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Inhibition of anion transport in the human red blood cell membrane with *para*- and *meta*-methoxyphenylglyoxal

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The positional isomers *para*-methoxyphenylglyoxal and *meta*-methoxyphenylglyoxal were newly synthesized and found to be potent inhibitors of sulfate exchange in the red blood cell membrane. The rate of inactivation of the transport system with both reagents obeys pseudo-first-order kinetics and increases with increasing pH and reagent concentration. The degree of inhibition of the transport system with the *meta*-isomer exceeds the inhibition caused by the *para*-isomer. At 2 mM 3-methoxyphenylglyoxal (3-MOPG) and 37°C the half-lifetime of the anion transporter is 5.4 min at pH 8.0. Under the same experimental conditions the half-lifetime of the transporter at 2 mM 4-methoxyphenylglyoxal (4-MOPG) is found to be 24.7 min. The binding site of these reagents is found to be the same as binding site of the reversibly acting phenylglyoxal derivative 4-hydroxy-3-nitrophenylglyoxal (HNPG). Chloride ions are able to protect the transporter against inhibition with both reagents. Anion transport inhibitors like 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS) and flufenemate, which are known to act on band 3 protein, are able to interact with the binding of the newly synthesised reagents. Phloretin and phloridzin show no interaction.

Introduction

In order to identify the essential amino acid residues that participate in anion transport across the red blood cell membrane, chemical modification studies have been undertaken [1,2].

A group of specific reagents, which have been first and extensively used in this laboratory, is the group of α-dicarbonyl reagents [3–11].

These compounds are known to react selectively with arginine residues in proteins [12].

In previous studies we have been able to show that 1,2-cyclohexanedione (CHD), 2,3-butanedione (BD), phenylglyoxal (PG) and its anionic and non-anionic derivatives are potent inhibitors of sulfate exchange in the red blood cell membrane [3–11].

The reversibly acting phenylglyoxal derivative HNPG is found to be a competitive inhibitor of both sulfate and chloride ions [10].

Our investigation on the chemical nature of the binding site of these reagents have shown that neither the hydrophobic character nor the electronic properties of these reagents seem to play a role in their inhibitory potency [11].

This work is the continuation of studies on the chemical nature of the essential arginine(s) in band 3. The inhibitory potency of two positional isomers of methoxyphenylglyoxal on anion transport has been examined. Also their interactions with other anion transport inhibitors have been studied.

Materials and Methods

Human blood (ORH^+) from apparently healthy donors was obtained from the blood bank in Frankfurt and stored at 4°C in acid/citrate/dextrose buffer. Cells were used within 3–5 days after withdrawal.

The experiments were performed with resealed red cell ghosts. Resealed ghosts were prepared essentially as described previously [2]. Cells were hemolysed at 3°C at a cell/medium ratio of 1 : 20 in medium containing 4 mM $MgSO_4$ and 1.45 mM acetic acid. 5 min after hemolysis, sucrose, gluconate, citrate, and Hepes were added from a concentrated stock solution to obtain a final concentration of 200 mM sucrose, 27 mM gluconate, 25 mM citrate, and 5 mM Hepes in the hemolysate. After centrifugation the ghosts were resus-

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Abbreviations: 3-MOPG, 3-methoxyphenylglyoxal; 4-MOPG, 4-methoxyphenylglyoxal; PG, phenylglyoxal; HNPG, 4-hydroxy-3-nitrophenylglyoxal; DNDS, 4,4'-dinitrostilbene-2,2'-disulfonate; 1,2-CHD, 1,2-cyclohexanedione; 2,3-BD, 2,3-butanedione.

pended and resealed 'in standard medium' containing (mM): 200 sucrose, 27 gluconate, 25 citrate, 5 Hepes, and 1 Na_2SO_4. The pH was either 7.4 or 8.0.

The resealing of the ghosts was essentially done as described by Zaki et al. [2].

The resealed ghosts were modified with either 3- or 4-methoxyphenylglyoxal at a hematocrit of 10% in standard medium at 37°C. In the protection experiments, sucrose was partly replaced by Cl^- ions. The resealed ghosts were incubated in standard medium at pH 8.0, the concentration of the reagents and the incubation time are indicated in the figures. $^{35}SO_4^{2-}$ equilibrium exchange was measured after removal of excess of the inhibitors by washing in a medium with the same composition as that had been used for resealing and reaction with the inhibitors. Flux measurements and calculations of the rate constants were done as described previously [2]. Transport is expressed as a percent residual activity relative to a control value measured in the same medium as used for the reaction but without the inhibitors. The lipophilic character of 3- or 4-methoxyphenylglyoxal was determined as previously described [11]. The computer modelling programs which have been used were Alchemy II and Sybyl Tripos Associate, a subsidiary of Evans & Sutherland.

