Tetrazolium salts as inhibitors of bacterial corrosion of metals

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Abstract

In this work, we made an attempt to determine the relationship between the ability of bacteria to reduce tetrazolium salts and their corrosion activity. The adsorption of tetrazolium salts on the surface of zinc and low-carbon steel has been shown to be accompanied by their reduction to give formazans. It has been revealed that the formation of corrosive products of oxygen bioconversion on the surface of metals under the influence of *Pseudomonas fluorescens* is hindered by adsorbed tetrazolium salts. Chemical kinetics methods were used to establish that the products of the reduction of tetrazolium salts by *Pseudomonas fluorescens* irreversibly block the redox centers of the electron transport chain of bacteria at low degree of reagent conversion. It is suggested that tetrazolium salts can inhibit the biogenic factor in the bacterial corrosion of metals. The adverse effects of steel exposure to *Pseudomonas fluorescens* bacteria were studied by determining the change in mechanical properties when the samples were torn at a strain rate of 0.1 mm/s. The observed decrease in the average tensile strength compared to the initial state is explained by highly localized pitting corrosion in the form of small, evenly distributed surface depressions.

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Introduction

The enhanced corrosion damage to metals used in warm and humid climates can be due to organotrophic bacteria [1–3]. The presence of oils, paints, and other organic-based protective coatings on the surface of metal structures is a factor facilitating the colonization of metals by organotrophic bacteria. In recent decades, a number of comprehensive studies have been carried out to identify the key factors of biogenic nature that drive the corrosion process [4–14]. A distinctive feature of the object studied in the works [4–14] is the use of the "metal–bacteria under optimal living conditions" system without their contact being affected by the liquid medium.

It has been clearly shown that the corrosive metabolites produced by organotrophic bacteria are products of oxygen bioconversion (OH^-, hydrogen peroxide). Ways of their

formation that are consistent with the principles of bacterial activity have been suggested [4–14].

Microstructural methods were used to establish a relationship between the stages of colonization by organotrophic bacteria of metals and visually observed changes in the state of the surface, on the one hand, and the content of hydrogen peroxide in the liquid-droplet exudate on local areas of the metal surface and its basicity, on the other hand [4, 9, 12–13].

In one of the first studies on bacterial corrosion, an attempt was made to identify an indicator of the corrosive activity of bacteria [15]. Tetrazolium salts were selected, since R. Kuhn's times [16], these substances, due to the formation of intensely colored formazans upon reduction, have been actively used in assessing the respiratory activity and, in general, the viability of bacteria [17, 18].

Using iodonitrotetrazolium chloride (INT) and bacteria suspended in physiological saline, it was found that the effective rate constants for the INT reduction by bacterial membrane components depended on the structure of the bacterial cell wall [19–21]. With rare exceptions, the kinetic curves of the accumulation of the iodomonoformazan (IMF) reduction product have the form of monotonically increasing dependences plateauing at various degrees of reagent conversion [19–21].

Based on the previously obtained data [19–21], we aim at determining the relationship between the reducing ability of bacteria with respect to tetrazolium salts and their corrosive activity, and identifying the capability of tetrazolium salts to reduce the role of the biogenic factor that stimulates biocorrosion.

Experimental

The gram-negative rod-shaped bacteria *Pseudomonas fluorescens* (isolated from the soil of the industrial zone of oil refineries located in Kstovo, Nizhny Novgorod region) was chosen as the biological object of study as the least examined ones in comparison with the bacteria described in other works [19–21].

The tetrazolium salts and formazans were used as commercial products: iodonitrotetrazolium chloride (95%, Sigma–Aldrich), methylthiazolyltetrazolium bromide (≥97.5%, Sigma–Aldrich), iodomonoformazan (crystalline, Sigma–Aldrich), and methylthiazolylformazan (MTF) (≥97%, Sigma–Aldrich). In kinetic experiments, we used: lysozyme ("Lizobakt", Bosnalijek), physiological saline ("Sodium chloride 0.9%", Mospharm, Russia), and ethyl acetate ("reagent grade") after preliminary purification according to the method reported elsewhere [22].

