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Characterization of chemically modified peat and coke residues as ion exchangers.

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SUMMARY

The products of chemically treated peat and coke present good cation exchanging abilities. The modification process requires relatively inexpensive chemicals in small amounts and only simple procedures are involved. This factor in conjunction with the availability of peat and coke residues makes these modified products well suited for large scale applications.

Key words: Peat, Coke Residues, Chemical modification, Ion exchangers

INTRODUCTION

In order to protect the environment methods for removing pollutants from industrial effluents are vital. These purification processes must be efficient but yet as simple and inexpensive as possible. A possible way of achieving this is to utilize naturally occurring materials, which are inexpensive due to their availability, or cheap industrial raw materials. With this in mind, chemically treated peat from Philippi (Kavala) in Northern Greece as well as residues of coke production have been investigated for their ability to remove impurities from water. In order to maintain the economic advantage of the peat and coke residues the modification procedures were kept relatively simple and utilized inexpensive reagents.

Peat may be roughly defined as the product resulting from a slow and partial decay of dead vegetation (1). It is usually formed in areas where water saturates or completely covers the dead plant material that has accumulated on the ground. The water blocks the action of aerobic bacteria and, in turn, greatly inhibits the rate of decay of the plant debris. Thus peat is a chemically heterogeneous and very complex material.

Approximately 1.5% of the earth's surface is covered with peat, while the largest deposits occur in the northern parts of the northern hemisphere (2,3). Peat, therefore, is an abundant material which is widely available and relatively easy to obtain.

Untreated peat has cationexchanger properties (4-8) and coke (9), however, a number of patents as well as papers have been published showing that peat exhibits

* The present work was financially supported by the General Secretary of Research and Technology in the frame of ΠΕΝΕΔ 91 ΕΔ 224 project.

remarkably enhanced cation exchanger abilities after treatment with sulphuric acid (10-14). This study involves the use of modified forms of peat as an inexpensive cation exchanger, which could ultimately be utilized in a flow system.
In this study, peat samples from Philippi (Kavala) in Northern Greece and residues of coke production were utilized and their characteristics were compared.
The approach undertaken in this work has been to optimize the modification processes not only in regard to exchange capacity but also with respect to the resulting physical and chemical characteristics. These are especially important as the product would ultimately be used in a flow system. Of course, the optimization must involve a compromise between the most desirable exchanger characteristics and the cost of production.

EXPERIMENTAL

Samples and sample characterization
The peat samples were collected from Philippi (Kavala) in Northern Greece, while coke powder was taken from residues of a coke producing factory in the industrial area of Thessaloniki.
The samples were undergone sieve analysis and the corresponding fractions -1000 +500, -500 +250, -250 +125, and -125 were collected.
The moisture was determined in both of these materials and was found to be as mean value of five independent measurements 5.7% for the peat and 2.3% for the coke. The moisture was determined by heating 80°C under vacuum for 2 h. Additionally, 5g of each sample were extracted by anhydrous xylene in a Soxhlet apparatus and by weighing the remaining solids the content in aromatic compounds was determined to be 0.5% for peat and 2.3% for coke.

Chemical treatment
The treatment of peat and coke by sulphuric acid, definitely, leads to esterification of the cellulose as well as to sulphurization of the aromatic compounds contained in these materials, thus introducing sulphuric functional groups ($-SO_3H$) acting as cation exchangers, as is evidenced by the reflectance FT-IR spectrum, showing peaks between 1000-1200 cm^{-1} figure 1.

Reagents
All the reagents used were Merck pro Analysi. Deionised water was used throughout the experimental procedures.
The determination of copper to calculate the recovery efficiency was performed by voltammetry using an E-506 Metrohm Polarecord equipped with an E-505 polarographic stand.

Procedure
5g of the sample were treated with 20 ml concentrated sulphuric acid in open 150 ml beaker. This amount of sulphuric acid was found to be the optimum, since larger amounts gave the same results, while smaller amounts resulted in lower exchange capacities. The treatment was taking place at different temperatures and times to find the optimum conditions. The mixture was stirred, by a glass rod mechanic stirrer every 5 min. After this treatment the mixture was allowed to cool down to room temperature. Then, it was quantitatively transferred into a 500 ml beaker, washed several times with water, filtrated through a G4 gooch funnel and again washed till the filtrate shows slightly acidic reaction.
The product was dried in an oven at 80°C and was left to get the moisture of the environment. All subsequent work was performed at room temperature and without drying the sample.

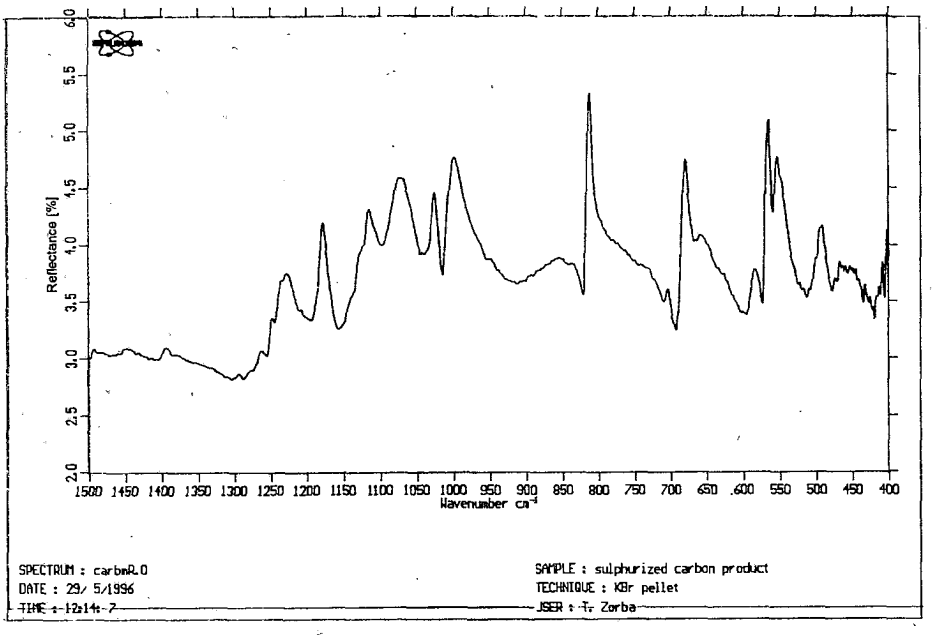

Figure 1. Reflectance FT-IR spectrum of the sulphurized carbon product.

1 g of the product was placed in a burette of 15 mm inner diameter above glass-wool forming the ion exchange column. Through the column 50 ml of 0.5 M sodium chloride were allowed to pass with a flow rate of 5 ml per minute. The effluent was collected in a 250 ml erlenmeyer flask, treated with a known amount of sodium carbonate, which was back titrated by 0.1 N sulphuric acid to calculate the ion exchange capacity of the column [3]. The same procedure was applied to, untreated with sulphuric acid, peat and coke and the result was indicating absence of ion exchange capacity. This led to the conclusion that the untreated products do not possess any ion exchange groups.

Example of the calculation of the ion exchange capacity

Into the effluent collected in the 250 ml erlenmeyer flask described previously, 0.05 g of sodium carbonate was added and the solution was back titrated by 0.1 N sulphuric acid using methyl orange as an indicator; 4.43 ml of the sulphuric acid were consumed to change the colour of the indicator from yellow to red. The non reacted with the liberated from the ionexchanger hydrochloric acid sodium carbonate was calculated to be 4.43mlx0.1 N =0.443 meq, corresponding to 0.443 meq Na_2CO_3 /0.053 g/meq Na_2CO_3 = 0.0235 g. Thus, the amount of Na_2CO_3 reacted with the liberated from the ion exchanger hydrochloric acid is 0.05-0.0235=0.0265 g corresponding to 0.0265/0.053=0.50 meq/g ion exchanger. By regeneration with 50 ml of 0.5 N hydrochoric acid the same ion exchange capacity was obtained for 10 successive procedures.

RESULTS AND DISCUSSION

Optimum temperature

The different fractions of the materials are treated with sulphuric acid for an hour at different temperatures and their effective sodium capacity was calculated. As the temperature was elevated the effective sodium capacity was increased reaching a maximum at 100° C. Indicative results are given in table I for 65° C, 100° C and 150° C.

TABLE I. Effect of sulphuric acid temperature treatment on the effective sodium capacity.

Particle size fraction (μ)	Effective sodium capacity ($meq.g^{-1}$)					
	65°C		100°C		150°C	
	Coke	Peat	Coke	Peat	Coke	Peat
-1000 +500	0:19	0.16	0.22	0.20	0.23	0.22
-500 +250	0.23	0.20	0.29	0.27	0.29	0.28
-250 +125	0.28	0.24	0.40	0.35	0.34	0.30
-125	0.35	0.31	0.50	0.39	0.38	0.34

Optimum time

At the optimum temperature (100° C) the different fractions of the materials are treated with sulphuric acid for various times and their effective sodium capacity was calculated. By increasing the time of the material treatment with sulphuric acid the effective sodium capacity was increased and reached a maximum at 2 hours. Indicative results are given in the table II for 1, 2 and 3 hours.

Comparing the results in the tables I and II is seen, that at optimum sulphuric acid treatment conditions (100° C, 2h), the smaller the material size the higher is the effective sodium capacity. Moreover, between the two materials, higher effective sodium capacities exhibit the coke residues.

Recovery efficiency

If modified peat or coke are to be used in environmental applications, their ability to efficiently remove trace levels of cations must be determined. In an attempt to demonstrate the recovery efficiency, 100 ml of a 10 ppm Cu^{2+} solution were passed through the H^{+}- form of the columns at a flow rate of 2 ml. min^{-1}. The recovery efficiency was then calculated from the percentage of copper eluted by 0.5 N HCl relative to total passed through the column. All of the samples shown in the table I had efficiencies of at least 95%. In a similar manner, a large volume (1.0 l) of a lower concentration of Cu^{2+} (1 ppm) was passed through these columns and good recovery efficiencies were again observed (>90%).

The maximum quantity of the copper ions retained on the columns was always

TABLE II. Effect of sulphuric acid time treatment on the effective sodium capacity.

Particle size fraction (μ)	Effective sodium capacity (meq.g^{-1})					
	1 h		2 h		3 h	
	Coke	Peat	Coke	Peat	Coke	Peat
-1000 +500	0.22	0.20	0.27	0.25	0.21	0.22
-500 +250	0.29	0.27	0.35	0.32	0.28	0.29
-250 +125	0.40	0.35	0.52	0.37	0.37	0.32
-125	0.50	0.39	0.72	0.45	0.42	0.36

higher than the ion exchange capacity of the sulphurized products, by an amount of 10 % for the peat product and 5 % for the coke product most probably due to the adsorption or compexation of copper ions; this complexation obviously is carried out through non acidic functional groups present on these products, like -OH or =C=O. The regeneration of the ion exchanger after copper retention was also successful by use of 0.5 N HCl ($\geq$ 95 %).

The effect of the flow rate on the recovery efficiency was determined by varying the flow rate used for passing 1 ppm Cu^{2+} solution through the columns. Figure 2A is a plot of the results for peat of -1000 to +500 μ size and indicates that relatively fast flow rates, although lower the recovery efficiencies, however, still give relatively high recoveries (20 ml/min above 40%). The recoveries are higher for coke than for peat as is seen in figure 3. The selectivity of the recovery efficiency is also shown in figure 2B. In this figure the recovery of Cu^{2+} (10 ppm) from 100 ml of a solution containing additionally an excess of Na^{+} (1 X 10^{4} ppm in Na^{+}) can be seen. The presence of the sodium ion lowers the efficiency slightly but good recoveries are still achieved. However, again the recoveries are better for coke than for peat as can be seen in figure 3.

The chromic acid treatment

The use of chromic acid in place of sulphuric acid for treating the materials presented no significant difference in the effective capacities, for the treatment of the peat and coke residues with either acids.

The nitric acid treatment

Samples of peat and coke were treated with nitric acid. Fuming began immediately and continued until the mixture cooled. Heating the reaction mixture to 150° C resulted in complete breakdown of the granules. Allowing the reaction to proceed at room temperature for 2 hours did leave some of the particles of the coke residue intact. However, these particles leached continuously. Thus, the oxidizing ability of nitric acid is apparently much too strong for the peat and coke matrix.

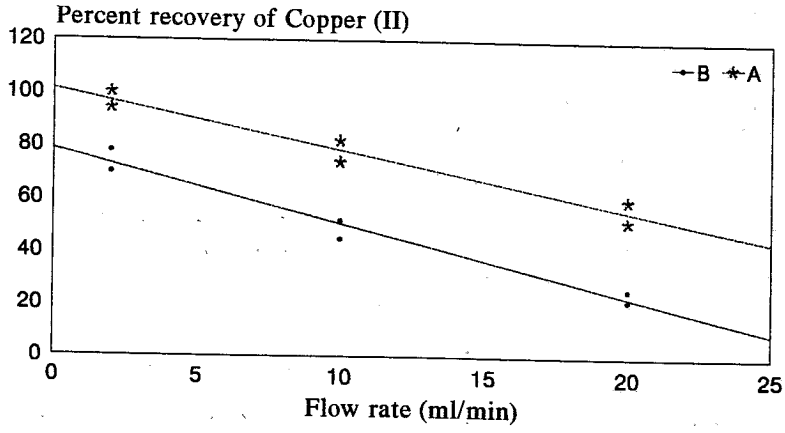

Figure 2. Effect of Flow Rate on the Recovery of Copper (II) form (A) 1.00 l ppm Cu (II) and (B) 0.10 l of 10 ppm Cu (II) and $1x10^4$ ppm Na, using the peat product (-1000 to 500 μ).

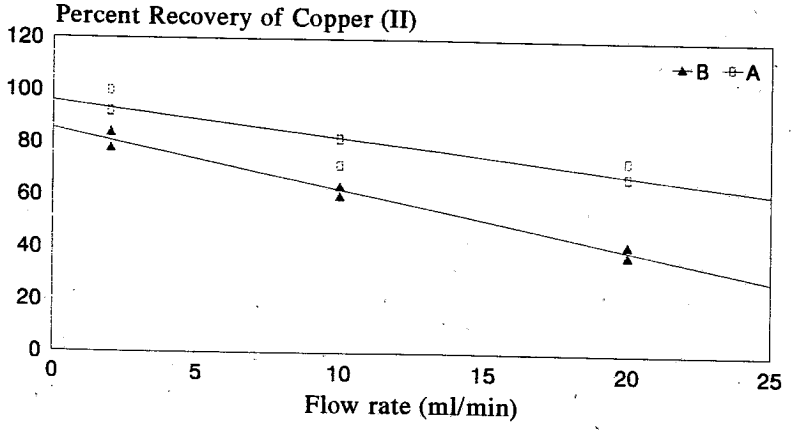

Figure 3. Effect of Flow Rate on the Recovery of Copper (II) form (A) 1.00 l ppm Cu (II) and (B) 0.10 l of 10 ppm Cu (II) and $1x10^4$ ppm Na, using the coke product (-1000 to 500 μ).

CONCLUSIONS

The data show that peat and coke treated with sulphuric acid have considerable potential for the removal of cationic species from water over a wide range of concentrations. The peat itself can be easily and cheaply obtained. The sulphuric acid treatment process is both inexpensive in its use of small amounts of acid and simple in the mere heating of the peat and coke with the acid. The resulting cation exchanger has good physical characteristics for use in a flow system, as well as relatively good capacities.

In applying the modified peat and coke to the removal of cations from water systems, it has two advantages which are also shared by the commercial organic ion

exchangers. One is that it can be easily regenerated by washing with strong acid. The other is that it could be ashed after use to a very small volume of material and then disposed of easily. With the lower cost of modified peat or coke, these procedures would be more economically feasible than if synthetic commercial resins were used. Thus, the application of modified peat and coke residues on an industrial scale appears to be highly feasible.

ΠΕΡΙΛΗΨΗ

Τα προϊόντα της Χημικής κατεργασίας τύρφης και κώκ παρουσιάζουν καλή κατιονανταλλακτική ικανότητα. Η διαδικασία της χημικής μορφοποίησης είναι απλή και απαιτεί σχετικά φθηνά χημικά αντιδραστήρια και σε μικρές ποσότητες. Το γεγονός αυτό σε συνδυασμό με την διαθεσιμότητα της τύρφης και των υπολειμμάτων του κώκ καθιστά τα μορφοποιημένα προϊόντα τους κατάλληλα για εφαρμογές ευρείας κλίμακας.

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CAPACITIVE DETECTION OF ADSORPTION/DESORPTION ON HYDROPHOBIZED GOLD ELECTRODES: AN APPLICATION FOR SURFACTANTS ADSORBTION AND PHOSPHOLIPASE ASSAY.

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Summary

Surfactant adsorption/desorption on/from polycrystalline gold electrodes, covered by a chemically adsorbed monomolecular layer of octadecanethiol, was studied by means of capacitance measurements. An initial capacitance jump at low concentrations occurs in the first adsorption cycle, later the curves show the usual saturation behaviour. The first surfactant layer could not be completely desorbed, only the electrode capacitance shown after the jump was reached. The following cycles do not show any capacitance jumps and were completely reversible. Adsorption isotherms in the homologous series of monoalkylacids are shifted according to the Traube rule. Adsorption kinetics also depends on the length of the hydro-carbon chains and varies from minutes for tetradecanoic acid to hours for decanoic acid. Fast desorption kinetics suggests an application of the capacitive method for the preparation of a capacitive biosensor for enzymes hydrolysing water insoluble substrates into water soluble products (phospholipases, lipases, etc.) The sensor is based on a sandwich like structure: $Au | S(CH_2)_{17}CH_3 |$ Substrate | Electrolyte. Hydrolysis of the substrate leads to the formation of water-soluble products and to the desorption of these compounds from the electrode and therefore to an increase of the electrode capacitance.

Key words: *adsorption, desorption, capacitance measurements, self-assembly, phospholipase assay*

Introduction

An essential stage in the development of affinity sensors is to find a suitable physical parameter, which is sensitive to ligand - receptor binding and can be easily measured. One of such physical parameters is the electrical capacitance. This approach to detect adsorption is well known in traditional electrochemistry - many hundreds of papers are devoted to capacitive measurements of adsorption on metal electrodes. But this adsorption is usually non-specific and the surface of these electrodes can hardly be modified by a receptor layer. This is probably a reason, why the capacitive method was used only very rarely during the history of biosensors development. The situation was changed during the last years, when self-assembled thiol layers on gold electrodes were discovered. Highly stable molecular films are formed on the gold electrodes, and the surface of these films can be modified by a receptor layer. This principle was used in the capacitive sensor for cholerotoxine with thioglycolipids as receptor [1]. In this paper we present some of our results obtained by the capacitive method to study non-specific adsorption of surfactants on gold electrodes, covered by a monomolecular alkylthiol layer [2], and an application of this approach [3] to measure the activity of phospholipases (Fig. 1).

Materials and Methods

All measurements were performed with a two electrode system. the reference electrode was an Ag/AgCl electrode with a surface of about 10 cm^2.