Chemicals

3- and 4-methoxyphenylglyoxal were synthesized from the corresponding acetophenone derivative by the method of Fodor and Kovacs [13]. The reagents were recrystallized from hot water as the monohydrates.

Analysis: (Department of Chemistry, Wolfgang von Goethe University, Frankfurt/Main)

3-MOPG: C, 59.46%, H, 5.55%. Calculated for $C_9H_{10}O_4$: C, 59.33%, H, 5.53%, O, 35.13%.

4-MOPG: C, 59.47%, H, 5.44%. Calculated for $C_9H_{10}O_4$: C, 59.33%, H, 5.53%, O, 35.13%.

4-Hydroxy-3-nitrophenylglyoxal (HNPG) was synthesized according to Julien and Zaki [10].

Phloretin and phloridzin were obtained from Carl Roth OHG, Karlsruhe, or from Sigma, Germany. DNDS from Pfaltz and Bauer, CT, USA. $Na_2{}^{35}SO_4$ from Amersham Buchler, Braunschweig, Germany. Hepes was obtained from Calbiochem, Boehringer. All other substances were from Merck, Darmstadt, Germany.

Results

Characteristics of the inhibition of sulfate self-exchange by 3- and 4-methoxyphenylglyoxal

Incubation of resealed ghosts at pH 7.4 and 8.0 with excess of the two reagents results in a time and concentration dependent inhibition of sulfate exchange. The time course of inactivation was found to follow pseudo-first-order kinetics up to less than 10% of the initial value (Figs. 1a, 2a).

Upon prolonged incubation complete inactivation was obtained, as would be expected if essential residues for substrate binding were modified.

The rate of inhibition increases by increasing the pH from 7.4 to 8.0 (results not shown).

At low concentrations of the two reagents where there is a linear relationship between the rate constant of inactivation and the concentration of the inhibitor (panels b in Figs. 1 and 2); the reaction order, n, with respect to either 3-MOPG or 4-MOPG is calculated according to the equation used by Zaki and Julien [7]. The reaction order n (the slope of the lines from the plots of ln K_{app} vs. ln [3-MOPG], Fig. 1c, and ln K_{app} vs. ln [4-MOPG], Fig. 2c) at pH 8.0 was found to be 0.9 for 3-MOPG and 0.7 for 4-MOPG.

These data show that n is close to one for both inhibitors, suggesting that one inhibitor molecule per one band 3 is involved in the rate-limiting step in the inactivation process. This is in agreement with our previous results for phenylglyoxal [7].

K_i as calculated at pH 8.0 according to Zaki and Julien [7] was 4.03 mM for 3-MOPG and 6.2 mM for 4-MOPG.

Table I shows the results of a comparison between the effect of different concentrations of both 4-MOPG and 3-MOPG. From the data in Table I, which were obtained under the same experimental conditions, it is evident that 3-MOPG is more effective than 4-MOPG. The half-time of inactivation of sulfate exchange by 3-MOPG is two to three times less than that for 4-MOPG.

Effect of chloride ions on the inactivation of sulfate transport by 3- and 4-MOPG

Chemical modification of resealed ghosts by either 3- or 4-MOPG was performed at pH 8.0 with the addition of chloride ions at the concentrations stated in Figs. 3a and b. The inactivation rates were markedly decreased indicating the protective effect of chloride ions. These results are in agreement with the results found for phenylglyoxal [7].

Similar results have been found with sulfate ions at pH 7.4, Betakis dissertation, Frankfurt/Main, 1992 [14]. These results are consistent with our results with phenylglyoxal [7].

The binding site of 3- and 4-MOPG

In order to find out whether or not these reagents act on the same binding site as phenylglyoxal, the following experiments were done.

Resealed ghosts were modified with the reagents in presence of the reversibly acting inhibitor HNPG at the concentration indicated in Fig. 4.