To prepare a dense nutrient medium for the growth and activity of microorganisms, a commercial formulation of nutrient agar for cultivation of microorganisms distributed by the Scientific Research Center for Pharmacotherapy (Russia) was used.

Low-carbon cold-rolled Q-panel steel of QD type (manufactured by Q-lab Corporation, USA) was employed for the corrosion studies. The chemical composition of the samples

was: 0.6%–manganese, 0.15%–carbon, 0.03%–phosphorus, 0.035%–sulfur, and 99.2%–iron.

To obtain zinc samples, zinc ingots measuring 210×70×8 mm were smelted from "analytical pure" grade granulated zinc, TU 6-09-5294-86. The melt was treated with anhydrous $ZnCl_2$ for refining. After the ingots were obtained, they underwent hot deformation with intermediate heating at 200°C to a thickness of 1.0 mm. Further, samples measuring 2.5×5 mm were cut out from the ingots.

To carry out metallographic studies, samples measuring 35×125 mm were polished, etched for 3 s with 4% solution of HNO_3 in glycerol, and then washed with ethyl alcohol.

The effect of bacteria on metal surfaces was studied as described elsewhere [4–14].

The concentration of hydrogen peroxide was determined according to the method used in [23], and that of ammonia, according to [24]. The exudate pH was determined using a HI 2210 pH Meter and a HI1083 microelectrode (HANNA Instruments).

Kinetic studies on the reduction of tetrazolium salts were carried out following the method described in [19–21]. The concentration of monoformazans was determined spectrophotometrically in cells 1 cm thick at wavelengths corresponding to the absorption maxima of the reduction products of the tetrazolium salts used: for IMF, 490 nm and for MTF, 558 nm. Extinction coefficients were determined from calibration curves plotted using pure commercial formulations.

The incubation of bacterial cultures and the corresponding studies using these cultures were carried out in a TS-1/80 SPU thermostat.

Microstructural studies of metal surfaces were carried out using a Tescam Vega II scanning electron microscope (Czech Republic), an MS-20 microscope from Micros (Austria), and a Keynce VHX-1000 digital optical microscope.

The mechanical properties of the samples after exposure to a controlled and inoculated environment were assessed according to GOST 1497 [25]. The samples were loaded on a versatile testing machine distributed by Tinius OIlsen Ltd., model H100KU; the sample deformation rate was 0.1 mm/s.

UV spectra were recorded with a 2802 UV/Vis Unico spectrophotometer.

Results and discussion

The effect of *Pseudomonas fluorescens* on the surface of metals is similar to that previously observed for other representatives of organotrophic bacteria [4, 9, 12–13]. Three to five days after the start of exposure, active colonization of the surface begins with the formation of a bacterial biofilm, Figure 1.

This process is accompanied by the local formation of a liquid-droplet exudate on the metal surface. The chemical composition of the exudate on the surface of zinc was analyzed for three parameters: pH and the content of hydrogen peroxide and ammonia, Table 1.

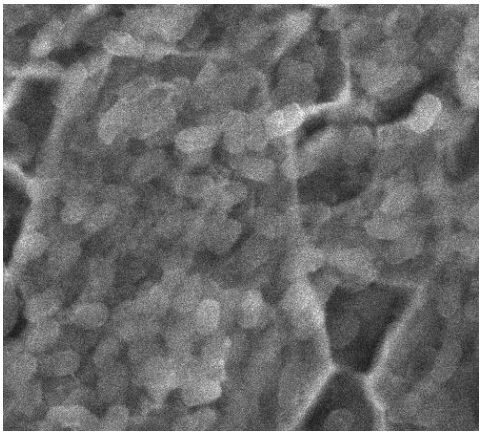


Figure 1. Microimage of the steel sample surface exposed to *Pseudomonas fluorescens* 5 days after the start of exposure (×1000).