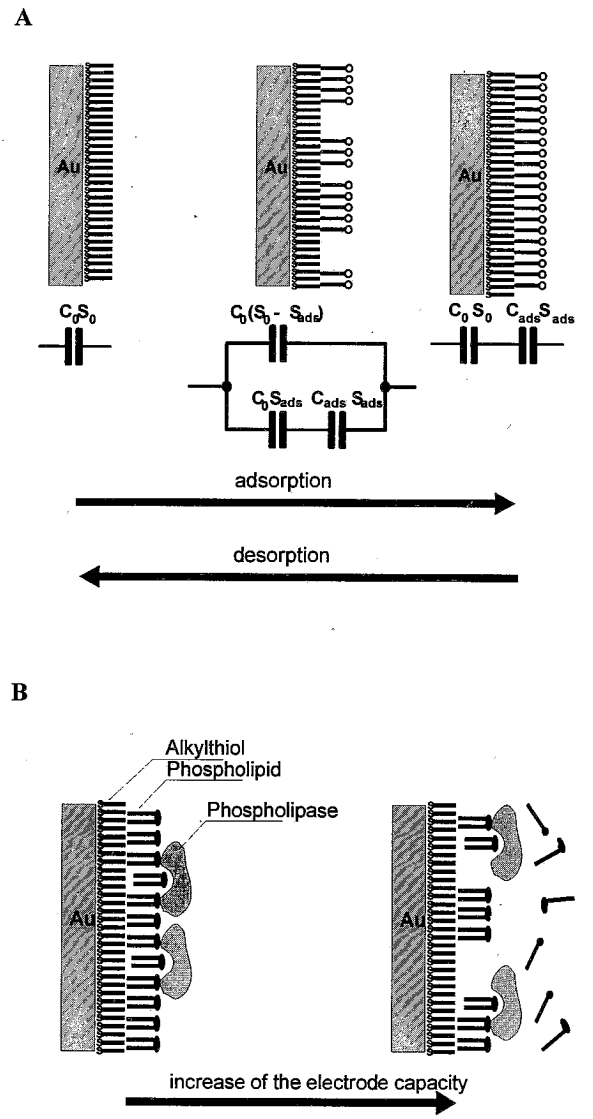

Fig. 1. Capacitive methods for the monitoring of adsorption and desorption: A) capacitive effects following adsorption of surfactants on the gold electrodes, coated by a monomolecular alkylthiol layer; B) an application of the capacitive method to measure phospholipase activity.

The working (sensitive) electrode was prepared from a gold wire (purity 99.995%) following a procedure described below. The wire was cleaned with a hot mixture of H_2SO_4/H_2O_2, rinsed by water and dried. Then the electrode was immersed into a 100 mM solution of octadecanethiol in chloroform for at least 12 hours and washed shortly with chloroform. The lipid layer deposition was done by subsequent immersions into 30 % (w/v) lipid solution in chloroform, usually 5 times for 2-3 seconds each, interrupted by pauses of 10 seconds to evaporate chloroform. The specific capacitance of the electrode was 1.2 $\mu F/cm^2$ before and approximately 1.0 - 1.1 $\mu F/cm^2$ after the lipid deposition. The macroscopic surface of the sensitive electrode was 6.3 mm^2. Cyclic voltammetric measurements in the presence of 3 mM $K_4[Fe(CN)_6]$ have shown a suppressing of the Faraday current by a factor of at least 10^5 for alkylthiol coated gold electrodes in comparison with uncoated gold electrodes (Fig. 2 A).

The two following methods were used to measure the electrode capacitance. The first one was a registration of the 90° component of the capacity current by means of a lock-in amplifier (PAR, Model 121) at 20 Hz. The amplitude of the sine voltage on the electrode was about 10 mV. The second methode was a registration of the current amplitude under application of triangle voltage with an amplitude of 5 mV at 1.5 Hz. A 10-bits digital storage oscilloscope (Philips, Model PM3320A) was used for these measurements as a stroboscopic voltmeter to measure the capacitive current at the moment of zero voltage. A home-made current amplifier or Keithley 427 was used, the typical amplification beeing 10^4 V/A for the lock-in method and 10^7 V/A for the oscilloscope method. A double channel 16 bit automatical lock-in amplifier (Stanford Research System, Model SR850) with its own

frequency sweep oscillator and current amplifier was used to measure the admittance spectrum. The sensitivity of the capacitance measurements was limited by capacity drift and was typically better than 0.1 %. All measurements (except several experiments especially described) were performed at the electrode potential +300 mV (ref. Ag/AgCl, 200 mM KCl) at room temperature in 200 mM KCl, 10 mM imidazole, pH 7.0. The electrolyte was degassed under vacuum before the experiment to prevent the formation of air bubbles.

Deionized water was additionally purified by letting it pass through the „Millipore-Mili-Q" system. All solvents, inorganic compounds and octadecanethiol were from „Merck". Surfactants, lipids and β-cyclodextrine were purchased from „Sigma". Phospholipase A_2 (isolated from Bee Venom) was from „Boeringer Mannheim". Octadecanethiol (98 %) was additionally recristallized from ethanol and Dodecylpiridiniumchlorid from acetone. The other chemicals (p.a.) were used without additional purification.

Results and discussion

The steady-state value of the electrode capacitance (1.2 $\mu F/cm^2$ for gold/octanedecanethiol electrode without lipid layer) did not depend on the electrode potential in the potential range from 50 mV to 450 mV. When the electrode potential did not exceed this range, both of the real (conductive) and the imaginary (capacitive) components of the electrode admittance were very stable (for example, during several days or even weeks under electrode potential of +300 mV). When the electrode potential was lower than 0 mV or higher than 800 mV, a strong increase of the real component of the electrode admittance was observed typically already in a few hours. Electrical properties

of the octanedecanethiol covered electrodes at the low frequencies (0.01 - 30 Hz) were practically purely capacitive (Fig. 2 B, C) - a phase angle between the electrode current and the applied sine voltage was near -90° and there was a linear dependence between the amplitude of the electrical current and the frequency of the applied sine voltage in the logarithmic scale.

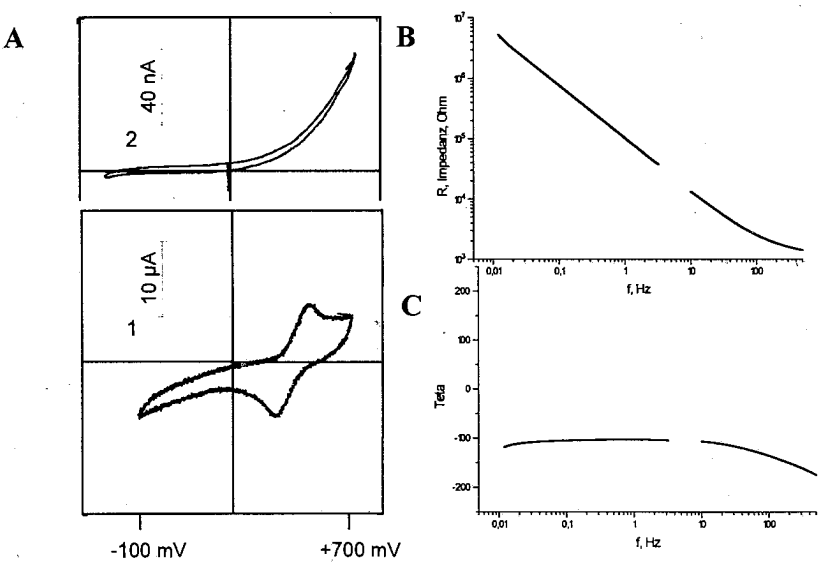

Fig. 2. Electrical properties of gold electrodes, covered by a monomolecular layer of octanedecanethiol. A) Voltammetry in the presence of $K_4[Fe(CN)_6]$: 1 - uncoated gold electrode, 2 - gold electrode, covered by octanedecanethiol B) and C) - Bode diagrams of the gold electrode, covered by octanedecanethiol. Electrolyte: A) 3 mM $K_4[Fe(CN)_6]$, 500 mM KCl, B) 200 mM KCL, 2 mM imidazol, pH 7.0.

The addition of a surfactant to the solution leads to the decrease of the electrode capacitance. The kinetic of this effect depends on the length of the hydrophobic chain of the surfactant and varies from minutes for tetradecanoic

acid to hours for decanoic acid. Such slow kinetics can not be explained by diffusion limitations. One could even expect the opposite dependence i.e., slower kinetics for larger molecules, in the case of diffusion controlled adsorption. This leads to the conclusion, that some other process limits the adsorption of surfactants on the hydrophobic surface. The existence of an energetic barrier, controlling the incorporation of surface active proteins into the lipid monolayer at the air/water interface, was postulated before [4, 5]. Probably, some similar barrier controls the adsorption of surfactants onto a solid hydrophobic surface.

The maximal capacitance decrease is about 30 % and this value is about the same for all surfactants studied. But the concentration dependence of the capacitance effect is determined by the length of the surfactant hydrophobic chain (Fig 3): surfactants with longer hydrophobic chains decrease the capacitance at lower concentrations. This effect can be explained as an adsorption of surfactants according to the Traube's rule. The fact, that the maximal capacitance effect was about the same for all of the surfactants studied means, that the real structure of the adsorbed layer may be more complicated than suggested by the simple model of a homogenious monolayer of surfactants with their alkyl chains pointing away from the aqueous phase (Fig. 1 A, [5]). For example, according to [7], there is a formation of hemicylindrical hemimicells as a result of adsorption of amphiphiles on a hydrophobic surface. Probably, only a few of the CH_2 groups of a surfactant, placed near to the hydrophobic surface of alkylthiol, are densely packed and highly oriented, the rest of the groups, beeing not so ordered, contains polarizable water molecules and therefore has a high dielectrical constant. This could also explain

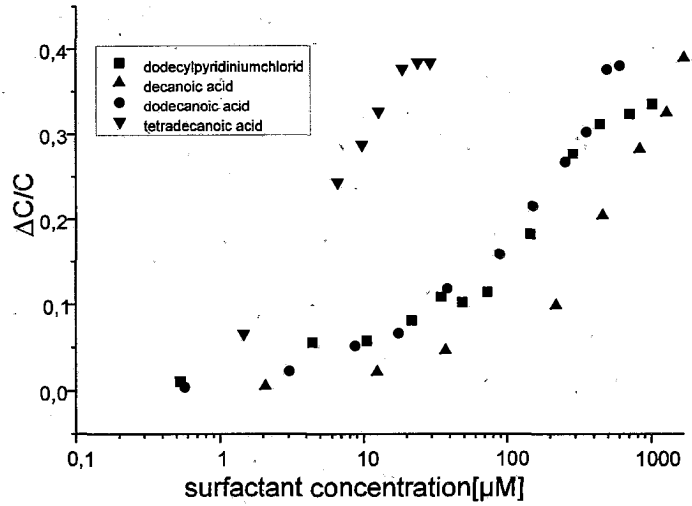

Fig. 3. Effect of surfactant adsorption on the electrical capacitance of the octanedecanethiol covered gold electrode. Electrolyte: 200 mM NaCl, 10 mM imidazole, pH 7.0.

approximately the same maximal capacitance effect for surfactants with a different length of hydrophobic chains.

The dependencies between changes of the electrode capacitance and concentration of surfactant were practically identical for anionic and cationic surfactants with similar hydrophobic chains (dodecanoic acid and dodecylpyridinium, Fig. 3), therefore a contribution of electrostatical effects into the surfactants adsorption is ignorable under the experimental conditions used.

Electrode behaviour in the first cycle of adsorption/desorption was totally different from that in subsequent cycles. In the first adsorption cycle, an initial capacitance jump in the low concentration range (<10 μM) occurs, later the curves show the usual saturated behaviour. The first surfactant layer could

not be completely desorbed even after many days of washing (by water or by aqueous solution of β-cyclodextrine, forming water soluble complexes with

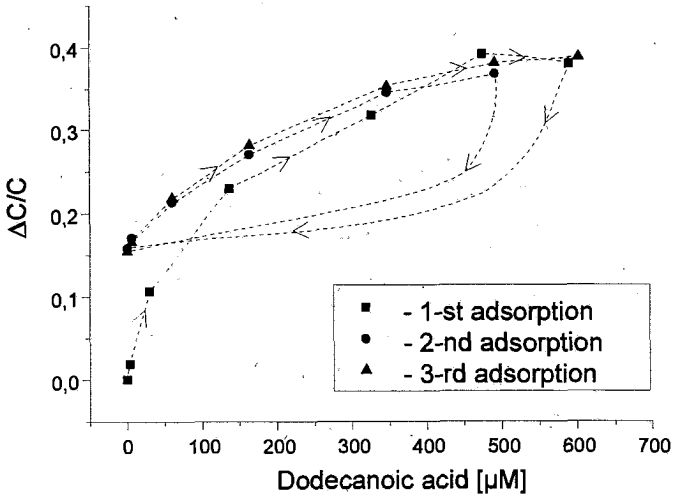

Fig. 4. Subsequent cycles of adsorption/desorption of dodecanoic acid on the octanedecanethiol covered gold electrode. Electrolyte: 200 mM NaCl, 10 mM imidazole, pH 7.0.

surfactants), only the electrode capacitance shown after the jump was reached (Fig. 4). The following cycles do not show any capacitance jumps and were completely reversible. The amplitude of this irreversible part of the capacitance effect is typically 35 % for dodecanoic acid. The reversible part of the adsorbed layer can be totally desorbed in the minute time scale and we were not able to resolve the kinetical constant, replacing the electrolyte with surfactant by the same electrolyte without surfactant. These effects mean formally that at least two populations of binding sites with quite different energy of adsorption exist on the gold electrode, coated by the monomolecular alkylthiol layer. Physically it probably means, that the ordered hydrophobic surface of alkyl chain end

groups forms the binding sites with low adsorption energy. The nature of the high energy binding sites is not quite clear yet. It could be defects or some structural irregularities of the alkylthiol layer, but it is surprising, that these defects are not visible in the voltammetry experiments. We conclude therefore, that these defects are smaller, than the size of the $Fe(CN)_6^{3-}$ ion. There is only one population of binding sites after the first adsorption of a surfactant, when practically only binding sites with high adsorption energy are occupied. The electrodes after such pretreatment show reversible adsorption behaviour and can in principal be used as non-selective sensors for water soluble surfactants. We have compared this method with a traditional method of surface tension measurements and have found that the sensitivities of both methods were similar [3].

The fast desorption of short chain surfactants from alkylthiol coated gold electrode suggests to use the capacitive method as basis for the preparation of a biosensor for phospholipases. The sensor is based on a sandwich like structure: Au | $S(CH_2)_{17}CH_3$ | Substrate | Electrolyte. Hydrolysis of the substrate leads to the formation of water-soluble products and desorption of these compounds from the electrode (Fig. 1 B). When the product formation is the rate - limiting step of this process, the desorption rate is predetermined by the enzyme activity. This desorption can easily be monitored as an increase of the electrode capacitance. By following this concept, a sensor to monitor continuously the phospholipase A_2 activity was prepared. The addition of phospholipase A_2 in the presence of calcium ions leads to an increase of the

electrode capacitance (Fig. 5 A). Subsequent addition of an excess of EDTA

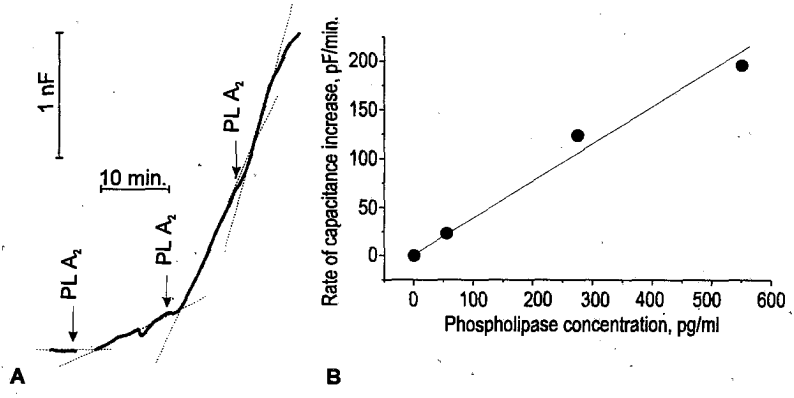

Fig. 5. Changes of the electrode capacitance after phospholipase A_2 (from bee venom) additions (A) and corresponding dependence of the rate of the capacity increase on the phospholipase A_2 concentration (B). Substrate (phospholipid) layer: dilauroylphosphatidylethanolamine. Electrolyte: 200 mM NaCl, 10 mM imidazole, 0.25 mM $CaCl_2$, pH 7.0.

completely inhibits the capacity increase. This cycle of first adding calcium and then an excess of EDTA can be repeated several times. Since it is well known that phospholipases A_2 are calcium-dependent enzymes, the experiment proves that the capacity increase after the phospholipase addition is really caused by the phospholipase action. When long-chain phospholipids were used as substrate (soy-bean phosphatidylcholine), no dependence of the rate of capacitance rise on the phospholipase concentration was observed. When short-chain phospholipids (dilauroyl- and didecanoyl- phosphatidylcholines and phosphatidylethanolamines) were used as substrate, the rate of capacitance rise increases with increasing enzyme concentration (Fig. 5 B). The reason of this difference in behaviour between short- and long chain lipids is certainly the slow desorption or low water solubility of the long-chain lipolytic products.

The monotonous dependence of the rate of capacity increase on the phospholipase concentration allows to use this rate as a measure of the enzyme activity. The detection limit (for phospholipase A_2 from bee venom) is about 50 pg/ml or about $5 \cdot 10^{-5}$ units/ml. This makes our method more sensitive than monolayer (10^{-3} units/ml) and electrostatical ($2 \cdot 10^{-4}$ units/ml) methods [8]. The reason for the high sensitivity of our method, compared with that of the monolayer method is, probably, the much higher volume/surface ratio in our system (300 ml/cm^2 comparing 1 ml/cm^2 for the monolayer method). The high surface activity of the phospholipase A_2 from toxins will lead to an enzyme accumulation on the small contact area of the electrode.

Using different electrodes, the value of their capacitance after lipid layer deposition and the values of the rates of the capacitance increase during lipid hydrolysis showed considerable scatter. One can expect an essential improve of the electrode behaviour by using another method for lipid layer deposition.

In 1971 Zografi, Verger and deHaas developed a Langmuir trough method to study lipolytic activity [9]. The method was then reproduced in many laboratories and successfully used to study phospholipases [10-11] and lipases [12]. The idea of that method is the measurement of the monolayer area decrease under a fixed surface pressure resulting from the formation of water soluble products, followed by their desorption from the monolayer to the aqueous subphase. Both the Langmuir trough method [9] and the capacitance method described here utilise the same ambivalent properties of the lipase and phospholipase substrates - solubility of the products and insolubility of the substrates. The advantage of the capacitance method can be found in low cost and simple devices such as an electrode, which can be further miniaturized.

The described idea could also be used for studying other enzymes which catalyse hydrolysis of lipophilic substrates into water soluble products.

Conclusion

The results show that monitoring of the electrical capacitance is a convenient way to study adsorption onto the electrode. In the present study a non-specific adsorption of surfactants was investigated. Reversible behaviour of the electrodes after strong binding of a surfactant allows to use these electrodes as capacitive sensors for surfactants. We have compared this method with the traditional method of surface tension measurements and the results have shown, that both these methods have about similar sensitivity [3]. Fast desorption of surfactants and extremely high sensitivity, easily reached with capacitive method, allowed to prepare an assay for lipolytic enzymes. The main advantage of the system Au | alkylthiol is the possibility to modify the surface by binding of a receptor layer. Therefore, an immunosensor, based one the system Au | alkylthiol | antibody will probably be the most attractive application of this system.

