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α-KETOALDEHYDES, SPECIFIC CATALYSTS FOR THIOL FORMATION FROM LEVAMISOLE

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Abstract—α-Ketoaldehydes, especially glyoxal and methylglyoxal, are very specific catalysts for the hydrolysis of levamisole into a thiol known as OMPI. The kinetics of this reaction are described. A mechanism is proposed involving a charge transfer complex followed by a concerted mechanism in which H_2O is carried from the hydrated aldehyde to levamisole with concomitant ring opening.. The possible significance of the reaction for the biochemical mode of action of levamisole is discussed.

One of the major metabolites of levamisole, a potent anthelmintic drug [1]with interesting immunomodulating properties [2], is a thiol, known as OMPI [(−)-2-oxo-3-(2-mercaptoethyl)-5-phenylimidazolidine]. The way in which it is formed *in vivo* has never been investigated. As part of our investigation of the biochemical effects of levamisole we tested the drug on the glyoxalase system (EC 4. 4. 1. 5 and EC 3. 1. 2. 6) and to our surprise detected thiol formation in an incubate with only methylglyoxal. This phenomenon prompted us to further characterize the kinetics of this new and interesting chemical reaction which might clarify the mode of action of levamisole.

MATERIALS AND METHODS

Levamisole, [(−)-2, 3, 5, 6-tetrahydro-6-phenylimidazo(2, 1-b) thiazole hydrochloride] and *dl*-OMPI are products of Janssen Pharmaceutica, Beerse, Belgium.

Glyoxalase I and II and methylglyoxal (MG) came from Sigma (St. Louis, USA). All other chemicals were obtained through Aldrich-Europe, Beerse, Belgium.

For measuring the cleavage of levamisole, two methods were chosen, both using the Beckman-25 K spectrophotometer.

Method A was based on the reaction between the thiol formed and 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB). Levamisole was dissolved in phosphate buffer (50 mM, pH 7.5 unless otherwise specified) containing 2 mM DTNB. The reaction was started by the addition of the aldehyde and the increase in absorbance at 412 nm was recorded. From the slope, the velocity was calculated as μmoles of thiol formed/min/(molar absorptivity: 13,600 $l/mole^{-1}\ cm^{-1}$). Due to its high sensitivity this method was only used for measuring initial velocities.

Method B was based on the spectral difference between levamisole and the reaction product at 250 nm allowing the process to be followed up without requiring any additional colour reaction. The change in molar absorptivity upon hydrolysis was found to be 1,800 $l.\ mole^{-1}\ cm^{-1}$.

Using a 1 mM solution of levamisole and glyoxal, both methods were closely comparable for up to 25 per cent of the conversion.

A Technicon Auto-Analyser system was applied for the parallel determination of thiol formation and the ethylenediamine (EDA) reaction. The incubate (levamisole–aldehyde buffer; 0.100 ml/min) was continuously diluted (1 to 10) in 0.05 N HCL and added (0.23 ml/min) to 0.1 M phosphate buffer (pH 7.5, containing 200 μl Brij-35/l; 1 ml/min). The colour reaction took place in a single mixing coil and was measured at 420 nm. Simultaneously, 0.23 ml/min of the incubate was added to a 5 mM solution of MG in phosphate buffer (50 mM, pH 7.2; 1 ml/min). After mixing in a double mixing coil, 0.23 ml/min of a 10 mM solution of EDA in water was added. The colour developed in one single and two double mixing coils and was measured at 505 nm.

All experiments were performed at 25° unless otherwise specified.

Thin-layer chromatography (t.l.c.) was done on precoated Silicagel plates (Merck-Darmstadt) using ethylacetate–acetic acid (90:5) as a solvent and iodine vapour for detection.

For mass spectrometry, the samples were introduced via the direct inlet system of the Varian MAT 311. A programmed evaporation of the samples was performed yielding the spectra of the identified compounds. The instrument settings were: electron energy; 70 eV; emission, 300 μA; electron multiplier gain: 2 kV.

RESULTS

Initial velocities and concentration of both reactants. The initial velocity of the reaction is directly related to the product of the concentration of both reactants as shown in Table 1 for 1 to 4 mM solutions of levamisole and the respective aldehyde. The reaction with glyoxal is almost 15 times faster than with MG.