Table 1. Chemical composition of the exudate on the surface of zinc in the presence of *Pseudomonas fluorescens* bacteria.

Parameter	Days after the start of exposure						
	7	**9**	**11**	**13**	**15**	**17**	**19**
pH	9.95	10.15	10.30	10.40	10.55	10.65	10.75
$[H_2O_2]$, μM/L	150	195	280	320	250	220	205
$[NH_3]$, mM/L	2.8	1.8	1.5	1.3	0.8	0.6	0.5

The data in Table 1 show that when zinc is exposed to *Pseudomonas fluorescens*, exudate in an amount sufficient for analysis is formed by the 7th day only. It has been previously shown that hydrogen peroxide exhibits corrosive properties toward metals [4–14]. In fact, after the oxide film is destroyed with involvement of biofilm metabolites, hydrogen peroxide can exhibit oxidizing properties toward zinc by the reactions:

$$Zn - 2e \rightarrow Zn^{2+}$$

$$H_2O_2 + 2e \rightarrow 2OH^-$$

or, in combined form,

$$Zn + H_2O_2 \rightarrow Zn(OH)_2$$

In addition to hydrogen peroxide, ammonia plays an important role in the biocorrosion of zinc. During long-term exposure to conditions of high pH and ammonia concentrations, $Zn(OH)_2$ is converted to water-soluble complex compounds:

$$Zn(OH)_2 + 2OH^-_{sol} \rightarrow [Zn(OH)_4]^{2-}_{sol}$$

$$Zn(OH)_2 + 4NH_{3,sol} \rightarrow [Zn(NH_3)_4](OH)_{2,sol}$$

Their transition into the exudate would favor the corrosion process due to an equilibrium shift towards the formation of $Zn(OH)_2$.

The ammonia released by *Pseudomonas fluorescens* can also serve as a buffer that favors the maintenance of pH at a sufficiently high level for a long time (Table 1).

The first signs of liquid-droplet exudate on the surface of steel appear, like on the surface of zinc, 2 to 3 days after the start of exposure; however, the exudate amount is much smaller, which hindered the quantitative analysis of its chemical composition. Nevertheless, during this period, the pitting nature of surface damage is clearly evident on local areas where the exudate was formed, Figure 2.