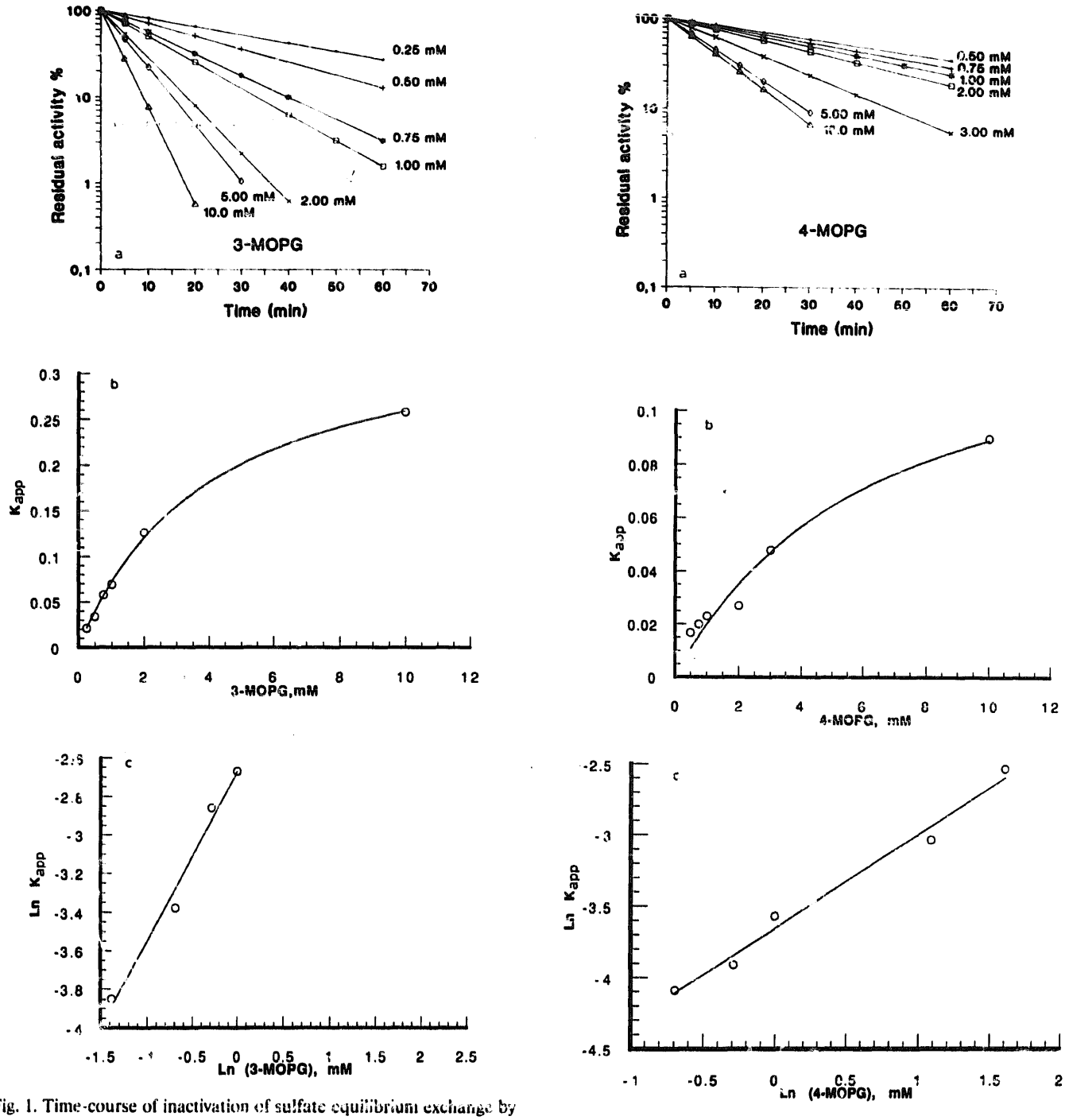

Fig. 1. Time-course of inactivation of sulfate equilibrium exchange by 3-MOPG. (a) Resealed ghosts were incubated in standard medium at pH 8.0, 37°C at the concentration of the reagent indicated in the figures. At the time indicated on the abscissa, aliquots were withdrawn, excess of the reagent was removed by washing and activity of $^{35}SO_4^{2-}$ equilibrium exchange was measured. The ordinate presents the residual flux as % of a control value without inhibitor. Panels b and c. First-order rate constant of inhibition versus concentration. (b) Linear scale, (c) double-logarithmic scale. The rate constants of inhibition (K_{app}) were determined from flux measurements by non-linear least-squares methods.