Acknowledgements

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ΠΕΡΙΛΗΨΗ

Μελετήθηκε η προσρόφηση/εκρόφηση τασιενεργού επί/από πολυκρυσταλλικών ηλεκτροδίων χρυσού, επικαλυμμένων με χημικώς προσροφημένη μονομοριακή στιβάδα οκταδεκανοθειόλης, με μετρήσεις χωρητικότητας. Κατά τον πρώτο κύκλο προσρόφησης λαμβάνει χώρα αρχικό άλμα χωρητικότητας σε χαμηλές συγκεντρώ-

σεις· αργότερα, οι καμπύλες δείχνουν τη συνήθη συμπεριφορά κορεσμού. Η πρώτη στιβάδα τασιενεργού δεν έγινε δυνατό να εκροφηθεί πλήρως, επιτεύχθηκε μόνο η χωρητικότητα του ηλεκτροδίου που παρουσιάσθηκε μετά το άλμα. Οι ακόλουθοι κύκλοι δεν έδειξαν άλμα χωρητικότητας και ήταν πλήρως αντιστρεπτοί. Οι ισόθερμες προσρόφησης στην ομόλογη σειρά των μονοαλκυλοξέων μετατοπίζονται σύμφωνα με τον κανόνα Traube. Η κινητική της προσρόφησης εξαρτάται, επίσης, από το μήκος της αλυσίδας του υδρογονάνθρακα και ο χρόνος ποικίλει από λεπτά για το τετραδεκανοϊκό οξύ σε ώρες για το δεκανοϊκό οξύ. Η κινητική ταχείας εκρόφησης οδηγεί στην εφαρμογή της χωρητικής μεθόδου για την παρασκευή χωρητικού βιοαισθητήρα για ένζυμα που υδρολύουν αδιάλυτα στο νερό υποστρώματα προς υδατοδιαλυτά προϊόντα (φωσφολιπάσες, λιπάσες κ.λπ.). Ο αισθητήρας βασίζεται σε δομή τύπου σάντουιτς: Au/S $(CH_2)_{17}CH_3$/Υπόστρωμα/Ηλεκτρολύτης. Η υδρόλυση του υποστρώματος οδηγεί στο σχηματισμό υδατοδιαλυτών προϊόντων και στην εκρόφηση των ουσιών αυτών από το ηλεκτρόδιο και, συνεπώς, σε αύξηση της χωρητικότητας του ηλεκτροδίου.

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KINETICS OF PHOSPHATE REACTIONS WITH CALCIUM-BENTONITE

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Surface run-off and soil erosion losses of P applied in fertilizers, manures and sewage sludge cause the greatest risk of environmental pollution through the contamination of surface water sources and increased concern that P saturation of soils may result in possible leaching of P, has resulted in legislation to control applications to soils in Europe.

Therefore studies of P sorption by soil and soil components based on equilibrium and kinetic models may be very important either for enviromental and/or fertilizing reasons.

The objective of the present investigation was to elucidate the effect of time, pH and initial P concentration on P reactions with Ca-bentonite and to suggest a possible mechanism of adsorption.

The phosphate adsorption by calcium bentonite at low phosphate concentrations in solution could be described by Langmuir adsorption isotherms, indicating that a monolayer of phosphate is formed on the surface.

The calculated maximum surface concentrations were 807.9, 545.7, 522.1 and 383.5 mg P Kg^{-1} for Ca-bentonite at 4.0, 6.0, 7.0, 8.0, pH values correspondingly.

A second-order kinetic equation was also applied, which considers both the change in phosphate concentration in solution and the surface saturation of the absorbent during the adsorption process.

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The rate constants of phosphate adsorption (K_1) and desorption (K_{-1}) increase with increasing phosphate concentration at pH 4.0, 6.0, and 8.0 and decrease at pH 7.0. The rate constant (K) and maximum adsorption (Xm) decrease with increasing pH values.

It was suggested that phosphate ions are adsorbed by Ca-bentonite displacing coordinated water molecules and/or coordinated anions.

Key Words : Kinetics, phosphate, adsorption, Ca-bentonite

Introduction

A number of researchers have investigated kinetic reactions on pure clays and soils [1-10]. Kuo and Lotse[2] pointed out that in developing a kinetic equation for phosphate adsorption, which has resulted from the Langmuir plot of the experimental data, the energy of adsorption does not vary with the surface coverage. Thus, the fact that the experimental points fit a straight line indicates that the equilibrium constant, obtained from the intercept, is indeed constant and so the activation energy which can be obtained from the Arrhenius equation is constant under isothermal conditions. Using fractions of surface uncoverage as the only factor governing the rate of adsorption leads to a first order kinetic equation. Ignoring fraction of surface uncoverage and considering concentration as the only rate determining factor, other researchers, found that the results follow a pseudo first-order reaction[1,11].

First-order kinetics were also found to describe the desorption of phosphate from kaolinite[12] and pseudo first order kinetics were found to describe the adsorption of phosphate from kaolinite, hematite and kaolinite-hematite system[6,13].The kinetics of phosphate adsorption and desorption in soils has also been shown to conform to first order reactions and to exhibit a diffusion-controlled exchange[3,14].

Chien and Clayton[15] working with soils suggested that the Elovich equation was superior to the first order rate approach because the former method tended to combine simultaneous first-order reactions into one linear slope.

Since the tend of increasing rate of lake eutrophication has been matter of public concern in recent years, P is an important nutrient in crop production. Except that surface run-off and soil erosion of P applied in fertilizers, manures and sewage sludge can create environmental pollution through the contamination of surface water sources, so increased concern that P saturation of soil may result in possible leaching of P , has resulted in legislation to control applications of P to soils in Europe. Therefore studies of P sorption by soil components based on equilibrium and kinetic models may be very important.

In this study , the effect of time, pH and P concentration on P reactions with Ca-bentonite is described.

Materials and Methods

Ca-bentonite was prepared by treating bentonite (Argilometalic No.7063) with 1N $CaCl_2$ solution .The clay was then washed until it was free of chloride. The cation exchange capacity (C.E.C.), exchangeable Na and K were determined by the methods described on Ioannou et al. (1994). Specific surface area (SSA) was determined by the BET method, using N_2 as adsorbate in a Sorptomatic 1900 Carlo Erba Surface area analyzer. ^{27}Al and ^{29}Si MAS (Magic Angle Spinning) NMR Spectroscopy was applied to establish the immediate chemical environmental of Ca-saturated bentonite. Control samples used for the ^{27}Al spectra and ^{29}Si spectra were Al_2O_3 and DDS, respectively. All spectra were obtained on a Brucker 400 MSL instrument. For silicon spectra we were used the inverse gated

heteronuclear decoupling (HPDEC. PC), the resonance frequency of 79.494 MHz, the high power mode with the acquisition time long enough to record FID (D7= 20 ms), the decoupler power (DP) IZH, the relaxation delay 104s, the data point (TD) 4K and the size (SI) of FID 4K. In the processing it was used trapezoidal multiplication (TM) (TM 1=0 and TM2= 400), and for the control sample was used only 8 scans while for the sample 2000 scans. For the aluminum we were used the quadropole one pulse acquisition with phase cycling (NUTATION. PC) and the resonance frequency of 104.262 MHz. The relaxation delay used was 1.0 s, TD 1K, SI 4K. The dead time used was 8.0×10^{-3} ms and for the processing TM D3 and D4 used were 8.0 $\times 10^{-3}$ ms and 0.6 $\times 10^{-3}$ ms, respectively. The number of scans used for the sample was 8.0.

For the kinetic studies 0.1g of Ca-bentonite was shaken with 10 ml of KH_2PO_4 of known P concentration at pH 4.0, 6.0, 7.0, 8.0 for various periods of time up to 6h. Initial P concentrations were 3.125 to 6.25 mg L^{-1}. After that, the Ca-bentonite suspensions were centrifuged at 14.500xg on a Varifuge 20 RS automatic superspeed refrigerated centrifuge. Aliquots were withdrawn for determination of the final phosphate concentration using the method of Murphy and Riley[17]. Amount of phosphate sorbed was calculated as the difference between the initial and final concentrations. The experiments were conducted at a constant temperature of 25±0.1°C in an agitated water bath, at four replicates, the reproducibility of the concentration data was ± 4%.

Results and Discussion

The Cation Exchange Capacity (C.E.C.) and the Specific Surface of Ca-bentonite are given in Table I.

Table I
Some chemical properties of Calcium-Bentonite

C.E.C cmol Kg^{-1}	Exchangeable Na cmol Kg^{-1}	Exchangeable K cmol Kg^{-1}	Specific surface m^2 Kg^{-1} Ca-b
81.4	2.56	0.64	45730

The ^{27}Al NMR Spectra , Fig. (1.A), shows two peaks corresponding to the tetrahedrally (T) coordinated (ca 40ppm) and octahedrally (O) (ca 10ppm) Al of Al_2O_3. The T/O ratio was found to be 1.8/6 measuring the peak intensities. Figure (1.B) of Ca-saturated bentonite appeared to clearly contain tetrahedral and octahedral Al. The calculated T/O ratio for this sample was 1.3/6. A second order effect is depicted in this sample (characteristic shape at 40 ppm). Figure (2.A,B) shows the ^{29}Si NMR Spectra of the control sample and of Ca-saturated bentonite. As a control sample for the silicon spectra was used the DDS (Fig 2.A).. Figure (2.B) of Ca-b showed peaks at ca-112 ppm and ca-96 ppm characteristic of $Si(0Si)_4$ grouping in high siliceous materials. The observed multiplicity in both sites arise from the crystallographically non-equivalent tetrahedral environments of the $Si(0Si)_4$ sites. Thus, Si(0Al), Si(1Al) appear in Q_4 area and Si(1Al) and Si(2Al) in Q_3 area. In Figure (2.B) of Ca-b seems to be a rather low content of Si-OH groups (nothing at ca-100 ppm). There is no effect from decoupling on the sample which appear to have a log T1.

Rate of Phosphate Adsorption

The basic idea of phosphate reaction with Ca-bentonite may be illustrated as follows:

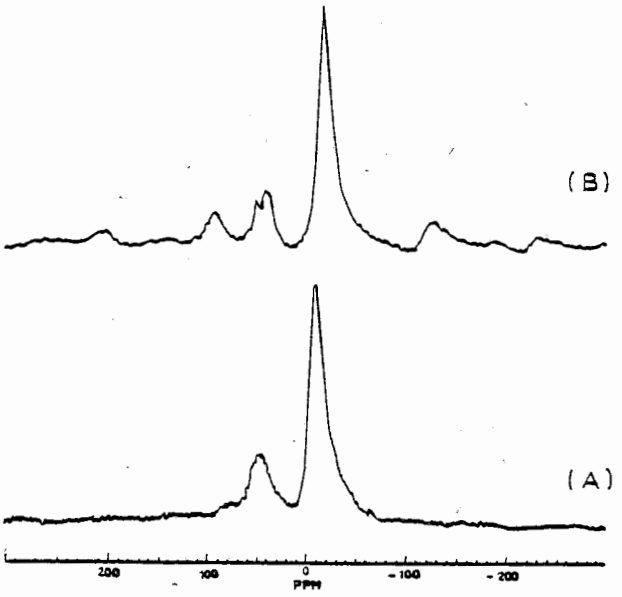


FIGURE 1: *^{27}Al MAS-NMR spectra at 104.262 MHz of Al_2O_3 (A) and Ca-bentonite (B)*

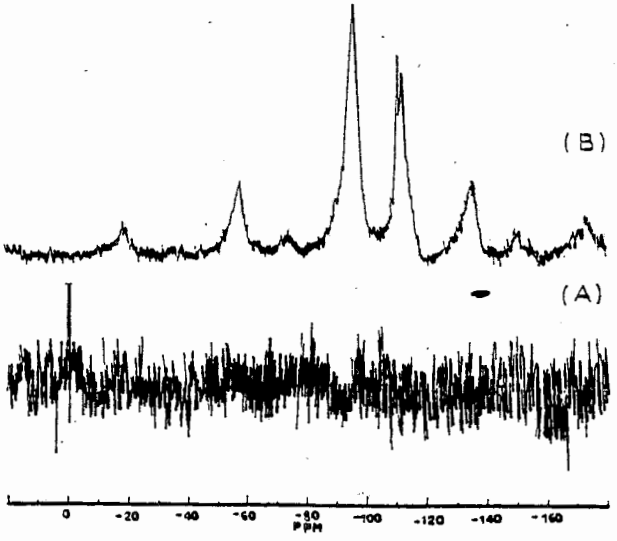
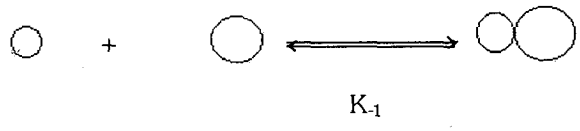


FIGURE 2: *^{29}Si MAS-NMR spectra at 79.494 MHz of DDS (A) and Ca-bentonite (B)*

$$P + B_e \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} PB_e$$



t=0 :	Co	Xm	0
t=t :	Co-X	Xm-X	X

When a phosphate ion in solution is brought in contact with the surface, an activated complex is formed.At time zero,the phosphorus concentration in solution is Co (mg P L^{-1}) and the surface unsaturation has the same numerical value as the maximum monolayer surface capacity, Xm (mg P Kg^{-1} of Ca-b).

After time t, when X amount of phosphate ions (mg P Kg^{-1} of Ca-b) has been adsorbed by the Ca-b surface, the concentration of phosphate ions remaining in solution is (Co-X) and the surface unsaturation is (Xm-X).

Thus the rate of phosphate adsorption by Ca-bentonite can be expressed by the equation:

$$\mathbf{dX/dt = K_1 (Co-X)(Xm-X) - K_{-1}X} \qquad (1)$$

where K_1, and K_{-1} are rate constants for adsorption-desorption processes, respectively. At equilibrium, the rate of adsorption would be equal to the rate of desorption (dX/dt= 0). Thus,

$$\mathbf{K_1\,(Co\text{-}X)_{eq}\,(Xm\text{-}X)_{eq} = K_{-1}X_{eq}} \qquad (2)$$

by rearranging equation (2) and expressing X and Xm in mg of P adsorbed Kg^{-1} of adsorbent, $(Co\text{-}X)_{eq}$= Ceq and $(Xm\text{-}X)_{eq}$= Xm-X, X_{eq}=X at equilibrium the Langmuir equation is obtained

$$\frac{\mathbf{1}}{\mathbf{KXm}} = \frac{\mathbf{Ceq}}{\mathbf{X}} - \frac{\mathbf{Ceq}}{\mathbf{Xm}} \qquad (3)$$

Where K is the rate constant (K_1/K_{-1}). Plotting Ceq/X as a function of Ceq (Fig. 3), the slope gives the inverse of the maximum monolayer surface capacity and the intercept is equal to 1/KXm.

The values of Xm and K have been given by Ioannou et al.[16] :

$$\mathbf{Xm = 12.323\text{-}1.061\ pH} \quad (r^2 = 0{,}999) \qquad (4)$$

$$\mathbf{K = 0.0275\text{-}0.0025\ pH} \quad (r^2 = 0{,}970) \qquad (5)$$

Findings by Ioannou et al.,[16] indicated that phosphate adsorption by Ca-bentonite at low phosphate concentrations can be described by the Langmuir isotherms and this is an indication that phosphate forms a monolayer on the surface of Ca-bentonite.

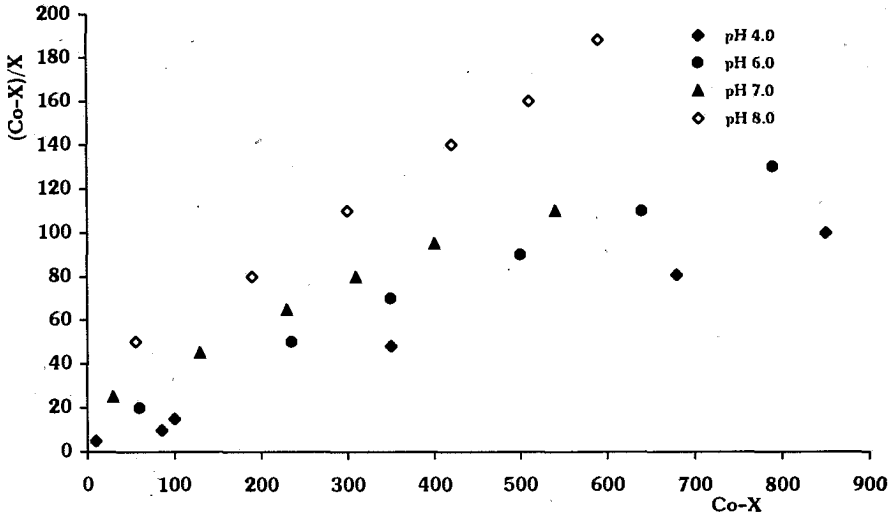


FIGURE 3: *Langmuir isotherms for P sorption on Ca-Bentonite at different pH values . X=mmolP/Kg of Ca-Bentonite, C=mmol P L^{-1} x10^{-3} of solution.*

The fact that the experimental points fit quite well a straight line (Fig. 3) is an indication also that the equilibrium constant, obtained from the intercept, is indeed constant and that therefore the activation energy which can be obtained from the Arrhenius equation, is constant under isothermal conditions. These findings are in agreement with earlier findings of phosphate adsorption by kaolinite[2,18].

The values of the maximum monolayer surface saturation calculated from the slope of the Langmuir isotherms for each studied pH are given in Table II.

By integrating Equation (1) the following expression is obtained:

$$\ln \frac{X - A - B}{X + B - A} = 2AK_1t + \ln \frac{B + A}{B - A} \qquad (6)$$

Table II

Constants of phosphate adsorption and desorption by Ca-bentonite at different pH, and phosphate concentrations.

Xm mgP Kg^{-1}	K	K_{-1} $L^2mgP^{-1}h^{-1}$	K_1 $L^2mgP^{-1}h^{-}$	A	B	pH	Co mg P L^{-1}
807.9	0.0175	1.497	0.026	2.487	5.611	4.0	3.125
		1.580	0.028	2.188	5.923	4.0	3.750
		1.754	0.031	1.881	6.275	4.0	4.375
		2.343	0.041	1.426	6.705	4.0	5.330
		3.314	0.058	0.981	7.173	4.0	6.250
595.7	0.0125	1.584	0.020	1.436	4.54	6.0	3.125
		1.840	0.023	1.132	4.86	6.0	3.750
		2.600	0.032	0.831	5.641	6.0	4.375
		5.384	0.067	0.414	6.110	6.0	5.330
		7.672	0.059	0.313	6.110	6.0	6.250
522.1	0.0108	2.574	0.028	1.069	4.178	7.0	3.125
		2.304	0.025	0.768	4.491	7.0	3.750
		1.674	0.018	0.571	4.80	7.0	4.375
		1.399	0.016	0.480	5.272	7.0	5.330
		1.275	0.014	0.234	5.741	7.0	6.250
383.5	0.0081	3.000	0.024	0.640	3.617	8.0	3.125
		2.977	0.024	0.517	3.929	8.0	3.750
		7.068	0.057	0.249	4.24	8.0	4.375
		8.548	0.069	0.239	4.711	8.0	5.330

where

$$A = \left[\frac{1}{4}\left(Co + Xm + \frac{K_{-1}}{K_1}\right)^2 - CoXm\right]^{1/2} \qquad (7)$$

$$B = \frac{1}{2}\left[Co + Xm + \frac{K_{-1}}{K_1}\right]$$

K_1 = rate constant of adsorption.

The parameters, A and B, are constants and contain a concentration unit.

By plotting ln (X-A-B/X+A-B) as a function of time a straight line is obtained.The slope of this line is $2AK_1$.