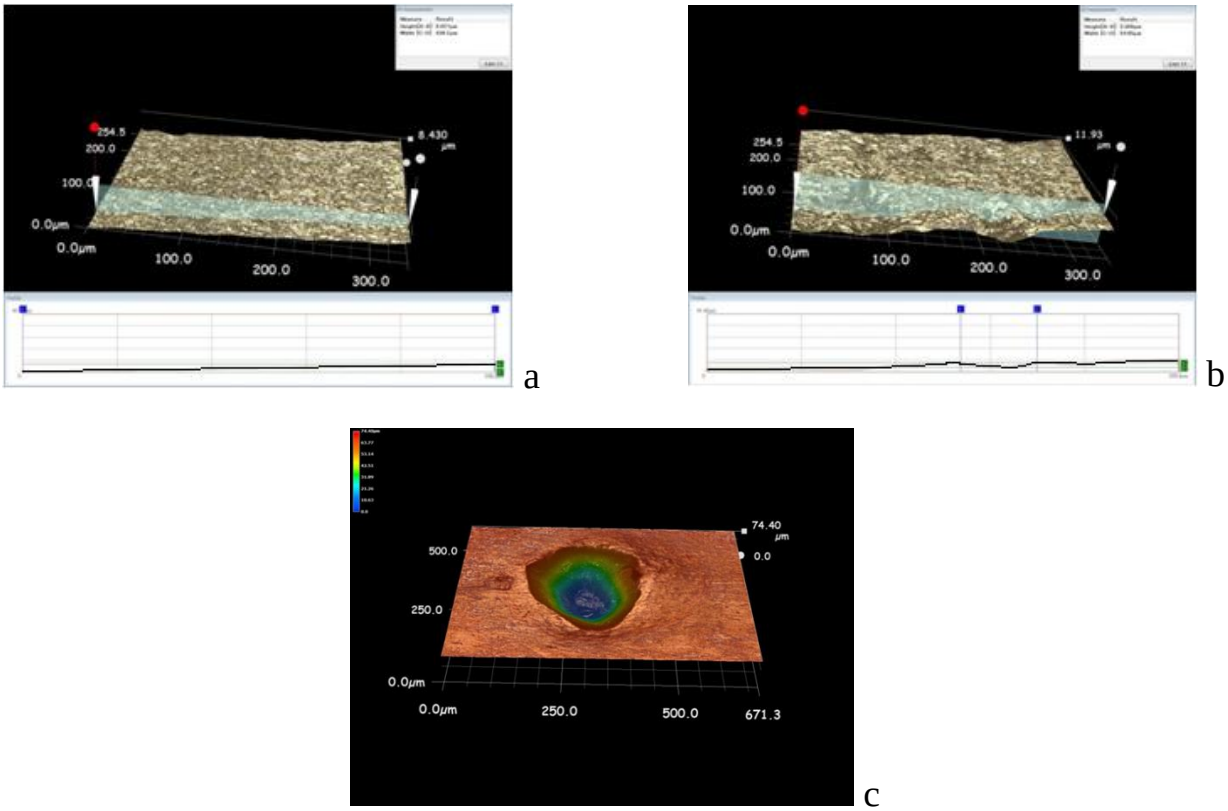


Figure 2. Surface of steel samples: a–initial state; b–after 3 days of exposure to *Pseudomonas fluorescens*; c–spot of pitting corrosion on the surface of a low-carbon steel sample (photographs were taken using a KeynceVHX-1000 optical microscope operating in 3D scanning mode, ×1000).

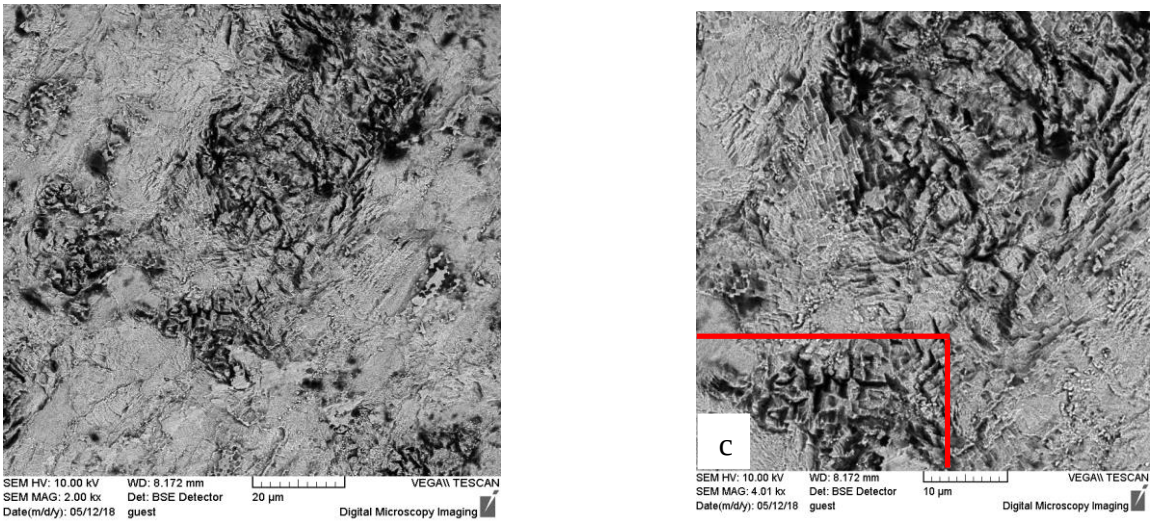
By the 10th day of exposure, the amount of the exudate on the steel surface increases and the concentration of hydrogen peroxide reaches 780 μmol/L, which leads to the development of corrosion damage:

$$Fe - 2e \rightarrow Fe^{2+}$$

$$H_2O_2 + 2e \rightarrow 2OH^-$$

$$Fe^{2+} + 2OH^- \rightarrow Fe(OH)_2$$

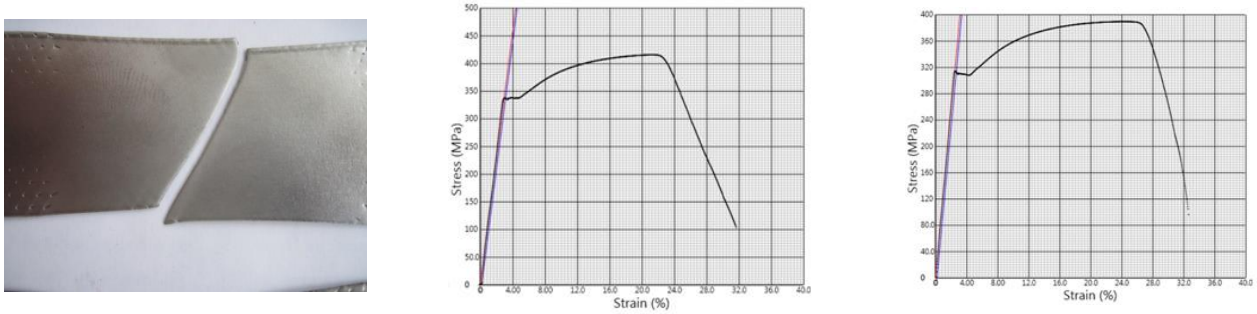
With increasing exposure time, spots of intergranular corrosion become visible on the surface of the samples under study along with pitting and ulcers, Figure 3c. Apparently, the intercrystalline nature of metal destruction is associated with the specific features of the surface colonization by the bacteria.



a b

Figure 3. Micrograph of a steel sample section exposed to *Pseudomonas fluorescens* for 12 days, with an intergranular corrosion area (c). Magnification: (a) ×2000; (b) ×4000

The negative impact of steel exposure to *Pseudomonas fluorescens* was studied based on the changes in mechanical properties upon rupture of the samples at a deformation rate of 0.1 mm/s, Figure 4. A typical stress-strain diagram for samples that underwent biocorrosion is shown in Figure 4c. The stress-strain curve features a yield drop. The samples behave in an almost purely elastic way until the yield point is reached, after which plastic deformation occurs, which is localized in shear bands and develops under almost constant stress.



a b c

Figure 4. Variation in the mechanical properties of steel under the influence of *Pseudomonas fluorescens:* a–steel sample exposed to bacteria for 20 days, after rupture; b–strain diagram in the initial state; c–strain diagram of the sample after exposure to bacteria.

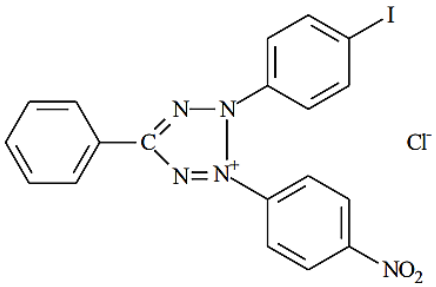
The average values of strength characteristics (for five samples) are presented in Table 2.

Table 2. Mechanical properties of steel samples after exposure to *Pseudomonas fluorescens*.

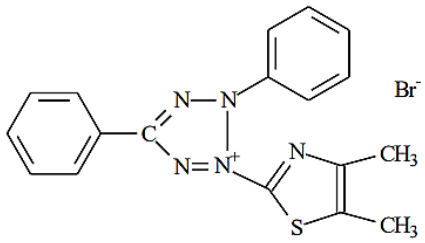
Sample	σ_S, mPa	$\sigma_{0.2}$, mPa	δ, %
Reference sample	420	339	32
12 days	397	316	31
20 days	386	308	30

As it can be seen from the data in Table 2, with increasing exposure time, the strength σ_S and the nominal yield strength $\sigma_{0.2}$ decrease by 6–8%. The observed decrease in average tensile strength compared to the initial state is explained by highly localized pitting corrosion in the form of small and uniformly distributed surface depressions, Figure 1.