Fig. 2. Time-course of inactivation of sulfate equilibrium exchange by 4-MOPG. For details see the legend to Fig. 1.

After an incubation time of 60 min at pH 8.0 the unreacted modifiers were removed by washing and $^{35}SO_4^{2-}$ efflux was measured. Fig. 4 shows that after such long incubation time the reversibly acting HNPG is still able to protect the transport system against inhibition with 3-MOPG and 4-MOPG.

Interactions between the binding site of 3- and 4-MOPG and the binding site of other anion transport inhibitors

A range of reversibly acting anion transport inhibitors were tested for their effects on the inactivation of sulfate transport by either 3-MOPG or 4-MOPG.

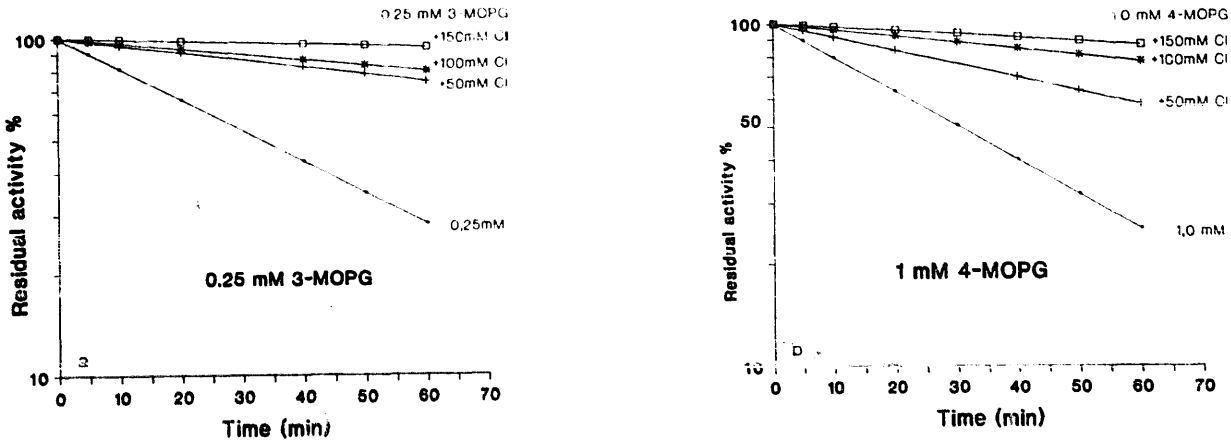

Fig. 3. Protection of sulfate equilibrium exchange against 3- and 4-MOPG inactivation. Resealed ghosts were incubated with 0.25 mM 3-MOPG (a) or with 1 mM 4-MOPG (b), in the presence of different concentrations of chloride ions. The ordinate presents the residual flux as % of a control value in the same medium (either standard or in a medium where sucrose was partly replaced by chloride ions (see Materials and Methods).

TABLE I

A comparison of the half-time of inactivation (min) of sulfate equilibrium exchange by 3- and 4-MOPG

The half-time of inactivation was calculated from the results in Figs. 1 and 2.

(mM)	$t_{1/2}$	
	4-MOPG	3-MOPG
0.25	–	33.1
0.50	41.5	20.6
0.75	35.5	12.5
1.00	30.8	9.8
5.00	9.0	4.6
10.00	8.0	2.6

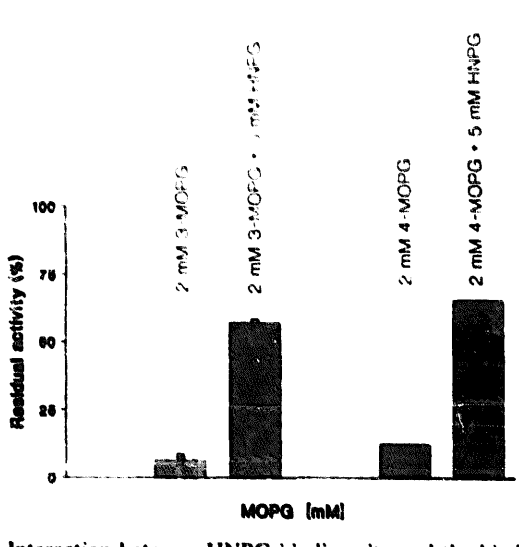

Fig. 4. Interaction between HNPG binding site and the binding site of either 3- or 4-MOPG. The ordinate presents residual activity in percent of control value without the inhibitors. The first and third column represent the effect of 2 mM of 3-MOPG and 4-MOPG. The second and fourth column show the protection caused by 5 mM HNPG when present during the incubation of the resealed ghosts with the inhibitors. The result is an average of 3–5 experiments, the error bars represent the standard deviations.