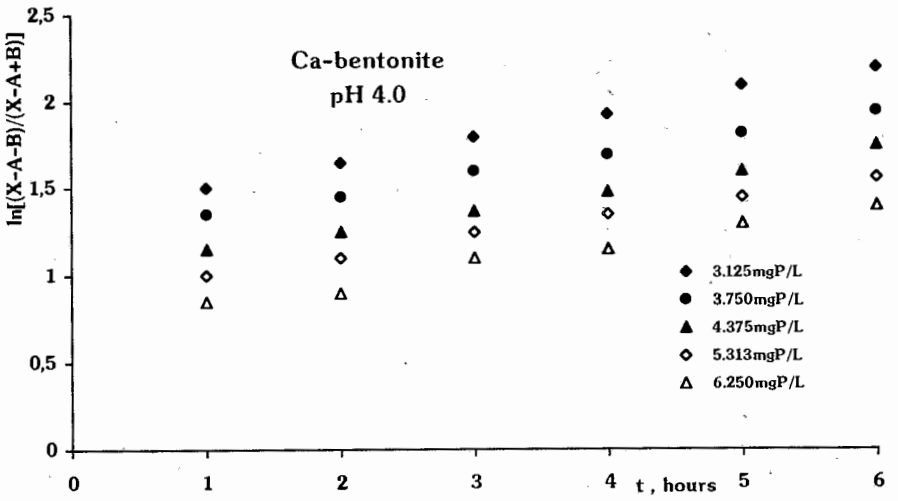


FIGURE 4:*Kinetic data of phosphate reactions with Ca-Bentonite at pH 4.0 and different initial concentrations,according to equation (6).*

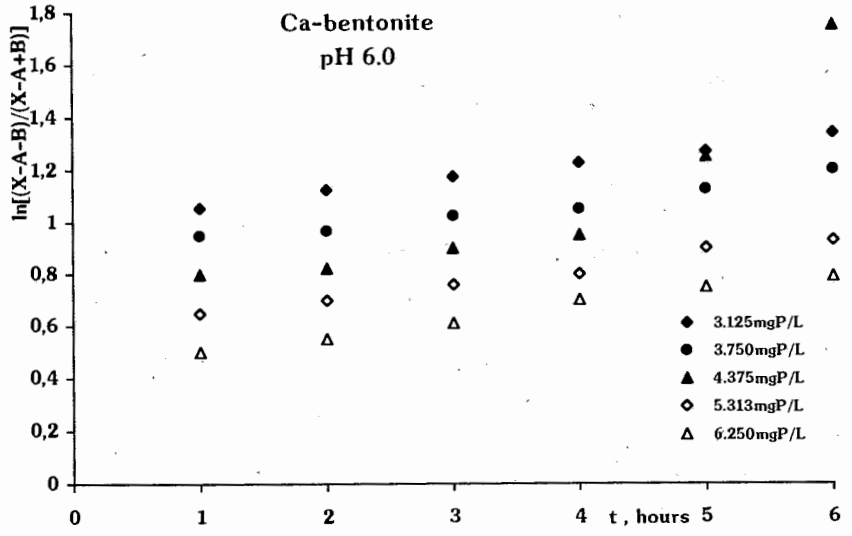


FIGURE 5: *Kinetic data of phosphate reactions with Ca-Bentonite at pH 6.0 and different initial concentrations,according to equation (6).*

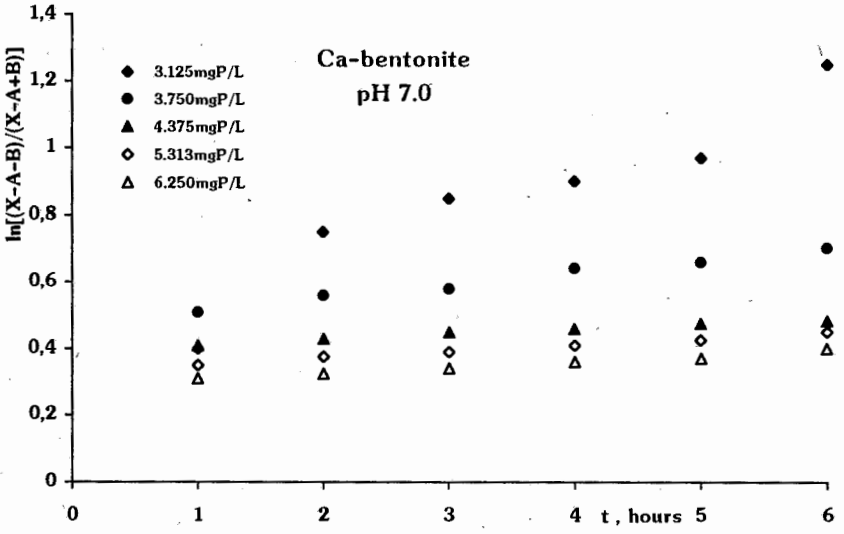


FIGURE 6: *Kinetic data of phosphate reactions with Ca-Bentonite at pH 7.0 and different initial concentrations,according to equation (6).*

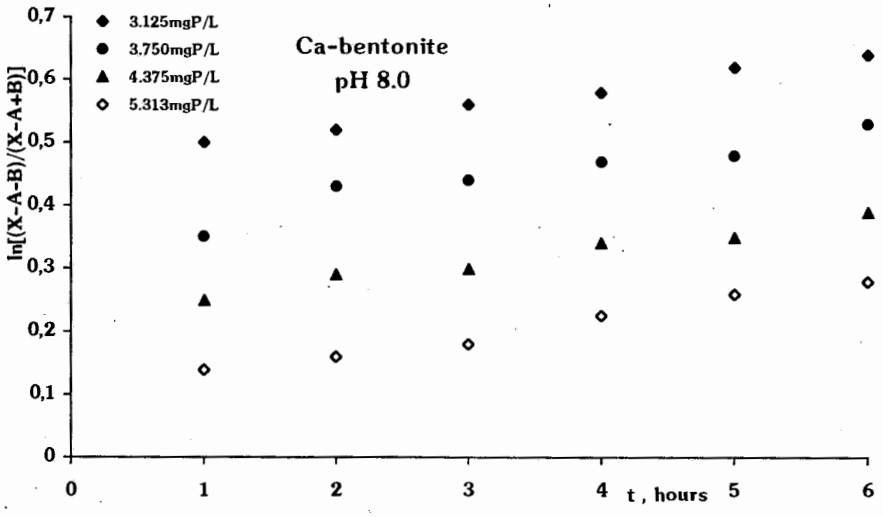


FIGURE 7: *Kinetic data of phosphate reactions with Ca-Bentonite at pH 8.0 and different initial concentrations,according to equation (6).*

Figures 4, 5, 6, 7 show the kinetic data plotted according to Equation (6). The correlation coefficient (r^2) of linear regression of all plots ranges between 0,997 and 0,999.

The values of maximum adsorption, the rate constants (K_1, K_{-1}), A and B constants for all pH and different initial concentrations (Co) are listed in Table II.

In Table IV the rate constants K_1, K_{-1} of phosphate adsorption and desorption by Ca-bentonite increase linearly (r=0.937-0.973) with increasing phosphate concentration at pH 4.0, 6.0 and 8.0 and decrease insignificantly linear (r=-0.940) with increasing initial concentration (Co) at pH 7.0. The equilibrium constant and maximum adsorption decrease with increasing pH values (Table II).

Table III

Constants A, B as a function of pH for each studied initial concentration calculated by Linear regression analysis.

	r	Co, mgP L^{-1}
A = 4.412-0.485 (pH)	r = -0.998	3.125
A = 4.08 - 0.479 (pH)	r = -0.998	3.75
A = 3.473-0.418 (pH)	r = -0.987	4.375
A = 2.119 - 0.23 (pH)	r = -0.75	5.33
A = 1.556 -0.165 (pH)	r = -0.75	6.25
B = 7.56 -0.49 (pH)	r = -0.998	3.125
B = 7.87 - 0.49 (pH)	r = -0.998	3.75
B = 8.589 - 0.518 (pH)	r = -0.90	4.375
B = 9.456 - 0.638 (pH)	r = -0.958	5.33
B = 9.09 - 0.485(pH)	r = -0.996	6.25

The values of A and B constants decrease linearly (r=-0.750 to -0.998 for A and r=-0,900 to -0.998 for B) with increasing pH (Table III).

Also constant A decreases linearly (r=-0.880 to -0.999) with increasing phosphate concentration while constant B increases linearly (r=0.980 to 0.999) with increasing initial concentration at pH 4.0, 6.0, 7.0 and 8.0 (Table IV).

Table IV

Rate constants (K_1, K_{-1}) and A, B constants of phosphate adsorption desorption by Ca-bentonite as a function of initial P concentration for each studied pH calculated by Linear regression analysis.

K_{-1}= -0.508+0.571Co	r = 0.937	
K=-8.512x10^{-3}+9.924x10^{-3}Co-	r = 0.937	**pH 4.0**
A = 3.933-0.482 Co	r = -0.999	
B = 4.051+0.499 Co	r = 0.999	
K_{-1}= -5.578+2.057Co	r = 0.973	
K =-6.964x10^{-2}+2.568x10^{-2}Co	r = 0.972	**pH 6.0**
A = 2.526-0.372 Co	r = -0.978	
B = 2.196+0.746 Co	r = 0.98	
K_{-1}= 3.77- 0.414Co	r = -0.95	
K_1 = 0.04075- 4.457x10^{-3}Co	r = -0.94	**pH 7.0**
A = 2.538-0.471 Co	r = -0.999	
B = 2.618+0.499 Co	r = 0.991	
K_{-1}= -10.5+ 4.46 Co	r = 0.97	
K_1 = -0.085+0.036 Co	r = 0.97	**pH 8.0**
A = 1.168 - 0.222 Co	r = -0.88	
B = 2.054+0.5 Co	r = 0.99	

The fact that phosphate adsorption increases with decreasing pH values probably indicates that phosphate ions are adsorbed by replacing H_2O groups rather than hydroxyl groups in agreement with Hsu[13].

Possible Mechanism of Phosphate Adsorption by Ca-bentonite.

Exchange of edge hydroxyl groups of the crystal lattice by phosphate ions was suggested by Low and Black[18] and Kafkafi et al.,[26]. Russel and Low[20] concluded that adsorbed aluminium precipitates the phosphate as an aluminium phosphate on the kaolinite surface. Olsen and Watanabe[21] suggested that phosphate ions become attached to exchangeable iron, aluminium and calcium ions or to these ions held in the outer edges of the lattice.

Hsu[22] suggested that adsorption of phosphate by soils is a special case of precipitation in which aluminium (or iron) remains as the constituent of the original phase but reacts with phosphate by use of residual force on the surface.

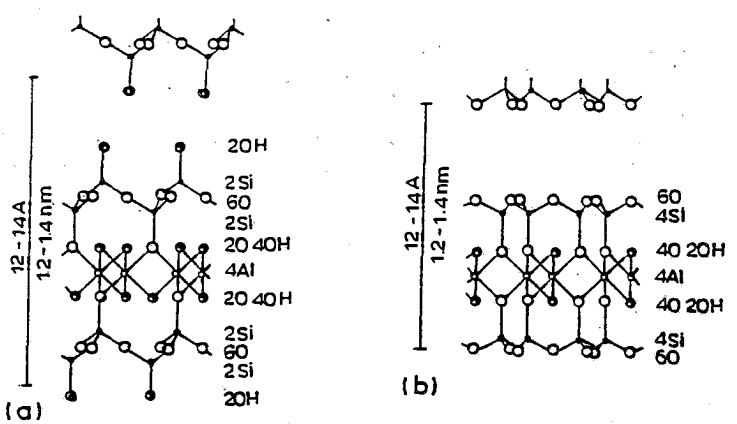


FIGURE 8 : *(a) Structural model according to Edelman and Favajee, and (b) structural model according to Hofmann and Endell . (From M.W.Wirjodihardjo, and K.H.Tan (1964). Ilmu. Tanah. Djilid. II. Publishers Pradnjaparamita II, Jakarta, Indonesia)*

According to Tan[23] the unit cell structure of bentonite is considered symmetric, with aluminium octahedral sheet sandwiched between two silica tetrahedral sheets (Fig. 8). The bonds holding the layers together are relatively weak, developing intermicellar spaces that will expand with increasing moisture content. In contact with water, the mineral exhibits interlayer swelling causing uniform increase in the volume of the basal spacing of bentonite with adsorption of water.

Under the conditions of the present experiment (pH 4.0, 6.0, 7.0, 8.0, temperature 25°C) calcium saturation of bentonite and low concentrations of phosphate dissolution of bentonite was probably very small. It was therefore unlikely that observed phosphate retention was caused by precipitation to form separate-phase phosphate crystals as proposed by Kittrick and Jackson[24]. The idea that phosphate was adsorbed by exchange of hydroxyl groups of the crystal lattices as proposed by Muljadi et al.,[25] and Kafkafi et al.,[26] was not favored by the present authors for the following reasons: First in this study the phosphate adsorption by bentonite did not significantly increase the pH. The adsorption of 1ppm P ($3.2x10^{-5}$ moles L^{-1}) by replacement of OH^- ions would increase the OH^- ion concentration in solution with $3.2x10^{-5}$ moles L^{-1}. At initial pH values of 5.0 to 7.0 this would mean a dramatic increase in pH.

Secondly the phosphate adsorption increases with decreasing pH[25], i.e. with increasing number of edge H_2O groups. The present authors therefore favor the idea[19] that phosphate ions are adsorbed by replacing H_2O groups, rather than hydroxyl groups. Kafkafi et al.,[26] suggested that the bond between the phosphate and the surface is at least partly covalent in that there is a sharing of a proton with the surface leaving a small additional negative charge on the $H_2PO_4^-$ grouping. It was suggested that the link of the phosphate with the surface is in effect a surface neutralization reaction.

Since the electronegative values of Al, H and O are 1.5, 2.1 and 3.5 respectively the present authors suggest that a covalent bond is formed between Al of the surface and O of the phosphate ion rather than between Al and H. In order to explain the increase in negative charge as a result of phosphate adsorption[27,28] it is proposed that phosphate ions replace coordinated H_2O groups and/or coordinated anions to form an outer octahedral complex of aluminum phosphate.

The proposed adsorption model is compatible with the observed relation between pH of clay suspensions and phosphate adsorption. It is also explained the increase in negative charge of the clay as a result of phosphate adsorption. The proposed second order kinetic equation is applicable only on adsorption processes which follow the Langmuir isotherm or not depend mainly on the nature and specific surface area of the adsorbent, the nature and concentration of the adsorbate, and the adsorption mechanism while the present work shows that phosphate adsorption by Ca-bentonite at low concentrations of P follows the Langmuir isotherm.

Περίληψη

Κινητική αντιδράσεων φωσφόρου με τον Ασβεστωμένο μπεντονίτη

Η προσθήκη του P στο έδαφος με χημική λίπανση , κοπριά ή αστικά απόβλήτα και η μεταφορά αυτού από την επιφάνεια ή μη του εδάφους και από τα διαβρωμένα εδάφη στα επιφανειακά νερά ή τα νερά διαφόρων πηγών αποτελεί έναν από τους μεγαλύτερους κινδύνους μόλυνσης του περιβάλλοντος.

Για το λόγο αυτό η Ε.Ε. προσπαθεί να ρυθμίσει νομοθετικά το θέμα αυτό. Επομένως η μελέτη της προσρόφησης του P από εδάφη και εδαφικά συστατικά στηριζόμενη σε μοντέλα ισορροπίας και κινητικής θα είναι ενδιαφέρουσα.

Η παρούσα εργασία σκοπό είχε να προσδιορίσει την προσρόφηση του φωσφόρου από τον ασβεστωμένο μπεντονίτη σε συνάρτηση με το χρόνο, την αρχική συγκέντρωση και το pH. Από τη μελέτη αυτή προέκυψε ότι:

1. Η προσρόφηση του P από τον ασβεστωμένο μπεντονίτη για χαμηλές συγκεντρώσεις ακολουθεί τις ισόθερμες προσρόφησης της Langmuir και αυτό αποτελεί ένδειξη ότι στην επιφάνεια του Ca-b σχηματίζεται ένα μονομοριακό στρώμα φωσφορικών. 2. Η μέγιστη προσρόφηση των φωσφορικών στα διάφορα pH (4.0, 6.0, 7.0, 8.0) είναι 807.9 , 547.7, 521.1 και 383.5 αντίστοιχα. 3. Η μελέτη της κινητικής ακολουθεί εξίσωση δευτέρου βαθμού που λαμβάνει υπόψη και την αλλαγή της συγκέντρωσης των φωσφορικών στα διάλυμα και τον κορεσμό της επιφάνειας του Ca-b κατά την προσρόφηση. 4. Οι σταθερές προσρόφησης (K_1) και εκρόφησης (K_{-1}) αυξάνονται με την αύξηση της συγκέντρωσης στα pH 4.0, 6.0 και 8.0 ενώ μειώνονται με την αύξηση της συγκέντρωσης στο pH 7.0. 5. Η σταθερά ισορροπίας (K) και η μέγιστη προσρόφηση (Xm) μειώνονται με την αύξηση του pH.

Η όλη μελέτη έγινε με την προυπόθεση ότι τα φωσφορικά ιόντα προσροφώνται με την αντικατάσταση μορίων νερού ή ανιόντων

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The Use of Diketones and Stabilized Phosphoranes in the Synthesis of Unsaturated Ketoesters and Diketones.

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Summary

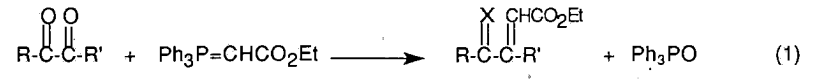
Wittig reactions of a variety of symmetrical and unsymmetrical diketones with the stabilized phosphoranes Ph_3P=CHCOR, where R=OEt (**1**), Me (**2**), and Ph (**3**), produced unsaturated ketoesters and diketones in 37-81% yields. Both *E* and *Z* isomers of the 1:1 Wittig adducts were formed; unsymmetrical diketones reacted faster at the less sterically hindered carbonyl function. Generally no isomerization of the newly created double bonds was observed in the examined cases and even highly enolizable ketones could be forced to react.

Introduction

Diketones are fairly readily available starting materials as several methods are known for the preparation of α–, β– and γ-diketones[1]. Similarly ethoxycarbonylmethylene-triphenylphosphorane (**1**) is readily available[2] and has been widely used in Wittig reactions. Acetylmethylenetriphenylphosphorane (**2**) and benzoylmethylenetriphenyl-phosphorane (**3**) have been previously used to synthesize 4-oxo-2-pentenoates from α-oxoesters[3]. Nicolaides and Litinas investigated the reactions of PhCOCOR where R=Ph, Me or H with the ylides Ph_3P=C(R)CO_2R' and found, for example, that benzil reacted with only one molecule of each ylide used to give isomeric 4-oxo-α,β-unsaturated esters.[4]

Results and Discussion

The present study aims at assessing the scope and limitations of the reaction of the Wittig reagents **1-3** with diketones to yield unsaturated carbonyl compounds. The first type of reaction studied is shown in equation 1.



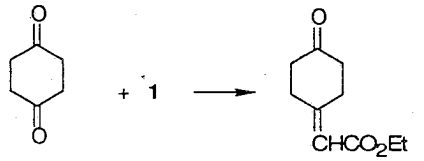
4; R=R'=Me (1)
5; R=R'=Et
6; R=Et, R'=Me
7; R=n-Pr, R'=Me
8; R=n-Bu, R'=Me

14; R=R'=Me, X=O
15; R=R'=Et, X=O
16; R=Et, R'=Me, X=O
17; R=Et, R'=Me, X=$CHCO_2Et$
18; R=n-Pr, R'=Me, X=O
20; R=n-Bu, R'=Me, X=O

It was found that where R'= Me ≠ R, there was complete selectivity for the reaction shown and the alternative product RC(=CHCOOEt)COMe was generally not formed. However, with diketone **6** the yield of the ketoester **16** was decreased because of the formation of the diester **17**.

