial potential E_1 = –0.35 V for: *4* 20 s; *5* 40 s; *6* 60 s; *7* 90 s. Scan rate 100 mV s^{-1}

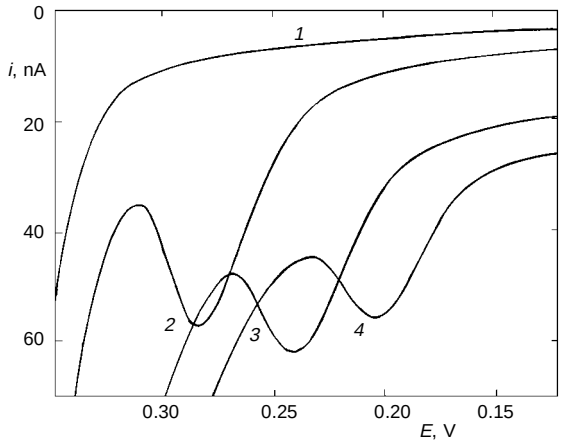

FIG. 8
Effect of 2-mercaptoethanol upon the voltammetric oxidation peak of Ca *N*(5)-methyl-5,6,7,8-tetrahydrofolate. *1* acetate buffer; *2* + Ca *N*(5)-methyl-5,6,7,8-tetrahydrofolate 9 . 10^{-6} mol l^{-1}; + 2-mercaptoethanol: *3* 5.9 . 10^{-5} mol l^{-1}; *4* 9.6 . 10^{-5} mol l^{-1}. Scan rate 100 mV s^{-1}

back in negative direction – this time presumably due to gradually increasing inhibitive effect of folates.

Electrooxidation of Folates

The interaction between folates and thiols following a reversible electrode reaction can be also induced by a procedure opposite to that described above – by generating the folates in a reversible reaction and adding the thiols to the solution. After consideration of the potential ranges of electroactivity and adsorptivity of the interaction partners we decided to study an electrooxidation of the *N*(5)-methyl-5,6,7,8-tetrahydrofolate which had been found to proceed in a primarily reversible step[26].

Figure 8 shows the shift of potential of the anodic peak, due to the oxidation of the reduced form of folate, upon addition of thiol. The shift occurs in negative direction, as expected according to our interpretation of effects of folates on the reversible reduction of disulfides. As in the previous case, upon addition of the interaction partner the peak current decreases, however, this time it is superimposed upon the current caused by the anodic reaction of thiols with mercury[25,27–29] starting at more negative potentials than the oxidation of tetrahydrofolate. In Fig. 9 the peak potential of the oxidation of folate is plotted as a function of concentration of added thiols.

The Type of Interaction

We found that folates, which do not react chemically with thiols in solution, interact with them when the two types of compounds are brought together at the electrode surface, and that the free enthalpy gain of this interaction is electrochemically well measurable by the shift of potential of reversible redox reaction – it amounts to tens of milivolts which is equivalent to several kilojouls per mol. The result of the interaction

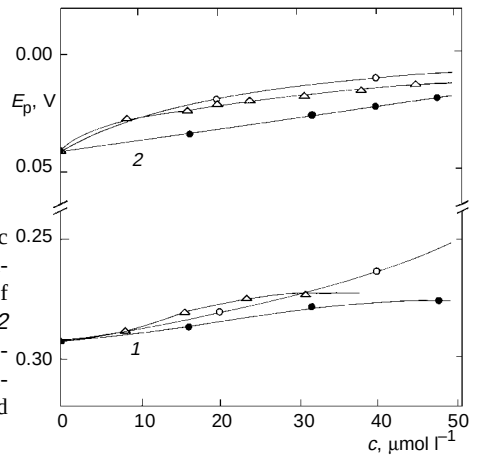

FIG. 9
Dependence of the potential of voltammetric peak due to electrooxidation of Ca *N*(5)-methyl-5,6,7,8-tetrahydrofolate, on concentration of added thiols, in solutions of: *1* acetate buffer; *2* borate buffer. Individual points pertain to: ❍ 2-mercaptoethanol; ● cysteine; Δ reduced glutathion. Concentration of folate between 16.7 and 8.1 . 10^{-6} mol l^{-1}

is apparently an adduct which has a definite stability in the adsorbed state. The experimental data presented in Fig. 1 indicate that the effect can be observed for various forms of folates and their main components, the pteridines, but to a much less extent with *p*-aminobenzoic acid, and practically not at all with glutamic acid. From a comparison of Figs 1, 2, 3 and 9 it follows that for the thiols to enter into interaction with folates, there is no special requirement, apart from the adsorbability and a favorable steric factor. Further, it is evident that the interactions do not follow a common pattern – each pair of partners in a given solution behaves in a different way. These observations lead to the conclusion that the main component of the forces holding the adduct together is charge–transfer (c–t) interaction between the sulfur atom of the thiols as electron donor and the pteridine ring system of the folates as electron acceptor. In the literature more examples are cited[30] where sulfur in organic sulfides or disulfides reacts as a donor, or pteridines appear as acceptors of electron in c–t interactions. In the case of *p*-aminobenzoic acid the role of the acceptor is presumbaly played by the benzene ring. Mutual orientation of the two partners at the electrode surface decides whether and to what extent the charge–transfer forces come into play in the interaction. Moreover, in the interface between electrode and solution these forces are affected by the electric field of the electrode double layer. Other factors contributing to the resulting interaction of folates with thiols are undoubtedly the electrostatic induction forces, the attraction and/or repulsion between parts of the molecules charged, according to pH of the medium and p*K*s of the pertaining groups, by dissociation and/or protonation, and the thereby connected hydrogen bridge formation. The relatively strongest interaction of folates with glutathione can be perhaps ascribed to the presence of several atoms which can participate in hydrogen bridge formation in the glutathione molecule.

In attempts to visualize the structure of the surface adducts of folates with thiols it should be borne in mind that in the case when the active folate is electrolytically generated from its reduced state (Fig. 9) the interacting partner is in the form of mercury thiolate adsorbed at the mercury surface[16,25].

Biological Analogy

Folates are water-soluble vitamins essential for basic metabolic reactions in cells, especially of fast proliferating tissues[31]. They are transported to the cell interior, and their metabolic products to the cell exterior, by highly specific carriers, the so-called folate-binding proteins[32] (FBP) some of which are membrane-associated. The way how folates are bound to the FBP is not exactly known; there are indications[13,14] that in the complex binding the sulfur atoms from the proteinic chains take also part. Our above described experiments in which folates interact with thiols at the surface of a mercury electrode can perhaps serve as a simplified model of the processes which take place in the microheterogeneous structure of living matter. The folate molecules, accumulated in a particular orientation at the sulfur atoms localized on the surface of the protein

attached to the mitochondrion or to the cell membrane, get presumably bound to the FBP by combined forces of weak interaction. the ensuing complex bond must be highly specific (*cf.*, *e.g.*, ref.[33]) and strong enough to stand the transport through the tissue medium to the site of the metabolic reaction. On the other hand, the bond must be weak enough to release the folate as soon as a concentration gradient is generated in the region of a particular site of the protein surface by the incipient enzymatic reaction.

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