Resealed ghosts were first incubated with one of the reversibly acting inhibitors: DNDS, flufenemate, phloretin or phloridzin at the concentrations indicated in Figs. 5 and 6. After an incubation time of 5 min, 3- or 4-MOPG was added and the ghosts were further incubated for 45 min at 37°C and pH 8.0.

After removal of the reversible-acting inhibitor and the excess of the PG derivative by washing, flux measurements were performed.

The results in Figs. 5 and 6 show that in presence of DNDS or flufenemate the inactivation of the transport system by 3- or 4-MOPG is strongly reduced, while

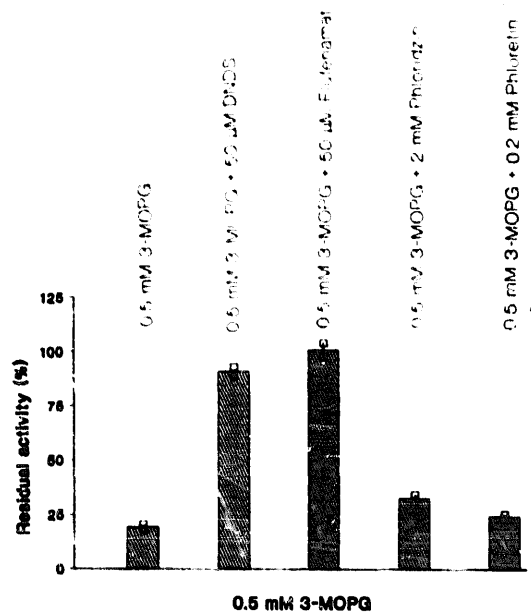

Fig. 5. Effect of reversible acting inhibitors on the inactivation of resealed ghosts by 3-MOPG. DNDS and flufenemate are able to protect the transport system against inhibition with the two phenylglyoxal derivatives. Phloretin and phloridzin have no effect. The result is an average of 3–5 experiments, the error bars represent the standard deviations.

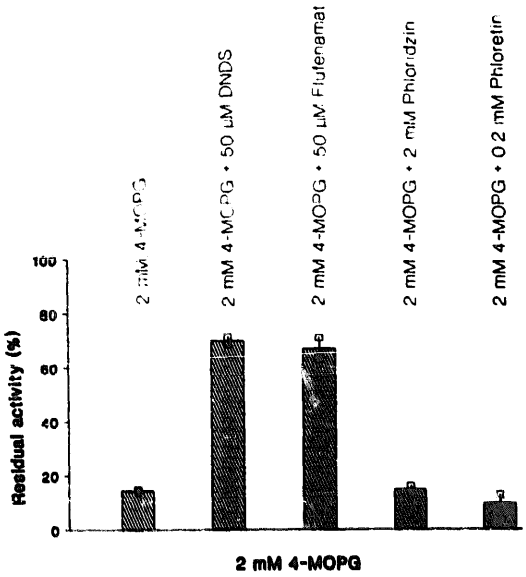

Fig. 6. Effect of reversible acting inhibitors on the inactivation of resealed ghosts by 4-MOPG. For details see the legend to Fig. 5.

phloretin and phloridzin have nearly no effect. These results are consistent with our previous results with phenylglyoxal and its derivatives [11].

Discussion

The rapid and complete inactivation of the sulfate self-exchange by 3- and 4-methoxyphenylglyoxal reported in the present study supports the essential role of guanidinum groups in anion transport in the red blood cell membrane. The binding site of these reagents is found to be the same as the binding site of the competitive anion transport inhibitor HNPG. The ability of chloride ions to protect the transport system against inactivation by the two reagents also indicates that this arginine(s) may be part of the anion binding site.

The results represented in Figs. 5 and 6 show that DNDS and flufenemate are able to protect the transport system against inactivation by 3- and 4-MOPG.

On the other hand previous results [3,4,9] have shown that after modification of the essential arginine(s) with cyclohexanedione, 1,2-butanedione or phenylglyoxal, H_2DIDS binding to band 3 had not been greatly altered. Such interactions most probably have allosteric character and can be explained according to Zaki's cascade model [8].