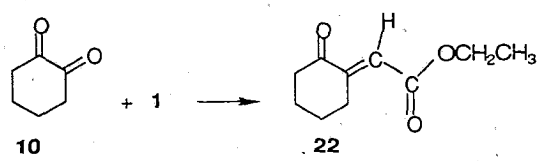
The cyclohexanediones were then investigated as starting materials. 1,3-Cyclohexanedione failed to yield an isolatable quantity of product. Reacton of the ylide **1** with this diketone, which readily enolizes, was very slow. This could be because the enolic hydrogen of 1,3-cyclohexanedione is more acidic than that of acyclic β-diketones and could possibly convert some of the ylide to the unreactive protonated form, thus slowing the reaction down. When the mixture obtained after removal of $Ph_3P=O$ was concentrated, it darkened on standing at room temperature.

Unusually 1,4-cyclohexanedione (**9**) reacted faster, and gave a better yield with **1**, than the 1,2-cyclohexanedione.



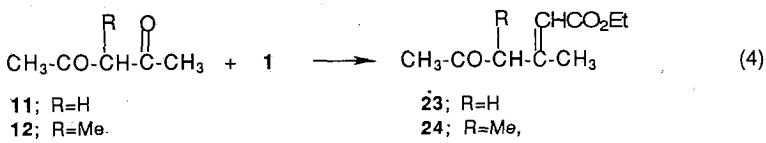
9 **21** (2)

In the case of 1,2-cyclohexanedione (**10**) the major product formed was the *E* isomer **22**, but the ^1H NMR of the crude product indicated the presence of small amounts of other isomers.



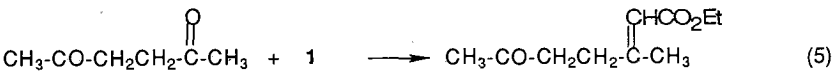
10 22 (3)

Reaction of **1** with other γ–diketones and with β-diketones was much slower than with the α-diketones but yielded the expected products.



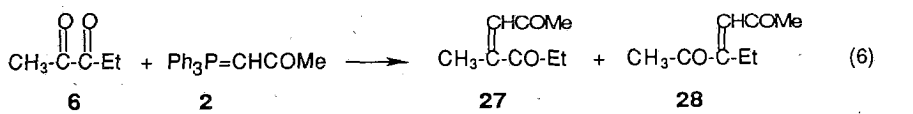
11; R=H
12; R=Me

23; R=H
24; R=Me, (4)

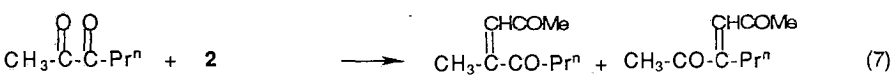


13 **25** (5)

When acetylmethylene triphenylphosphorane (**2**) was used, it was less selective towards unsymmetrical diketones than was **1**. For example the reactions depicted in equations 6 and 7 occurred. In both reactions the major products **27** and **29** respectively, resulted from nucleophilic attack at the less hindered carbonyl function.



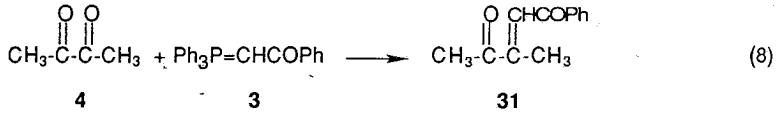
6 **2** **27** **28** (6)



7 **29** **30** (7)

These products darkened on exposure to air and were therefore characterized as their corresponding 2,4-dinitrophenylhydrazones. For reaction 6 the 2,4-dinitrophenylhydrazone of **27** was the major product and for reaction 7 that of **29** was the major product. Obviously however, one cannot deduce exact ratios of **27:28** or of **29:30** from the ratios of precipitated derivatives.

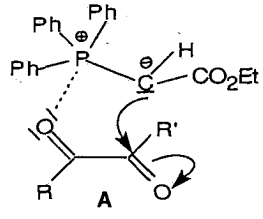
Benzoylmethylenetriphenylphos-phorane (**3**) failed to react with benzil, but reacted with diacetyl to give mainly (*E*)-3-methyl-1-phenyl–pent-2-ene-1,4-dione **31** (equation 8). Further reations of **3** are planned.



(8)

In the reactions studied the relative reactivities of the examined Wittig reagents towards a given diketone were **1>2>3**. In general, the α-diketones reacted with Wittig reagent **1** faster than did the β- and γ–diketones, which had to be used in excess to drive the reactions to completion. However, as exceptions to these generalizations, 1,4-cyclohexanedione reacted slightly faster than 3,4-hexanedione, and 1,2-cyclohexanedione, although much more reactive than β– and γ-diketones, was by far the least reactive of the α-diketones studied. Clearly the enhanced reactivity generally exhibited by α-diketones is not purely the result of the inductive effect which increases the susceptibility of the carbonyl function to attack by the nucleophilic Wittig reagent, as this would not explain all the above observations. Possibly, coordination intermediates of the type shown below (**A**) are easily achieved sterically for most α-diketones but not for 1,2-cyclohexanedione.

IR studies of 1,4-cyclohexanedione indicate that it may exist to the extent of 20% in the twist boat conformation.[5] It is therefore possible to suggest that a twist boat conformation may be attained in coordination structure, shown as **B** below, during the Wittig reaction.



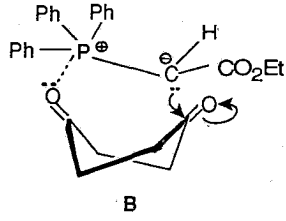


Table 1 summarizes the experimental results, including the *Z* : *E* ratios where these were determined. However, products from Wittig reagents **2** and **3** were not

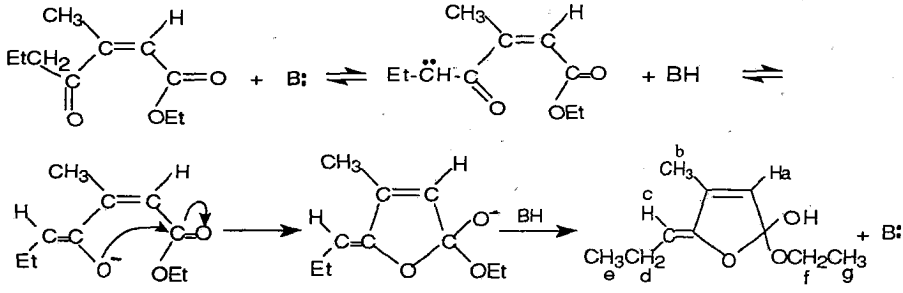
particularly stable, generally darkening was observed within one hour at room temperature, and their *Z* : *E* ratios were not determined. ^{1}H NMR spectra of the products were used to determine *Z* : *E* ratios. The δ values for vinyl, and for allylic methyl hydrogen atoms were calculated and the *Z* and *E* isomers were assigned on the basis of lower δ values for *Z* than for *E* isomers in each case. In later work we observed that, in GLC analysis of isomers, *E* isomers invariably had lower retention times than correspoding *Z* isomers.

Differentiation between MeCOC(=CHCOOEt)R and RCOC(=CHCOOEt)Me (R= Et, *n*-Pr and n-Bu) was easily achieved by use of the iodoform test. The unsaturated diketones gave the colour reactions (see experimental) previously noted in the literature. [6]

Two interfering reactions were observed, namely reaction of the initially formed product with a second molecule of the Wittig reagent, and cyclization. The first reaction was evident in many cases, but the resulting product was formed in sufficiently large amounts for isolation in only one case (entry 3 in table 1). This is in agreement with previous work in which the compounds formed from reaction of only one carbonyl group of a dicarbonyl compound with a Wittig reagent were readily isolatable. Thus, benzocyclobutenedione reacts with one molecule of $Ph_3P{=}CHCO_2Me$ to give the corresponding 4-oxo unsaturated ester in 93% yield, whereas 2 molecules of this ylide gave an 85% yield of the diester.[7]

In our present work the diester **17** EtC(=CHCO$_2$Et)C(=CHCO$_2$Et)Me is obviously a mixture since the *Z* : *E* ratio is 58 : 42. Four isomers *ZZ, EE, 2Z 4E,* and *2E 4Z* are possible, but we were unable to separate isomers or to determine exactly which ones were present. It is interesting to note that in the same case the *E* isomer of the 1:1 adduct was formed exclusively indicating that either the *Z* isomer was not formed at all, which is highly unlikely considering similar reactions, or that the latter reacted much faster than the *E* isomer with a second molecule of the reagent. Possibly a coordination structure, involving ester group participation, and simillar to those shown in structures **A** and **B**, could have been formed from the *Z* isomer.

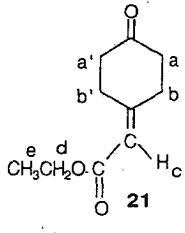
Cyclizaton of the initially formed adducts might be expected under the basic conditions of the reactions, but in fact was only observed in one case (entry 4 in table 1), and must have occurred during isolation of products since the initial distillate obtained after the reaction of diketone **7** with ylide **1**, showed no sign of OH absorption in its IR spectrum. Cyclization to give isomer **19** could have been brought about during the isolation procedure by a small amount of base. A possible mechanism is as follows:



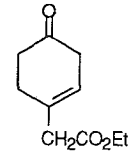
Z-**19**

Obivously the *E* isomer of **19** could readily be formed in the same way. Although **20** (entry 5 in table 1) would be expected to behave in a similar way we did not observe this in our experiments. This may be because no trace of base was present during the isolation procedure.

Double bond migration has been previously observed for exocyclic ketones in the presences of bases,[8] but does not appear to have occurred with the exocyclic ester **21** in entry 6 of table 1.

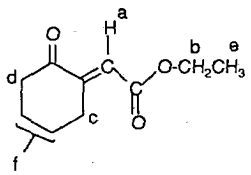


21

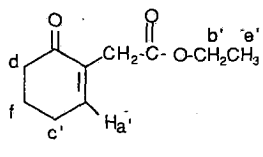


21a (not formed)

However, reaction of 1,2-cyclohexanedione **10** with phosphorane **1** gave the *E* isomer **22** containing an impurity, possibly **22a**.



22 (*E* isomer)



22a

In conclusion diketones can be used to prepare unsaturated ketoesters and unsaturated 1,4-diketones by the use of Wittig reagents. Whereas in some other syntheses predominately *E* isomers were obtained[9], this was not necessarily the case in this work. Cis or *Z* selectivity has been previously reported, but mainly of reactive ylides.[10]

Experimental Section

General ^{1}H NMR spectra were recorded on either 60MHz or 80MHz spectrometers using TMS as an internal standard and $CDCl_3$ as solvent. IR spectra were recorded on Perkin Elmer 157 or 1430 or SP3-200 instruments. Melting points are uncorrected. TLC were run on Merck plates coated with Kieselgel 60 and visualization was performed by iodine, acidified permanganate or dilute 2,4-dinitrophenylhydrazine solution (2% in acidified MeOH). Unless otherwise stated, the solvent used for the development of TLC was diethyl ether (DE) / petroleum ether (PE) 30-40° (1 : 3). All reactions were carried out under anhydrous conditions and under an atmosphere of dry nitrogen. Solvent was removed from reaction mixtures using a rotary evaporator at 40° or less. Reactions were monitored by TLC and by titration of unreacted Wittig reagents. The phosphoranes used in the work were prepared by the normal procedures,[11] and the % purity of each was determined by titration. Other chemicals were obtained from commercial sources and were thoroughly dried according to the standard procedures. In general the reactions were carried out by dissolving the phosphorane in a solvent, benzene or dichloromethane (DCM), and then adding the diketone solution to it. The resulting solution was then heated under reflux until the phosphorane was consumed. The solvent was evaporated, PE was added and the precipitated triphenylphosphine oxide was filtered yielding the crude ester.

A. Reactions of ethoxycarbonylmethylenetriphenylphosphorane (1)

1 With 2,3-butanedione (4) to give ethyl 3-methyl-4-oxo-2-pentenoate (14)

Phosphorane **1** (1.777 g, 5.1 mmol) dissolved in benzene (20 ml) was added to a solution of diacetyl **4** (0.678 g, 7.88 mmol) in benzene (5 ml). After heating the resulting solution under reflux for 2.5 hours, 93.5% of **1** had been consumed. After leaving overnight at room temperature (rt), solvent and unreacted **4** were evaporated, PE was added, and the precipitated triphenylphosphine oxide (TPPO) (m.p. 149°C, m.m.p. 149-151°C, 92%) was filtered off. The filtrate yielded more precipitate (ppt) on standing at rt and it was discarded. Evaporation of the solvent yielded ester **14** as an oil (0.50 g, 66%) Ester **14**, obtained as mixture of *E* and *Z* isomers had n_D^{23} 1.4403, lit.[12] n_D^{20} 1.445 , R_f 0.55 and 0.64, R_f ($CHCl_3$ / MeOH 17 : 2) 0.60 and 0.70. $C_8H_{12}O_3$ (156.184) requires C, 61.52; H, 7.74. Found C, 61.54; H, 7.54. IR (neat): ν 1724, 1695. and 1640 cm^{-1}. ^{1}H-NMR (60 MHz) δ 1.27 and 1.37 (two t, *J* 7 Hz, 3H, *Z* and *E* $OCH_2\underline{CH_3}$ respectively), 1.97 (d, *J* 1 Hz, 1.41 H, *Z* $=C-CH_3$), 2.17 (d *J* 1 Hz, 1.59 H, *E* $=C-CH_3$), 2.33 and 2.37 (two s, 3 H, CH_3CO), 4.12 and 4.23 (two q, *J* 7 Hz, 2H, *Z* and *E* $O\underline{CH_2}CH_3$ respectively), 5.68 - 5.71, (m, 0.46H, *Z* =C-H), 6.55 - 6.65 (m, 0.54 H, *E* =C-H). The *Z* : *E* ratio was 47 : 53. Ester **14** yielded a 2,4-dinitrophenylhydrazone as yellow crystalline needles, m.p. 202-3°C dec., lit.[12] m.p. 202-202.5° C R_f ($CHCl_3$ / MeOH 17 : 2) O.81. IR (nujol): ν 1712-1690, 1190 and 1104 cm^{-1}.

2 With 3,4-hexanedione (5) to give ethyl 3-ethyl-4-oxo-2-hexenoate (15)

Phosphorane **1** (5.06 g, 14.52 mmol) was dissolved in DCM (25 ml) and diketone **5** (1.84 ml, 15.3 mmol) was added to it. The resulting solution was heated under reflux until the reaction was complete. Work up as for **14** followed by distillation, yielded ester **15** as a mixture of the *E* and *Z* isomers (2.16 g, 81% yield). Ester **15** had a b.p 224-5°C and 54-55°C/0.1 mm Hg, $n_D^{32.5}$ 1.4505, R_f 0.38 and 0.53. $C_{10}H_{16}O_3$ (184.238) requires: C, 65.19; H, 8.75. Found: C, 64.76; H, 8.66. IR (neat): ν 1720, 1700-1690, 1640, 1230, and 1140 cm^{-1}. ^{1}H-NMR (80 MHz): δ 1.116, 1.150 and 1.256 (three t, *J* 7.3,

7.5 and 7.0 Hz respectively, 9H, $CH_2C\underline{H}_3$), 2.27-2.67 (br. q., 3H, *Z* =C-CH_2 and CH_2-CO), 2.77 - 2.92 (m, 1H, *E* =C-CH_2 and CH_2-CO), 4.0-4.4 (two q, *J* 7.1 Hz, 2H, OCH_2), 5.67 (br. s, 0.76 H, *Z* =C-H,), 6.47 (s, 0.24 H, *E* =C-H). The *Z* : *E* ratio was 76 : 24. Ester **15** yielded a 2,4-dinitrophenylhydrazone as mustard yellow crystals with m.p. 132 - 133°C, R_f ($CHCl_3$ / MeOH 8 : 1) 0.83. $C_{16}H_{20}N_4O_6$ (364.363) requires: C, 52.74; H, 5.53; N, 15.38. Found: C, 52.47; H, 5.51; N, 15.33. IR (nujol): ν 3320 (w), 1720, 1620, 1600 and 1505 cm^{-1}.

3 With 2,3-pentanedione (6) to give ethyl (*E*)-3-methyl-4-oxo 2-hexenoate (16) and diethyl 4-ethyl-3-methyl-2,4-hexadiene-1,6-dicarboxylate (17)

Phosphorane **1** (8.21 g, 23.6 mmol) was dissolved in DCM (25 ml) and diketone **6** (3.3 ml, 32 mmol) was added. The resulting solution was heated under reflux until **1** was completely consumed. Unreacted **6** could not be removed efficiently by periodate oxidation, whereas distillation resulted in some decomposition. Therefore, after initial removal of most of the TPPO, the crude product was chromatographed on a silica gel column (height: 52 cm, diameter: 2 cm), using PE/DE (3 : 1) as eluant to give ester **16**, giving (1.45 g, 36% yield) and diester **17** (0.71 g, 25% yield). Ester **16**, which was obtained as an oil, gave a negative iodoform test. $C_9H_{14}O_3$ (170.211) requires: C ,63.51; H, 8.29. Found: C , 64.21; H, 8.38. IR (neat): ν 1725, 1690 and 1630 cm^{-1}. ^{1}H-NMR (80 MHz) δ 1.22 (t, *J* 7.3 Hz, 3H, $C\underline{H}_3CH_2CO$), 1.32 (t, *J* 7 Hz, 3H, $C\underline{H}_3CH_2O$), 2.25 (d, *J* 1.4 Hz, 3H, =C-CH_3), 2.74 (q, *J* 7.3 Hz, 2H, $CH_3C\underline{H}_2CO$), 4.25 (q, *J* 7.0 Hz, 2H $CH_3C\underline{H}_2O$), 6.56 (br. s, 1H, =C-H). Ester **16** formed a crystalline orange ppt with 2,4-dinitrophenylhydrazine with m.p. 127-130°C. $C_{15}H_{18}N_4O_6$ (350.336) requires C ,51.43; H, 5.176; N, 15.99. Found: C, 51.16 ; H, 5.18; N, 15.8. IR (nujol): ν 3305, 1710, 1620 and1595 cm^{-1}. Diester **17** was also obtained as an oil. $C_{13}H_{20}O_4$ (240.303) requires: C, 64.98; H, 8.39. Found: C, 64.83; H, 8.46. This product gave no colour on the TLC plate with the 2,4-dinitrophenylhydrazine solution nor did it yield a ppt with this reagent. IR (neat): ν 1715, 1705 and 1640 cm^{-1}. ^{1}H-NMR (80 MHz) δ 1.03 -1.36 (overlapping triplets, *J* 7 Hz, 9H, $CH_2C\underline{H}_3$), 1.98 (d, *J* 1.4 Hz, *Z* =C-$C\underline{H}_3$), 2.35

(s, 3H, *E* =C-C<u>H</u>$_3$, overlapping with a multiplet due to protons CH_2-C=C- at 2.78-2.5, probably *Z*), 2.63 (q, *J* 7.3 Hz, probably *E* CH_2-C=), 4.15 and 4.17 (two q, *J* 7.0 Hz, 4H, *E* and *Z* O-CH_2), 5.63-5.73 (br. s with fine structure, 2H, *E* and *Z* =C-H).