Since hydrogen peroxide of biogenic nature was detected at the stage that stimulates the corrosion of zinc and steel, tetrazolium salts were used to analyze the respiratory activity of *Pseudomonas fluorescens* as the most common non-selective indicators of bacterial viability [17, 18]. It was shown in [19–21] that the reducing ability of bacteria towards tetrazolium salts can be determined quantitatively by kinetic methods. INT and MTT, which have a similar ability to undergo reduction, but differ in the polarity of substituents in the five-membered rings, were used as the tetrazolium salts.

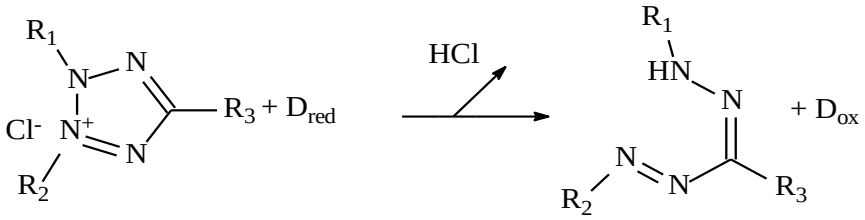


2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride (INT)



3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT)

The reaction of cellular electron donors with tetrazolium salts can be presented as follows:



where D is an electron donor of biogenic nature in the reduced (D_{red}) and oxidized (D_{ox}) forms, respectively.

Figure 5 shows the kinetic curve of IMF accumulation in the presence of *Pseudomonas fluorescens* cells at their optimal metabolism temperature (27°C). As it can be seen from Figure 5, on long exposures (more than 5 hours), the kinetic curve plateaus, but the reagent conversion does not exceed 55%. The same phenomenon was observed in the reduction of INT by other bacteria in other works [19–21] where it was suggested that the decrease in the rate may be affected by IMF associated with membrane components of the cell and hinders the delivery of the reagent to the reducing agents in the membrane.

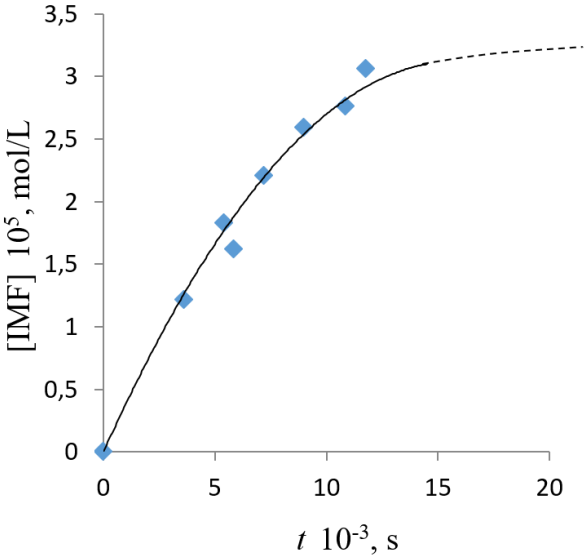


Figure 5. Kinetic curve of iodomonoformazan accumulation in the presence of *Pseudomonas fluorescens*: $[INT]_0 = 5.88 \cdot 10^{-5}$ M; t=27°C (experimental data are presented as an averaged result of 6–7 independent experiments; the average statistical error is 10–15%).

When MTT reacts with *Pseudomonas fluorescens* suspended in physiological saline, a short induction period appears on the kinetic curves, see Figure 6. The degree of the reagent conversion before the kinetic curve plateaus does not exceed 34%.

As it can be seen in Figures 5 and 6, tetrazolium salts are indicators of bacterial viability only until the moment when the product exhibits it inhibitory effect. Moreover, the higher

the hydrophobicity of neutral formazans, the lower the tetrazolium salt conversion at which the reduction product blocks the redox centers of bacteria. For example, reduction stops when the MTT conversion is about 35%, and that of INT is about 55%. The time to reach a plateau on the kinetic curve of MTT reduction is almost 5 times smaller than in INT reduction.