The differences in the inactivation rates between the *meta*- and *para*-methoxyphenylglyoxal cannot be explained by the hydrophobic character of the molecules. The 3-MOPG which is less hydrophobic than 4-MOPG (Betakis dissertation, Frankfurt/Main, 1992) [14] is 2–3-times more effective.

Also the electronic properties cannot explain their inhibitory potencies. 4-MOPG has the methoxy-group in the *para* position which should increase the partial negative charge of the carbonyl-group and hence should increase its affinity to the positively charged groups in the membrane. This is not found to be the case. The *para*-isomer is less effective than the *meta*-isomer.

Takahashi [15] has found that phenylglyoxal reacts with arginine residues under mild conditions to give a product that contains two phenylglyoxal moieties per guanidino group. The first phenylglyoxyl molecule condenses reversibly with the guanido group to form a glyoxaline ring which then reacts rapidly with a second molecule of phenylglyoxal to form the final and stable product.

The complex between 3-MOPG or 4-MOPG and the guanidino residues has been investigated for their size and steric characters using computer modelling approaches [16].

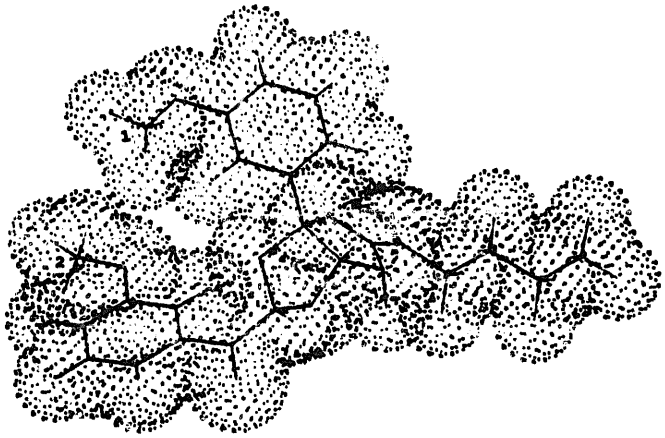

Fig. 7. A display of surfaces and a simulated energy minimized structure of the complexes between two molecules of 3-MOPG and the arginine as obtained from the Sybyl Molecular Modeling Software. In this procedure all atoms of the molecules are surrounded with van der Waals surface.

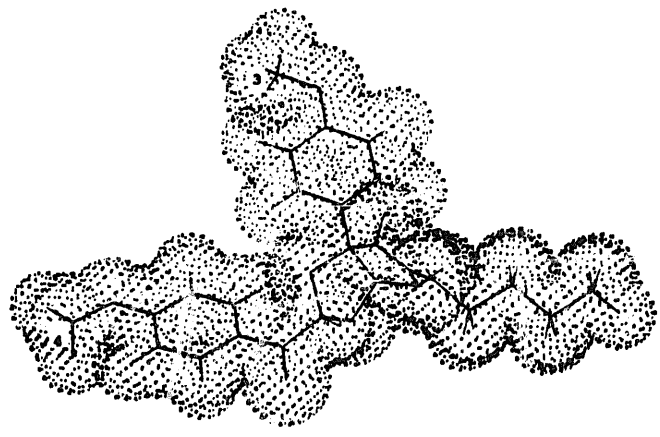

Fig. 8. A display of surfaces and a simulated energy minimized structure of the complexes between two molecules of 4-MOPG and the arginine as obtained from the Sybyl Molecular Modeling Software. In this procedure all atoms of the molecules are surrounded with van der Waals surface.

In this modelling approach the molecular mechanics optimizes the 3D structure of a molecule input, generally moving to the nearest local minimum energy structure. In this simple type of analysis neither solvent nor environmental effects were taken into account.

An energy minimized structure of the complex between the essential arginine and either 3- or 4-MOPG is shown in Figs. 7 and 8.

The distance between the two carbon atoms of the methoxy group in the case of 3-MOPG 1 and 2 was found to be 7.7 Å or 8.3 Å, that of 4-MOPG 3 and 4 was found to be 11.8 Å or 11.2 Å, depending on the energy minimizing technique. The difference of about 3.5 Å, which is about that of a water molecule, may explain the difference in their reactivities. The essential arginine may be located in a narrow cleft which could restrict the formation of a stable complex between the arginine residue and the second 4-MOPG molecule at the binding site in the pocket. This could explain its lower affinity.

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