4 <u>With 2,3-hexanedione (7) to give ethyl (*E*)-and (*Z*)-3-methyl-4-oxo 2-heptenoate (18)</u>

Phosphorane **1** (7.90 g, 23 mmol) was dissolved in DCM (33 ml) and diketone **7** (2.92 ml, 24 mmol) was added to it. The resulting mixture was refluxed until **1** had disappeared. Work up in the usual way yielded a mixture (3.7 g) of four components, by TLC. Distillation at 80-85°C/0.3 to 0.4 mm Hg failed to separate the components. The distillate gave a negative iodoform test. Its IR spectrum had bands at ν = 1725, 1690, 1640, 1220, 1190 and 1160 cm^{-1} and it showed no OH absorption. A portion (1.67 g). of the distillate was chromatographed on a silica gel column as in experiment No.3. Elution removed 54% of the material from the column giving an overall yield of 42% of the three isolated products. The major products formed, which were isolated as an inseparable mixture were : *Z*- **18** and possibly **19.** The mixture of these two products had the following properties: R_f (DE / PE, 1:3) 0.49-0.58. $C_{10}H_{16}O_3$ (184.238) requires: C, 65.19; H, 8.75. Found: C , 65.12; H, 8.66. IR (neat): ν 3400 (br), 1720, 1680 and 1640-1635 cm^{-1}. ^{1}H-NMR (80 MHz) δ 0.96 (t, *J* 6.8 Hz, 3H, of H-e and C<u>H</u>$_3$$CH_2CH_2$), 1.26 -1.27 (two t, *J* 7 .0 and 7.2 Hz, 3H, C<u>H</u>$_3$$CH_2O$), 1.47-1.75 (m, 2H, CH_3C<u>H</u>$_2$$CH_2$), 1.98 (d, *J* 2 Hz, 3H, *Z* =C-CH_3), 2.27 (m, 2H, H-d), 2.36 (s, 3H, H-b), 2.61 (t, *J* 7.0 Hz, C<u>H</u>$_2$CO-), 3.28 (s, 1H, OH), 4.17 (br. q, *J* 7.0 Hz, 2H, H-f), 5.65-5.70 (unresolved broad signals with fine structure, =C-H and H-a of **19**), 5.90 and 6.15, (slightly broadened singlets, H-c of *E* and *Z* isomers). From the integration of signals at δ = 3.28 and 4.17, which had relative intensities of 1 :4.1 respectively, the ratio of **19** to **18** appears to be approximately 49 to 51. Isomer *E* -**18** was also isolated and had the following properties: R_f (DE / PE, 1:3) 0.365. $C_{10}H_{16}O_3$ (184.238) requires: C ,65.19; H, 8.75. Found: C, 65.05; H, 8.69. ^{1}H-NMR

(80 MHz) δ 0.95 (t, *J* 7.0 Hz, 3H, $C\underline{H_3}CH_2CH_2$), 1.32 (t, *J* 7.0 Hz, 3H, $C\underline{H_3}CH_2O$-), 1.66 (m, 2H, $CH_3C\underline{H_2}CH_2$-), 2.22 (d, *J* 1.5Hz, 3H, =C-CH_3), 2.60 - 2.82 (br. t, *J* 7.0 Hz, 2H, CH_2-CO), 4.24 (q, *J* 7.0 Hz, 2H, OCH_2) : 6.54 (poorly resolved q, *J* 1.5 Hz, 1H, =C-H). The mixture of *E* and *Z* esters **18** yielded a 2,4-dinitrophenylhydrazone as a yellow solid with m.p. 120-122°C. IR (nujol): ν 3300, 3120, 3080, 1710, 1620, 1600, 1500, 850 and 840 cm^{-1}.

5 With 2,3-heptanedione (**8**) to give ethyl (*E*)- and (*Z*)-3-methyl-4-oxo-2-octenoate (**20**)

Phosphorane **1** (2.198 g, 6.31 mmol) in DCM (20 ml) was added to a solution of diketone **8** (0.845 g, 6.59 mmol) also in DCM (5 ml) and the mixture was heated under reflux until **1** had disappeared. Work up in the usual way followed by distillation, yielded *E* -and *Z* -**20** (0.71 g, 60% yield), b.p. 144°C/19 mm Hg, n_D^{21}= 1.4620, R_f 0.44 and 0.54. The product gave a negative iodoform test. $C_{11}H_{18}O_3$ (198.265) requires: C, 66.64; H, 9.15. Found: C, 67.03; H, 8.92. IR (neat): ν 1730-1700, 1685,1642, 1290 and 1190-1150 cm^{-1}. ^{1}H-NMR (80 MHz) δ 0.85 - 1.4 (m, 10 H, $C\underline{H_3}CH_2O$ and $C\underline{H_3}C\underline{H_2}C\underline{H_2}$), 1.92 (d, *J* ≈ 1 Hz, 1.23H, *Z* =C-CH_3), 2.17 (d, *J* ≈ 1Hz, 1.77 H, *E* =C-CH_3), 2.36 (two t, *J* ≈ 6 Hz, 2 H, CH_2-CO), 4.08 and 4.19 (two q, *J* ≈ 7 Hz, 2H, *Z* and *E* OCH_2), 5.65 (m, *J* ≈ 1 Hz, 0.36 H, *Z* =C-H) : 6.48 (q., *J* ≈ 1 Hz, 0.64 H, *E* =C-H). The *Z* : *E* ratio was about 36 : 64. On treatment with 2,4-dinitrophenylhydrazine and allowing the mixture to stand these esters yielded the corresponding hydrazones as long yellow needles, m.p. 145-146°C. $C_{17}H_{22}N_4O_6$ (378.39) requires: C, 53.96; H, 5.861; N, 14.81. Found: C, 53.71; H, 6.17; N, 14.74.

6 With 1,4-cyclohexanedione (**9**) to give 4-ethoxycarbonylmethylidene-cyclohexanone (**21**)

Phosphorane **1** (8.28 g, 23.8 mmol) was dissolved in DCM (38. ml) and diketone **9** (2.93g, 26.1 mmol) was added. The resulting mixture was heated under reflux until **1** had been consumed. TPPO was removed by work up in the usual way and the refrigerated petroleum ether solution yielded white crystals of ester **21** (2.81 g, 65% yield), m.p. 54-56°C, R_f ($CHCl_3$ / MeOH 20 : 1) 0.69.

$C_{10}H_{14}O_3$ (182.222) requires C, 65.91; H, 7.74. Found C ,66.13; H, 7.71. IR (nujol): ν 1725 and 1650 cm^{-1}. ^{1}H-NMR (80 MHz) δ 1.29 (t, *J* 7 Hz, 3H, H-e), 2.40 - 2.72 (m, 6 H, a, á, b), 3.11-3.29 (br. distorted t, 2 H, H-b'), 4.17 (q, *J* 7 Hz, 2H, H-d), 5.84 (br. s, 1H, H-c). The product gave a 2,4-dinitrophenylhydrazone as a bright yellow crystalline solid, m.p. 195-9°C, R_f ($CHCl_3$) 0.46. $C_{16}H_{18}N_4O_6$ (362.35) requires C, 53.04; H, 5.007; N, 15.46. Found: C ,52.84; H, 5.08; N, 15.45. IR (nujol): ν 3325, 1705,1655, 1620 and 1595 cm^{-1}

7 With 1,2-cyclohexanedione (**10**) to give (*E*)-2-ethoxycarbonylmethylidene-cyclohexanone (**22**)

Phosphorane **1** (7.32 g, 21 mmol) was dissolved in DCM (20 ml) and a solution of diketone **10** (2.47g, 22 mmol), also in DCM (40 ml), was added to it. The resulting mixture was heated under reflux until **1** disappeared. Work up in the usual manner gave crude ester **22** (1.49, 37% yield) containing some impurities, possibly the isomeric ester **22a**. Crude ester **22** had b.p. 218-220°C or 78-84°C/0.2 mm Hg or 95-100°C/0.3 mm Hg, n_D^{22} = 1.4865. Lit.[13] bp of ester **22** (isomers not stated) 134°C/14 mm Hg, of pure *Z* isomer of **22** only 82-85°C/0.05 mm Hg[14], of pure the *E* isomer of **22** 66-67°C/0.02 mm Hg[14], and of a mixture of **22** and **22a** 80-90°/0.5 mm[15], lit.[15] n_D^{20} for **22**: 1.4923 and for a mixture of **22** and **22a**: 1.477 -1.487. IR (neat): ν 1720, 1690 and 1625 cm^{-1}. ^{1}H-NMR (80 MHz) δ 1.21 - 1.39 (overlapping triplets, *J* ≈ 7 Hz, 3H, H-e), 1.82 - 1.90 (m, 4H, H-f), 2.54 (t, *J* 6.6Hz, 2H, H-d), 3.18 - 3.08 (m, 2H, H-c), 4.21 (br q, *J* 7 Hz, 2H, H-b), 6.47 (t, *J* ≈ 2 Hz, 1H, H-a); impurity peaks at 6.89 (broad signal) and 3.35 (singlet) due to isomers. The assignment of ester **22** as *E* isomer was based mainly on the observed *J* value of H-a being about 2 and the multiplicity of the corresponding signal being a triplet. The 2,4-dinitrophenylhydrazone was a yellow-orange solid with m.p. 95-97°C. IR (nujol): ν 3320, 1710, 1620 and 1595 cm^{-1}.

8 With 2,4-pentanedione (**11**) to give ethyl 3-methyl 5-oxo 2-hexenoate (**23**)

Because a 1 : 1 molar ratio of ylide **1** to diketone **11** reacted very slowly, a 1 : 15 molar ratio was used. Thus, phosphorane **1** (4.422 g, 12.7 mmol) in DCM (40 ml) was added to diketone **11** (19.07 g, 190.5 mmol) and the resulting mixture was heated under reflux for one day, when **1** had disappeared. The mixture was extracted with 5% aqueous NaOH solution until the acidified extract gave a negative $FeCl_3$ test. The organic layer was then washed with water and dried. Work up in the usual manner yielded ester **23** (1.06 g, 51% yield), b.p. 120°C/20 mm Hg, lit.[16] b.p. 72°C/2 mm Hg, $n_D^{21.6}$ = 1.4580, lit.[16] $n_D^{21.6}$ = 1.4573. $C_9H_{14}O_3$ (170.211) requires: C, 63.51; H, 8.29. Found: C, 64.09; H, 8.22. IR (neat): ν 1740-1700, 1645, 1625, 1255 and 1190-1140 cm^{-1}. ^{1}H-NMR (60 MHz) δ 1.24 (t, *J* 7 Hz, 3H, $OCH_2C\underline{H}_3$), 1.92 (br. s, *Z* =C-CH_3), 2.18 (br.s, *E* =C-CH_3 and CH_3CO), 3.10 -3.80 (4 peaks, 2H, *Z* and *E* $C\underline{H}_2CO$), 4.10 and 4.14 (two q, *J* 7Hz, 2H, *Z* and *E* $OC\underline{H}_2$-), 5.87 (br s, *Z* =C- H), 6.17 (br. s, *E* =C-H). The *Z* : *E* ratio as obtained from the vinyl H peak ratios, was 7 : 3

9 With 3-methyl-2,4-pentanedione (**12**) to give ethyl 3,4-dimethyl-5-oxo-2-hexenoate (**24**)

Phosphorane **1** (2.258 g, 6.48 mmol) in DCM (20 ml) was added to diketone **12** (4.571 g, 40 mmol). Reaction and work up, as in the previous procedure yielded, on distillation (a) a colourless oil, probably impure diketone **12** (0.19 g) b.p. 68-70°C/20 mm Hg, lit.[17] b.p. of **12** 86°C/60 mm Hg, n_D^{24} = 1.4324, lit.[17] n_D^{20} = 1.4437 and (b) ester **24** (0.53 g, 49% yield), b.p. 128°C/20 mm Hg, n_D^{24} = 1.4453. $C_{10}H_{16}O_3$ (184.238) requires: C, 65.2; H, 8.75. Found: C ,65.10; H, 8.82. IR (CCl_4): ν 1730-1700, 1690 sh., 1640, 1590, 1220 and 1170 - 1150 cm^{-1}. ^{1}H-NMR (CCl_4, 60 MHz) δ 1.0-1.3 (overlapping t and d, 6 H, $C\underline{H}_3CH_2O$ and $C\underline{H}_3CH$), ~ 1.8 (br. s, *Z* CH_3C=C), 1.92-2.1 (overlapping s, *E* CH_3C=C and *E* and *Z* CH_3CO), 3.20-3.80 (overlaping q, 1H, *E* and *Z* $CH_3C\underline{H}$), 3.97 (q, *J* 7 Hz, *Z* OCH_2), 4.00 (q, *J* 7 Hz, *E* OCH_2), 5.29 (br s, *Z* =C-H), 5.68 (br s, *E* =C-H). The *Z* : *E* ratio, as obtained from the integrals of the vinyl H

signals, was about 40 : 60. Ester **24** yielded a 2,4-dinitrophenylhydrazone as brown-yellow needles with m.p. 200-202°C.

10 With 2,5-hexanedione (**13**) to give ethyl 3-methyl-6 oxo-2-heptenoate (**25**)

Phosphorane **1** (1.828 g, 5.25 mmol) in DCM (20.0 ml) was added to diketone **13** (10.15 g, 88.6 mmol) and the mixture was heated under reflux until 95% of **1** had been consumed. Work up in the usual way followed by distillation yielded ester **25** (0.36g, 42% yield) b.p. 130°C /17 mm Hg, lit.[18] b.p. 113-120°C/5 mm Hg and 94°C/1 mm Hg, n_D^{22}= 1.4640, lit.[18] n_D^{20}=1.4648. $C_{10}H_{16}O_3$ (184.238) requires: C, 65.2; H, 8.75. Found: C, 65.1; H, 8.87. This ester yielded orange needles of its 2,4-dinitrophenylhydrazone from ethanol, m.p. 119- 121°C, lit.[18] m.p. (from n-octane) 121-122°C, R_f (ethyl acetate) 0.70. $C_{16}H_{20}N_4O_6$ (364.36) requires: C, 52.7; H, 5.53; N, 15.38. Found: C, 52.8; H, 5.68; N, 15.53.

B Reactions of acetylmethylenetriphenylphosphorane (**2**)

1 With 3,4-hexanedione (**5**) to give 4-ethyl 3-heptene 2,5-dione (**26**)

Phosphorane **2** (3.8 g, 12 mmol) was dissolved in DCM (25 ml) and diketone **5** (1.53 ml, 12.6 mmol) was added to it. Heating under reflux, until 92% of **2** had been consumed, and subsequent work up, yielded **26** (1.3 g, 70% yield) as a pale yellow liquid. TLC of **26** using DE/PE (2 : 3) showed 2 spots with R_f 0.3 and 0.38, which darkened on standing. Diketone **26** gave positive iodoform and Br_2 in CCl_4 test. In the latter case, the colour was first discharged and then went green with a small quantity of Br_2, but turned violet and then dark red with excess reagent. Diketone **26** also gave a red colour with an alkaline sodium nitroprusside solution which changed within a few minutes to green upon acidification; other unsaturated 1,4-diones behave similarly.[6] Preparation of a 2,4-dinitrophenylhydrazone using excess reagent yielded the bis derivative of **26** as a soft red powder, m.p. 228-230°C, R_f ($CHCl_3$/MeOH, 8 : 1) 0.89. $C_{21}H_{22}N_8O_8$ (514.45) requires: C, 49.03; H, 4.31; N, 21.78. Found: C, 48.85; H, 4.31; N, 21.64. IR (nujol): ν 3310, 1645 and 1600 cm^{-1}. ^{1}H-NMR (80 MHz) δ 1.30 (t, J ≈ 7.4 Hz, 6H, $CH_2C\underline{H_3}$), 2.32 (s, 3H, CH_3-C=N), 2.74 (q, J 7.4 Hz,

2H, $CH_2C{=}N$), 3.04 (q, $J \approx 7.5$ Hz, 2H, $CH_2C{=}C$), 6.45 (s, 1 H, =C-H) ; 7.92 - 9.2 (m, 6 H, Ar-H), 11.34 and 11.52 (two s, 2H, NH).

2 With 2,3-pentanedione (**6**) to give 4-methyl-3-heptene-2,5-dione (**27**) and 3-ethyl 3-hexene 2,5-dione (**28**)

Diketone **6** (1.95 ml, 19 mmol) was added to a solution of phosphorane **2** (5.7 g, 18 mmol) in DCM (25 ml) and the resulting mixture was heated under reflux until **2** had completely disappeared. Work up in the usual manner gave a crude mixture (1.6 g, 46% yield) of **27** (major product) and **28** as a liquid with R_f 0.26 and 0.40. The product instantly decolourized Br_2 in CCl_4 and the resulting solution then went pink and finally cherry red. The product gave negative periodate and positive iodoform tests. IR (neat): ν 1700 -1690 and 1620 cm^{-1}. The bis-2,4-dinitrophenylhydrazone of the product was a dark orange-red solid, m.p. 240°C, R_f ($CHCl_3$/MeOH, 3 : 1) 0.85. $C_{20}H_{20}N_8O_8$ (500.43) requires: C, 48.00; H, 4.03; N, 22.39. Found: C, 47.72; H, 4.02; N, 22.17. IR (nujol): ν 3310, 1620 and 1600 cm^{-1}. ^{1}H-NMR (80 MHz) δ 1.24 -1.41 (two overlapping t, 3H, $C\underline{H}_3CH_2$), 2.33 (s, ~3H, $CH_3C{=}N$), 2.49 (s, ~3H, $CH_3C{=}C$), 2.68 -2.78 (m, 2H, $C\underline{H}_2CH_3$), 6.42 (minor) and 6.52 (major) (two s, =C-H), 7.79 -9.15 (m, 6 H, Ar-H), 11.32 and 11.50 (two s, 2H, NH). Peaks at 2.9 -3.2 ppm, due to the methylene protons $CH_2C{=}C$, indicated the presence of the bis-2,4-dinitrophenylhydrazone of **28**. Measurement of the $CH_3C{=}C/CH_3C{=}N$ ratio, which was 0.77 : 1, showed that **27** was the major product.

3 With 2,3-hexanedione (**7**) to give 4-methyl-3-octene-2,5-dione (**29**) and 3-(1-propyl)-3-hexene-2,5-dione (**30**)

Diketone **7** (2.29 ml, 19 mmol) was added to a solution of phosphorane **2** (5.76 g, 18 mmol) in DCM (30 ml). The mixture was heated under reflux until **2** had been consumed. Work up in the usual manner yielded the crude product **29** (1.6 g, 58% yield), containing minor quantities of its isomer **30** as a pale yellow liquid. This mixture, which darkened on standing, had R_f (PE / DE, 3 : 2) 0.33 and 0.47. Both spots darkened on standing with that at R_f 0.33 more rapidly. The product gave a positive iodoform test and rapidly decolourized

Br_2 in CCl_4, but then the resulting solution went pink and finally orange-brown. IR (neat): ν 1700-1680 and 1615 cm^{-1}. The product formed a blood red bis-2,4-dinitrophenyl-hydrazone with m.p. 232-4°C, R_f ($CHCl_3$ / MeOH, 3 : 1) 0.88. $C_{21}H_{22}N_8O_8$ (514.453) requires: C, 49.05; H, 4.30; N, 21.78. Found: C, 48.75; H, 4.31; N, 21.63. IR (nujol): ν 3300, 1640 and 1590 cm^{-1}. ^{1}H-NMR (80 MHz) δ 1.3 (poorly resolved m, $J \approx 7$ Hz, 3H, $C\underline{H_3}CH_2CH_2$), 1.5-1.8 (m, 2H, $CH_3C\underline{H_2}CH_2$), 2.33 (s, 3H, $CH_3C{=}N$), 2.49 (s, 3H, $CH_3C{=}C$), 2.61 -2.81 (m, 2H, $CH_2C{=}N$), 2.91-3.17 (very weak peaks due to the methylene protons $CH_2C{=}C$ in the derivative of **30**), 6.55 (s, 1H, =C-H), 8.01- 9.18 (M, 6H, Ar-H), 11.32 and 11.52 (two s, 2H, NH).