As we have shown in [26], tetrazolium salts can also accept metal electrons upon reduction. When nitroblue tetrazolium chloride was adsorbed from an aqueous solution, an intensely blue formazan was visually detected on the surface of zinc.

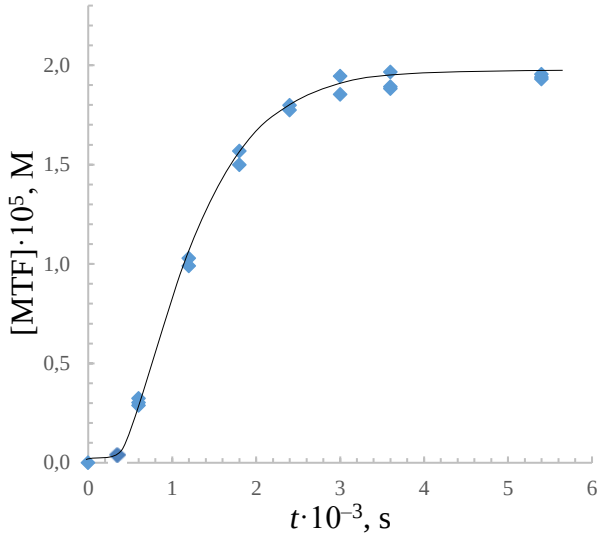


Figure 6. Kinetic curve of MTT-formazan accumulation under the influence of *Pseudomonas fluorescens*: $[MTT]_0 = 5.88\ 10^{-5}$ M, $t = 27$°C (experimental data are presented as averaged results of 6–7 independent experiments; the average statistical error is 10–15%).

Taking into account these two factors, *i.e.*, inhibition of the respiratory activity of bacteria by formazans (Figures 5 and 6) and the capacity of tetrazolium salts to accept electrons from metals, it was assumed that tetrazolium salts could perform as biocorrosion inhibitors. If a molecule contains adsorption-active nitrogen-containing groups, tetrazolium salts are more active adsorbates than hydrogen peroxide and oxygen. As a result, the corrosion process can occur by the tetrazolium depolarization mechanism. For example, on the surface of zinc:

$$Zn - 2e = Zn^{2+} - \text{anodic process}$$

$$\text{tetrazolium salt} + 2e = \text{monoformazan} - \text{cathodic process}$$

Formazans do not undergo oxidation in air and can form claw-shaped complex chelate-type surface compounds with metals.

The dynamics of the corrosion process after adsorption of INT and MTT on zinc and steel were studied visually by observing the changes in the surface state and formation of a liquid-droplet exudate on local areas of the surface, Figure 7.

a b

Figure 7. State of steel surface in the presence of INT adsorbed on the steel surface (a) under the influence of bacteria on day 31 of the experiment; (b) non-treated steel sample with corrosion products.

It has been found that the time to the appearance of the exudate on zinc surface shifts upward (>20 days). The amount of the exudate is so small that even 20 days after the moment of its visual detection, it is not possible to determine hydrogen peroxide quantitatively in the exudate. Inhibition of biocorrosion by INT and MTT occurs similarly on steel where the inhibition time is much longer than on zinc, and the first signs of a liquid-droplet exudate appear after 35 days of exposure of a steel sample to *Pseudomonas fluorescens*.

Conclusion

Summarizing, it has been found that tetrazolium salts adsorbed on a metal surface suppress corrosion for several reasons:

- tetrazolium salt, as a stronger electron acceptor, replaces oxygen and hydrogen peroxide in the cathodic corrosion reaction;
- it forms an efficient long-acting inhibitor, formazan, in the reduction process on the metal surface;
- it blocks the respiratory activity of bacteria, which ultimately leads to a decrease in the amount of hydrogen peroxide in the exudate.

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