C- Reaction of benzoylmethylenetriphenylphosphorane (**3**) with diacetyl (**4**) to give (*E*) 3-methyl-1-phenyl-2-pentene-1,4-dione (**31**)

Diacetyl (2.41 ml, 2.45 g, 28.4 mmol) was added to a solution of phosphorane **3** (3.49 g, 9.5 mmol) in DCM (35 ml). Heating until **3** had disappeared, followed by work up in the usual manner, yielded a pale yellow oil which consisted mainly of diketone **31** (*E*-isomer) (1.08 g, 61% yield). This oil gave a single spot on TLC and had $n_D^{22.5}$ 1.5610, lit.[19] n_D^{20} 1.5518 for impure **31**. IR (neat): ν 1685, 1665, 1610, 1600 and 1580 cm^{-1}. The product **31** rapidly decolourized Br_2 in CCl_4 but the solution turned to green and then to blue. Diketone **31** turned an alkaline sodium nitroprusside solution a dirty red colour and this on acidification gave a green ppt. The product appeared to isomerize on standing as the IR spectrum showed several changes, amongst which was the appearance of new bands at 3350 and 1010 cm^{-1}. The ^{1}H-NMR spectrum also showed the presence of isomerized material. ^{1}H-NMR (80 MHz) δ 2.41 (s, 3H, *E* $CH_3C{=}$), 2.47 (s, 3H, CH_3CO), 6.24 (s, 1H, *E* =C-H), 7.45 - 8.01 (m, 5H, Ph-H) and peaks due to isomerized material at δ 2.36 (d), 2.28 (s), 3.97 (s), 5.94 (s). IR (fresh distillate, neat): ν 1685, 1670, 1610, 1600 and 1580 cm^{-1}. Reacton of diketone **31** with an excess of 2,4-dinitrophenylhydrazine gave at rt, the mono derivative as an orange solid with m.p. 200°C.

$C_{18}H_{16}N_4O_5$ (368.35) requires: C, 58.69; H, 4.38; N, 15.21. Found: C, 58.54; H, 4.25; N 15.16 IR (nujol): ν 3340, 1650, 1630 and 1600-1580 cm^{-1}.

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Table 1: Products from the reaction of the Wittig reagent Ph_3P=CHCOR and some diketones

Entry	Wittig Reagent	Diketone	Products	Isolated %Yield[a]	*Z* : *E* ratio[b]	δ *Z*=C-H	δ *E*=C-H
1	**1** (R=OEt)	**4**	**14**	66	47:53	5.7	6.6
2	**1**	**5**	**15**	81	76:24	5.7	6.5
3	**1**	**6**	**16**	36	100% E,		6.56
			17	25	58:42[c]	5.6-5.7	5.6-5.7
4	**1**	**7**	**18+19**	42	*d*		
5	**1**	**8**	**20**	60	36:64	5.65	6.50
6	**1**	**9**	**21**	65	-		
7	**1**	**10**	**22**	37	100% E		6.47
8	**1**	**11**	**23**	51	70:30	5.87	6.17
9	**1**	**12**	**24**	49	40:60	5.29	5.68
10	**1**	**13**	**25**	42	*d*		
11	**2** (R=Me)	**5**	**26**	70[e]	*d*		
12	**2**	**6**	**27+28**	46[e]	*d*		
13	**2**	**7**	**29+ 30**	58[e]	*d*		
14	**3** (R=Ph)	**4**	**31**	61[e]	*d*		

(*a*) Based on the of Wittig reagent used. (*b*) Ratios are correct to within ±5%. (*c*) *Z*:*E* ratio based on observation of the signals due to allylic methyl hydrogens at d 1.98 for *Z* isomer and 2.35 *E* isomer. (d) *Z*: *E* Ratio could not be determined (e) Yields of crude product containing traces of triphenylphosphine oxide.

ΠΕΡΙΛΗΨΗ

Οι αντιδράσεις Wittig μιας ποικιλίας συμμετρικών και μη συμμετρικών δικετονών με σταθεροποιημένα φωσφοράνια Ph_3P=CHCOR, όπου R=OEt (1), Me (2) και Ph (3), παρήγαγαν ακόρεστους κετοεστέρες και δικετόνες με αποδόσεις 37-81%. Σχηματίσθηκε αμφότερα τα E και Z ισομερή των 1:1 προϊόντων Wittig. Οι μη συμμετρικές δικετόνες αντέδρασαν ταχύτερα με το στερεοχημικώς λιγότερο παρεμποδισμένο καρβονύλιο. Στις περιπτώσεις που εξετάσθηκαν δεν παρατηρήθηκε, γενικώς, ισομερείωση των νεοδημιουργηθέντων διπλών δεσμών και ακόμη και οι ισχυρώς ενολοποιήσιμες κετόνες ήταν δυνατό ν' αντιδράσουν.

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ELECTROCHEMICAL BIOSENSORS BASED ON ENZYMES IMMOBILIZED IN ELECTROPOLYMERIZED FILMS

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SUMMARY

An electropolymerization method for the immobilization of various enzymes such as: glucose oxidase, lactate oxidase, in different polymers (polypyrrole, poly-*o*-phenylenediamine, poly-*N*-methyl-pyrrole and polyaniline) was applied to obtain electrochemical biosensors sensitive to glucose, lactate, and urea. Amperometry was chosen as a detection method giving the possibility to detect the above species indirectly *via* hydrogen peroxide or ammonia, being the products of enzymatic reaction. H_2O_2 oxidation on platinum electrode or ammonia oxidation on PPy layer in the case of urea sensitive biosensor was utilized. The obtained biosensors exhibited fast response, sufficient to employ them in flow-injection analysis for the determination of lactate or urea in blood serum samples. The attractiveness of electropolymerization as a convenient method for biocomponents immobilization at the electrode surface to obtain suitable biosensors was fully confirmed.

Key words: *amperometry, electrodeposition, polypyrrole, poly-o-phenylenediamine, poly-N-methylpyrrole, polyaniline, glucose oxidase, lactate oxidase, choline oxidase, urease.*

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INTRODUCTION

Enzymatic reactions are widely used in modern chemical analysis. However, the use of soluble enzymes has numerous limitations. Enzyme can be used in a single determination which made the analysis costly, especially in the case of expensive enzymes. Additionally, some enzymes are unstable in the solution due to their sensitivity to pH changes or the presence of inhibitors, causing rapid lost of catalytic activity of the enzyme.

These drawbacks can be eliminated by the immobilization of enzymes. The advantages of immobilized enzymes, such as reusability, better stability, less sensitivity to inhibitors, and broader pH range and combined with the high selectivity provided by the enzyme itself, account for their broad application in clinical chemistry and biotechnology. The enzymes can be immobilized in flow-through enzymatic reactors or directly at the detector surface.

Recently, one of the most common techniques of enzyme immobilization is entrapment into electropolymerized conducting or non-conducting films[1]. This procedure exhibits considerable advantages. It is usually simple, one-step procedure, that allows the accurate control of polymer film thickness and hence - the amount of enzyme entrapped. It is very often carried out in mild conditions (aqueous solutions, neutral pH) suitable for biomolecules. As occurring locally at the electrode surface it can be used to confine an enzyme precisely only on the electrode surface giving the possibility of fabrication of arrays of enzyme microelectrodes[2], and also to build up multilayer structures[3] with one or more enzymes[4]. Good detectability of response may be obtained as enzymatic reaction takes place in the bulk of polymer layer, which can form also a barrier for interfering species due to sieve effect (size exclusion)[5] or electrostatic interaction (ion-exclusion)[6]. This layer may also act as a catalytic layer for direct electron transfer between the electrode surface and active centre of the enzyme via molecular wiring[7].

There are also few drawbacks of this kind of enzyme immobilization. Often relatively short long-term stability of the biosensor caused by the decrease of the enzyme activity is observed. It is sometimes difficult to immobilize effectively enzymes other than oxidoreductases. Significant chemical and electrochemical activity of the polymer matrix due to its sensitivity toward ion-exchange processes and redox equilibria may be a source of signal disturbance.

Conducting polymers are first off all a convenient matrix for enzyme immobilization. Most of the enzymes at the pH value corresponding to that of physiological environment are negatively charged due to the predominance of aspartate and glutamate over lysine and arginine. They can therefore play a role of negative species neutralizing positively charged polymer and be incorporated into the polymer matrix due to electrostatic interactions[8]. It was, however, also suggested that enzyme may be simply entrapped by occlusion during the elctropolymerization[9]. This mechanical immobilization stabilizes the enzyme[10], but of greater benefit is to bound enzyme covalently to properly modified polymer, e.g. forming peptide bonds by carbodiimide activation[8].

The mechanism of the electron transfer between the prosthetic group of enzyme and the electrode surface, and the role played by conducting polymers in this process, essential for the operation of amperometric biosensors, still remains unclear. One can find rather large number of papers reporting the correct operation of biosensors with enzymes immobilized in non-conducting polymers or only after the addition of appropriate mediator. In the last case the mediator coimmobilized with the enzyme in polymer layer plays a fundamental role in "molecular-wiring" appearing also when so called "redox polymers" are used[11].

The aim of the present work was to compare the response of glucose biosensors with glucose oxidase immobilized in various polymers, to examine the possibility of removal of interferences using multilayer polymer films for lactate biosensor with lactate oxidase immobilized in PPD, and to explore the possibility to utilize the sensitivity of PPy to ammonia for the biocatalytic determination of urea.

EXPERIMENTAL

Reagents

The following monomers were used for the electropolymerization: pyrrole from Sigma (Cat.No. P 4892) or Aldrich (Cat.No. 13,170-9), *o*-phenylenediamine from Sigma (Cat.No. P 9029) or Aldrich (Cat.No. 27,578-6), *N*-methylpyrrole from Aldrich (Cat.No. M7,880-1), aniline from Aldrich (Cat.No. 13,293-4) and phenol from Sigma (Cat.No. P 3653).

The enzymes used were as follows: glucose oxidase (GOD) from *Aspergillus niger* type II-S (activity 18 U/mg) from Sigma (Cat.No. G 6641) and type X-S (activity 166 U/mg) from Sigma (Cat.No. G 7141), lactate oxidase (LOD) from *Pediococcus* sp. (activity from 16 to 40 U/mg) from Sigma (Cat.No. L 0638), and urease type VI from Jack Beans (activity 60-120 U/mg), also from Sigma (Cat.No. U 2125).

Apparatus

Electrochemical measurements and electropolymerization were carried out using AUTOLAB system from Eco Chemie B.V. or voltammograph model CV-37 from Bioanalytical Systems and potentiostat/galvanostat model 363 from PAR, connected to streap-chart recorder model BD 111 from Kipp & Zonen.

The flow-injection set-up consisted of a rotary injection valve (model 5020) from Rheodyne, a peristaltic pump from Gilson (model Minipuls 2) and a large-volume wall-jet cell.

The measurements were made with Pt disk of various diameter or Pt wire working electrodes, Pt foil auxiliary electrode and saturated calomel (SCE), (model K401) or Ag/AgCl, (model K801) electrodes from Radiometer as reference ones.

Before electropolymerization the platinum working electrodes were polished with alumina suspension, rinsed thoroughly with water, then cleaned by cycling the potential for 30 min. between -0.1 and +1.1 V *vs.* Ag/AgCl in 1 M sulphuric acid and finally rinsed with water.

Flow-injection system

Flow-injection system used was a two-line manifold (Fig.1.). The samples or standards were injected into a stream of deionized water. This stream was then merged with a stream of 0.05 M phosphate buffer (pH 7.2-7.4) when glucose or lactate were determined or with a stream of 0.05 M borate buffer (pH 9.2) for the determination of ammonia or urea. The optimal experimental conditions for each determination are given in the text.

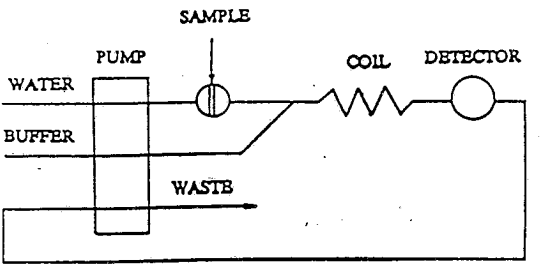

Fig.1. Schematic diagram of the used flow-injection manifold.

Procedures for the biosensors preparation

Glucose biosensors with GOD immobilized in various polymers

Polypyrrole (PPy). The first layer of polypyrrole was deposited by 20 s polymerization from solution containing 0.2 M monomer in 0.1 M KCl at +0.8 V *vs.* SCE. After 5 min. conditioning in 5 mM hydrogen peroxide solution, the electropolymerization was continued from the solution used for an electropolymerization containing 200-1000 U/ml GOD during 0.5 to 5 min.

Poly(*N*-methylpyrrole (PMPy). The first layer of polymer was obtained by 5 s polymerization from 0.2 M monomer in 0.1 KCl solution at +0.8 V *vs.SCE* and then this process was continued in the presence of GOD (200 U/ml) during the time ranged from 1 to 10 min.

Poly(*o*-phenylenediamine) (PPD). In a two-step procedure the polymer layer was deposited for 1 min. from 5 Mm monomer solution in 0.2 M acetate buffer (pH 5.2) at +0.75 V. Then GOD (200 U/ml) was added and electropolymerization was continued during 0.5 to 30 min.

Polyaniline (PAn). In the case of GOD immobilized in PAn, a three-step procedure was applied. First PAn was deposited by 10 min. electropolymerization from 0.2 M aniline in 1.2 M HCl solution at +0.7 V. The obtained layer was polarized cathodically at -0.5 V in 0.2 M acetate buffer (pH 5.4) during 15 min., then a layer of PAn containing GOD was obtained from 0.2 M acetate buffer with 500 U/ml GOD at +0.65 V during 15 min electropolymerization.

Lactate multilayer biosensor

For the preparation of lactate biosensor, three layers of polymer were formed consecutively. The first one was a PPy layer obtained by 1 min. electrodeposition from 0.4 M monomer solution in 0.05 M phosphate buffer (pH 7.3) at +0.8 V. Then, the second layer of polyphenol was formed from 5 mM monomer solution in phosphate buffer in the same conditions as the PPy layer. The third layer containing LOD was obtained from by 30 min. electropolymerization from 5 mM monomer solution in 0.2 M acetate buffer (pH 5.2) with addition of 200 U/ml of enzyme at +0.8 V.

Urea biosensor with urease immobilized in PPy

For the urea biosensor he polymer layer containing immobilized urease was obtained by electropolymerization of 0.1 M pyrrole solution in 0.1 M NaCl or 0.1 M sodium dodecylsulphate or in phosphate buffer in the presence of 1000 to 5000 U/ml of urease at the potential shifting from 0.8 to 1.2 V *vs.* Ag/AgCl reference electrode in the time period varying from 3 to 60 min.

RESULTS AND DISCUSSION

Glucose biosensors with GOD immobilized in various polymers

For the immobilization of glucose oxidase, few polymers of different properties were examined. The comparison of calibration plots of biosensors with GOD immobilized in polypyrrole, poly-*N*-methylpyrrole, poly-*o*-phenylenediamine and polyaniline is presented in Fig.2.

For polypyrrole, the described earlier procedure of immobilization gave a black layer of polymer with entrapped enzyme. Electropolymerization from KCl solution is, however, non-reproducible. The reproducibility of this process was highly increased by covering a Pt electrode surface with thin layer of PPy from the solution without enzyme. The conditioning of enzyme containing layer in 5 mM hydrogen peroxide improved the response stability of the sensor response both for hydrogen peroxide as well as for glucose, especially at higher concentrations. The response of the PPy/GOD biosensor for these species is presented in Fig.3, where signals recorded for the electrode of 3.5 mm diameter covered for 1 min. with GOD containing layer from a solution of 1000 U/ml are shown together with corresponding calibration plots.

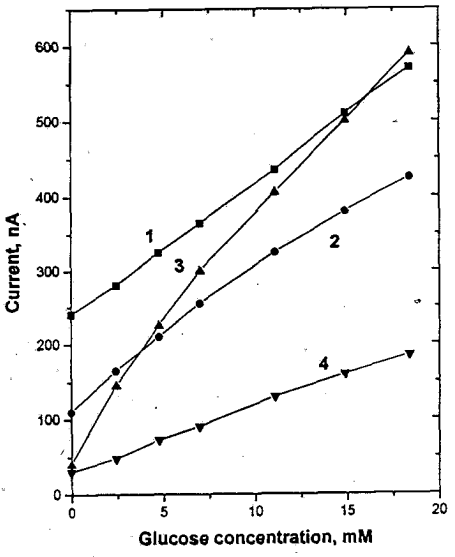

Fig.2. Calibration plots obtained in non-flow measurements for glucose biosensor obtained by the immobilization of GOD in different polymers:
1 - PPy, 2- PMPy, 3 - PPD, 4 - PAn.
Conditions of measurement: 0.05 M phosphate buffer, pH 7.2, potential of the measurement: +0.8 V *vs.* SCE.

The biosensor exhibited satisfactory stability by the time when stored between measurements in 0.05 M phosphate buffer (pH 7) at +4°C. The detection sensitivity and the lifetime of the detector depended strongly on the time of electropolymerization. Increasing this time up to 90 s resulted in an increase of sensitivity of glucose response, but for longer deposition times the sensitivity decreased leading to complete loss of response for 10 min electropolymerization. After two weeks the decrease of sensitivity was less than 30% of the initial value for biosensor with sensing layer deposited during 1 min.

The deposition of poly-*N*-methylpyrrole layer containing GOD gave a colourless transparent film. For the polymerization of *N*-methylpyrrole the use of a shorter polymerization time (1 min compared to 10 min reported earlier as optimum [7]) improved the sensitivity of detector by about 40%. The observed sensitivity, expressed as a slope of calibration curve, was comparable to that obtained for PPy/GOD biosensor (Fig.2, plots 1 and 2). After one day conditioning of the electrode a 40% decrease of response was observed being then stable for at least one week.

As a result of electropolymerization of poly-*o*-phenylenediamine from the solution containing monomer and GOD in acetic buffer also a colourless transparent film of the enzyme-containing polymer layer was obtained. The sensitivity of biosensor with the polymer layer deposition time 5 min towards

glucose was the largest one (Fig.2, plot 3); it was also accompanied by a lower background current in phosphate buffer solution. The pronounced increase of response was observed after overnight conditioning and followed by a decrease of sensitivity and functioning for at least one week.

The most complicated procedure was used for GOD entrapment in polyaniline layer. The obtained blue polymer showed good sensitivity to hydrogen peroxide. However, further deposition of the layer containing GOD from the solution without aniline gave biosensors exhibiting evident response to glucose, but with much lower sensitivity than those of other polymer matrices (Fig.2, plot 4). The long-term stability of this sensor was not investigated.

The recordings of the amperometric response of several glucose biosensors with GOD entrapped in polymer films as shown in Figure 3, indicated satisfactory dynamic properties of these sensors. Therefore they were also used for glucose detection by fast flow-injection measurements in a simple two-line set-up, where 100 ul of glucose solutions were injected into distilled water stream merged with another one containing 50 mM phosphate buffer of pH 7.2. The obtained flow-injection peaks were well shaped (Fig.4) and the linear response for glucose within the range 2-20 mM was observed for flow

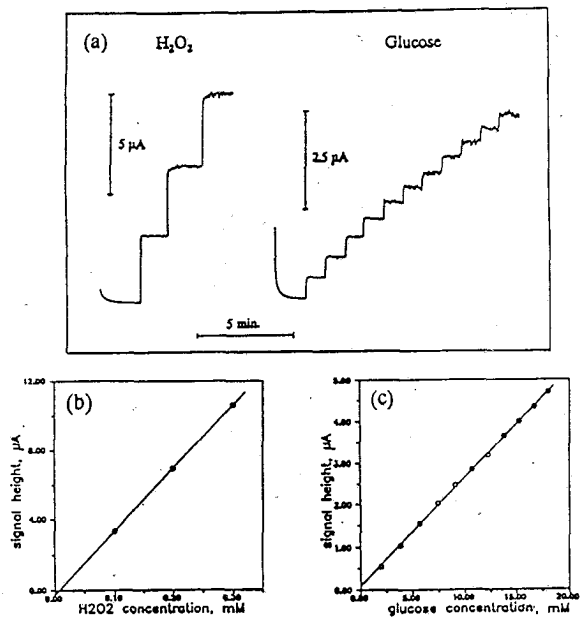

Fig.3. Recordings of the amperometric response (a) and the corresponding calibration plots obtained for hydrogen peroxide (b) and glucose (c) for PPy glucose sensor.
Conditions: to 50 ml of 50 mM phosphate buffer (pH 7.2) adequate portions of 0.1 M H_2O_2 or 1 M glucose were added and the signal was recorded at +0.8 V *vs.* Ag/AgCl reference electrode.

rate in the range from 0.8 to 2.8 ml/min. The about 2% (RSD) precision obtained for repeated injections (n = 4-5) for the whole concentration range tested can be considered as satisfactory. The detection limit obtained for S/N = 3 during a 10 days testing was estimated as 10-50 μM glucose.

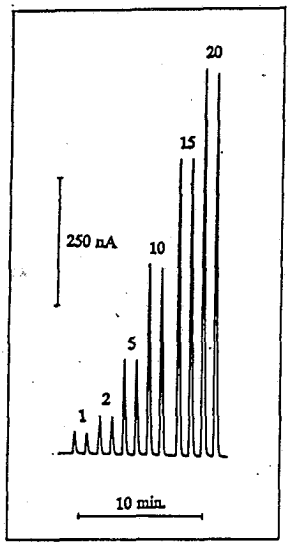

Fig.4. Recording of the flow-injection measurement of glucose in a two-channel system using a biosensor with GOD immobilized in PPy. Concentrations in injected solutions shown in mM.
Conditions: flow rate: 2.8 ml/min, injection volume: 100 μl. Measurement made at +0.8 V *vs.* Ag/AgCl reference electrode.

Lactate biosensor with LOD immobilized in poly-o-phenylenediamine

Poly-*o*-phenylenediamine which formed the matrix for GOD entrapment giving the most sensitive biosensor for glucose was also used for the entrapment of another enzyme, the lactate oxidase. To obtain the sensor for lactate, three layers of polymers were used according to the procedure described in the Experimental section. It was designed in flow-injection mode for practical application in routine analysis of blood serum. Under these conditions its long-term stability as well as its selectivity of response were tested.

The effect of interferences (0.2 mM ascorbate, 1.0 mM urate, 0.2 mM paracetamol and 0.2 M NaCl) on the response of PPD/LOD lactate biosensor was investigated. The concentration of ascorbate and urate were 2.5 times larger than upper limit of the normal concentration range of human blood serum and the NaCl was investigated as the species introducing matrix effects due to ionic interactions with polypyrrole.

The PPD layer itself eliminates to some extent the interferences from species such as ascorbate, urate and paracetamol, which may be oxidized on the electrode surface at the measuring potential (+0.8 V *vs.* Ag/AgCl). This elimination is better as thicker is the polymer layer. However, the increase of the thickness of PPD film also decreases the signal of lactate which causes this discrimination of electroactive interferences insufficient. Especially paracetamol interferences are relatively high. Also the formation of other polymers were tested to suppress the response of electroactive interferences. Among them the most effective for the elimination of paracetamol influence was polyphenol. It was found that polyphenol practically eliminates the interference of paracetamol, but the suppression of the anionic species influence (urate, ascorbate) was in turn not complete. The other polymer tested for the elimination of electroactive interferences was polypyrrole in its non-conducting overoxidized form. We have observed, that in the case of this polymer the interferences of ascorbate and urate as well as those of NaCl were practically eliminated, but paracetamol still caused a large positive error (>20%).

The above investigations led to the use of both polymers, PPh and PPy to eliminate effectively the interferences. The three-layer biosensor obtained according to this procedure showed good selectivity (Fig.5) in the experimental conditions. The flow-injection set-up was the same as used for glucose determination with the PPy/GOD sensor.

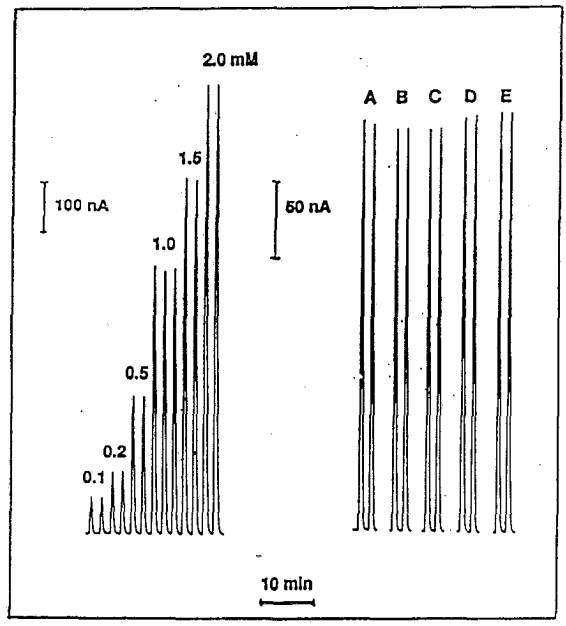

Fig.5. Recording of flow-injection response obtained with the optimized three-layer lactate biosensor for different concentrations of lactate and for 0.5 mM lactate: A - without added interferences; B, C, D and E with added ascorbate, urate, paracetamol and NaCl, respectively. For experimental conditions see Fig. 4.

In Figure 5 also the signals obtained during example calibration of the biosensor in the range from 0.1 to 2.0 mM are presented. The calibration plot was linear only in the range up to 0.2 mM, but for higher concentrations it can be fitted very well with 2nd or 3rd order polynomials with correlation better than 0.9990.

The long-term stability of the lactate sensor is presented in Figure 6. It can be seen that during the first 10 days a significant loss of the sensitivity is observed, down to 10% of the initial value, after intensive everyday use of the biosensor. After this period of time, the sensitivity was stabilized and the sensor may be used without further loss of sensitivity for up to 2 months. It can be seen from Fig. 6, that the additional layers of PPh and PPy decreased slightly the signal for lactate. However, these additional layers had to be used to eliminate interferences and they did not change the profile of the dependence of signal height *vs.* time.

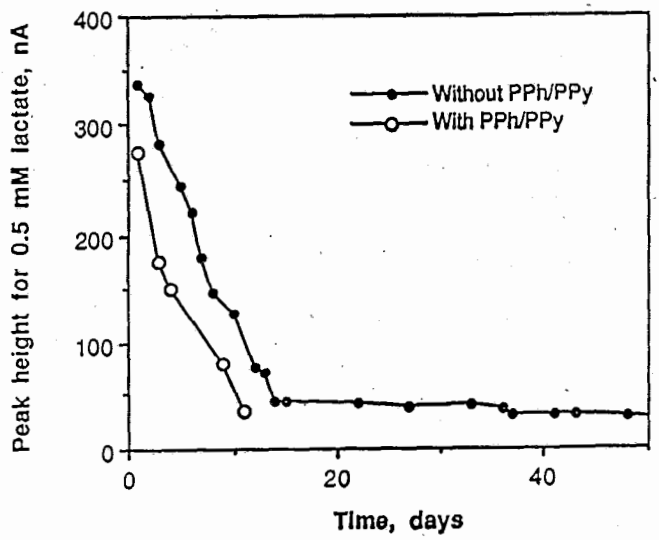

Fig.6. Change of flow-injection response in time for 0.5 mM lactate for PPD/LOD biosensors without and with inner PPy/PPh layer.

The developed biosensor was used for the determination of known amounts of lactate in human blood serum samples. The reference method was the determination with clinical analyzer Dimension from DuPont. The flow-injection assays were performed both for samples undiluted and diluted 1:5 or 1:10 with deionized water. In the last case the linear calibration plot in the range from 0.02 to 0.2 mM was used. The example correlation plots are shown in Figure 7. The results can be considered as satisfactory in spite of large decrease of sensitivity between 2nd and 11th day of the biosensor functioning (Fig.6). The increase of the intercept value in the 11th day comparing to the 2nd day may be attributed to the increase of contribution of interferences in the total signal with time.

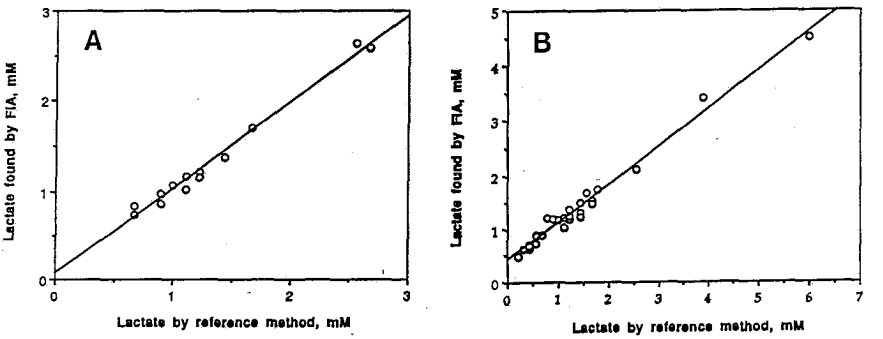

Fig.7. Correlation plots obtained for flow-injection determination of lactate with the optimized lactate biosensor in undiluted blood serum samples. (A): 2nd day of biosensor functioning, (B): 11th day of biosensor functioning. The equations of correlation plots are as follows: (A): $y = 0.958x + 0.071$ ($r = 0.993$, $n = 13$), (B): $y = 1.01x + 0.21$ ($r = 0.966$, $n = 24$)

Pt/PPy ammonia sensor

The first application of ammonia detection with polypyrrole modified electrode for the design of an amperometric urea biosensor, using an air-gap ammonia gas microelectrode, was already reported[12]. We have observed, that the increase of anodic current of PPy covered Pt electrode in the presence of ammonia in potentiostatic conditions is favoured by the increase of anodic polarization potential of the working electrode, but this was accompanied by rapid decrease of the detection sensitivity by the time at optimum potential of +0.3 V *vs.* Ag/AgCl. Also a decrease of detection sensitivity was observed with the increase of the concentration of the ammonia concentration. However, stable and practically reversible amperometric response to ammonia was obtained in submilimolar range of concentration. The calibration in flow-injection conditions ensuring a possibly short time period of the interaction of ammonia with the working electrode was chosen.

Several parameters affecting the signal magnitude, the stability of response and selectivity of detection were investigated. As optimum conditions 3 min electrodeposition time from the solution of 0.1 M KCl and 0.1 M pyrrole at +0.8 V *vs.* Ag/AgCl were chosen without stirring, using freshly distilled monomer.

The calibration plot of the Pt/PPy ammonia detectors of 3.5 mm (a) and 1.5 mm (b) is presented in Fig.8. The response in both cases was linear in the range of 10 to 100 uM ammonium ion.

A slow decrease of the detection sensitivity of the sensor was accompanied with the increase of noise amplitude of the base line caused by the decrease of conductivity of polymer layer. The precision for several electrodes estimated as RSD was ranging from 0.9 to 2.0% at 100 μM NH_4Cl.

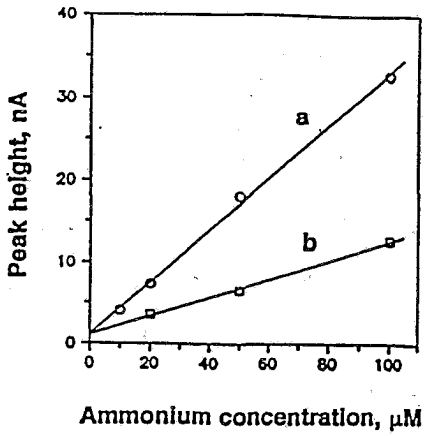

Fig.8. Flow-injection response to ammonia of Pt/PPy electrodes of 3.5 mm (a) and 1.5 mm (b) diameter obtained by 3 min electropolymerization at +0.8 V of pyrrole from a solution containing 0.1 M NaCl.

Urea biosensor with urease immobilized in the polypyrrole

The sensitivity of polypyrrole to ammonia was utilized to obtain biosensors with enzymes producing ammonia during an enzymatic reaction. Among them the most popular and cheapest is urease. Similarly to glucose oxidase the attempts to immobilize urease in PPy layer were undertaken to obtain a urea biosensor. However, in one-step procedure, regardless of the electrolyte used for electropolymerization (KCl, sodium dodecylsulphate, phosphate buffer), the electropolymerization potential (between +0.8 and +1.2 V *vs.* Ag/AgCl), the electropolymerization time (from 3 to 60 min), and enzyme concentration (1000-5000 U/ml) always in the presence of urease a yellow polymer was obtained instead of dark blue or black, showing much smaller response to ammonia and with practically no response to urea. Also the preadsorption of urease during 10 to 90 min at +0.5 V prior to electropolymerization was unsuccessful. The amperometric urea biosensor of very small sensitivity was obtained in two-step procedure, where first during 30 s PPy layer was electropolymerized only from monomer solution in KCl, and then the next layer was deposited according to procedure described in Experimental section. Figure 9 shows flow-injection signals recorded for such a sensor and a corresponding calibration plot.

Satisfactory results were obtained with PPy detector and urease immobilized on the dialytic membrane by simple cross-linking with glutaraldehyde. This procedure provides the biosensor of satisfactory sensitivity to urea and dynamic properties good enough for the application in flow-injection measurements (Fig.10). This biosensor was applied for the determination of urea in blood serum samples diluted 1:10 (see Fig.10) with good precision (RSD = 0.8%, n = 5 for sample S6). The correlation plot for another series of 24 samples was described by the following equation: $y = 1.008x - 0.115$ with correlation coefficient 0.980. Urea content values determined with Beckman Astra 8 clinical analyzer were used as a reference.

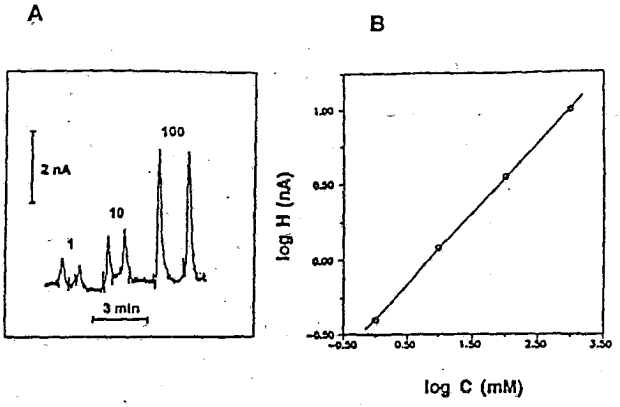

Fig.9. Recording of the flow-injection signal (A) and the calibration plot (B) obtained for urea using urease immobilized in PPy. Concentration in (A) shown in mM of urea. Conditions: flow rate: 2.8 ml/min, injection volume: 100 µl. Measurement at +0.8 V *vs.* Ag/AgCl reference electrode.

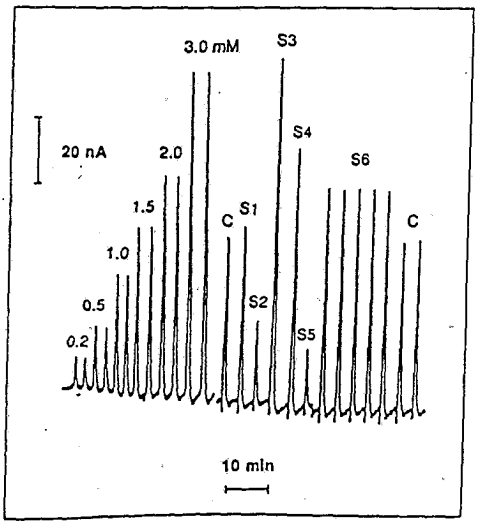

Fig.10. Flow-injection signals recorded for the determination of urea in blood serum. C: control serum sample; S1-S6: human blood serum samples.

CONCLUSIONS

The results presented in this work show the usefulness of the electropolymerization method for the immobilization of enzymes in polymer layers at the electrode surface to obtain biosensors sensitive to various species being of clinical analysis interest. It is especially attractive when one-step procedure for biocomponent immobilization can be applied with exact control of the parameters of the polymer layer based on the measurement of current passed during the electrodeposition process. However, it indicates also the requirement of the careful optimization of enzyme entrapment procedure for each polymer used. This study indicated that among polymers under investigation the best biosensors containing enzymes from oxidases group were obtained with poly-*o*-phenylenediamine. It was also shown, that enzymes other than oxidases may be difficult to be immobilized, without a loss of activity, in polymer to obtain properly working biosensors. The biosensors obtained may be successfully used in clinical analysis for the determination of lactate or urea in blood serum samples.

ΠΕΡΙΛΗΨΗ

Εφαρμόσθηκε μέθοδος ηλεκτροπολυμερισμού για την ακινητοποίηση διάφορων ενζύμων, όπως π.χ. οξειδάσης γλυκόζης και οξειδάσης γαλακτικού άλατος, σε διάφορα πολυμερή (πολυπυρρόλη, πολυ-ο-φαινυλενοδιαμίνη, πολυ-Ν-μεθυλο-πυρρόλη και πολυανιλίνη) για να ληφθούν ηλεκτροχημικοί βιαισθητήρες ευαίσθητοι στη γλυκόζη, γαλακτικό άλας και ουρία. Ως μέθοδος ανίχνευσης επιλέχθηκε η αμπερομετρία που δίνει τη δυνατότητα ν' ανιχνευθούν οι ανωτέρω ουσίες, εμμέσως, μέσω του υπεροξειδίου του υδρογόνου και της αμμωνίας που είναι τα προϊόντα της ενζυματικής αντίδρασης. Χρησιμοποιήθηκε η οξείδωση του H2O2 επί του ηλεκτροδίου λευκόχρυσου ή η οξείδωση της αμμωνίας επί στιβάδας PPy στην περίπτωση του ευαίσθητου στην ουρία βιοαισθητήρα. Οι λαμβανόμενοι βιοαισθητήρες έδειξαν ταχεία ανταπόκριση και ικανοποιητική για να χρησιμοποιηθούν σε ανάλυση flow-injection για τον προσδιορισμό του γαλακτικού άλατος ή της ουρίας σε δείγματα ορού αίματος. Επιβεβαιώθηκε πλήρως η καταλληλότητα της μεθόδου του ηλεκτροπολυμερισμού για την ακινητοποίηση των βιοσυστατικών στην επιφάνεια του ηλεκτροδίου και τη λήψη κατάλληλων βιαισθητήρων.

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ΕΡΕΧΘΕΙΟΝ

ΕΛΛΗΝΙΚΟΣ ΟΡΓΑΝΙΣΜΟΣ ΤΟΥΡΙΣΜΟΥ