Concentration–time curve—Catalytic effect of glyoxal. The reaction between 1 mM levamisole and 1 mM glyoxal was followed to completion using method B. The decrease in the concentration of levamisole with time is shown in Fig. 1. A detailed analysis of the results reveals that we have a first-order reaction at the beginning (up to 20 per cent conversion) followed by a period where the log (concentration of levamisole)

Table 1. Initial velocity (μmoles hydrolyzed/min/l ± S.D.) and concentrations of levamisole [LMS] and glyoxal or MG (m

[LMS]	Glyoxal mM 1	2	3	4	Methyl-glyoxal mM 1	2	3	
1	24.33 ± 1.58	44.31 ± 2.42	65.56 ± 3.74	87.79 ± 4.19	1.51 ± 0.07	2.90 ± 0.07	4.16 ± 0.11	5.4
2	45.83 ± 4.22	87.70 ± 4.49	123.47 ± 6.94	160.17 ± 8.99	2.87 ± 0.14	5.51 ± 0.19	7.99 ± 0.21	10.2
3	66.49 ± 3.74	120.08 ± 4.80	182.97 ± 9.22	234.54 ± 7.17	4.21 ± 0.13	7.97 ± 0.20	11.23 ± 0.31	15.0
4	86.59 ± 3.88	162.74 ± 5.78	242.53 ± 9.51	321.57 ± 8.85	5.38 ± 0.22	10.31 ± 0.40	14.61 ± 0.51	19.2

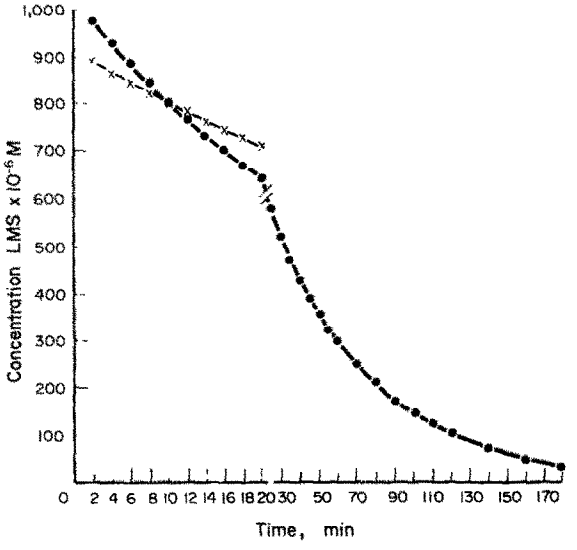

Fig. 1. Concentration–time curve for levamisole—glyoxal 1 mM. pH 7.5. Method B—Mean values from 7 experiments. x—x: disappearance of levamisole. 0.9 mM. added after completion of the reaction between 1 mM of the reactants.

vs time curve is not linear. From 80 per cent conversion on, it is once more a first-order reaction. This is also illustrated by the time needed for a 5 per cent decrease of the remaining levamisole, which is nearly constant between 1 and 0.7 mM (2 min 06 sec–2 min 18 sec), then gradually increases and again becomes constant at 0.25 mM levamisole and less (3 min 12 sec–3 min 40 sec).

When, after complete conversion, levamisole (0.9 mM) is added to the mixture, the reaction starts again as shown in Fig. 1. The initial velocity of the second reaction (59 μmoles $min^{-1}l^{-1}$) is nearly half the original velocity (115 μmoles, $min^{-1}l^{-1}$). From the relation $V_{initial} = k$ [LMS] [K], where [LMS] and [K] are the molar concentrations of levamisole and glyoxal, the free glyoxal concentration at the end of the first reaction can be calculated and was found to be 0.57 mM. The remainder (0.43 mM) is bound as hemimercaptal (see further).

Effect of pH and temperature. The velocity of the reaction between levamisole and glyoxal is strongly dependent of the pH, being very low at a pH below 7.0, rising to a rather sharp optimum between pH 8.8 and 9.0 and then rapidly falling to almost zero at pH 10.0 (Fig. 2). There is no reaction in 0.1 or 1 N NaOH.

The reaction proceeds equally well in imidazole buffer as in phosphate, pyrophosphate and bicarbonate–carbonate buffers or in water. In the latter howeve the pH has to be monitored by continuous addition NaOH or ammonia solutions in order to prevent th drop in pH accompanying the reaction. In *N*-ethy aminoethanol or borate buffers the reaction is very slo or even absent at pH values above 8.6

The effect of the temperature was studied on th slower reaction between MG (10 mM) and levamisol (1 mM) at pH 7.5.

The initial velocities were calculated from the slop of a continuous record of the DTNB reaction using th procedure on the Auto-Analyzer. The results are show in Table 2. From an Arrhenius plot the activatio energy was estimated to be 46.610 J/mole.

Specificity. The reaction with levamisole is ver specific for the α-keto aldehydes as shown in Table where the initial rates of thiol formation are compare for several related compounds. Since it could be imag ined that for several aldehydes the DTNB reaction i masked, the reaction was also checked by recording th u.v.-spectra at several time intervals (1 to 48 hr) afte initiation of the reaction. No spectral changes wer detected in those cases where the DTNB reaction wa negative.

When levamisole base is dissolved in acetonitril containing 20 per cent of water, there is a pronounce hydrolysis upon addition of glyoxal. No thiol formatio occurs when 2, 3,-dichloro-5, 6-dicyano *p*-benzoqu none is added instead of glyoxal.

The reaction between levamisole and glyoxal (2 mM each) at pH 8.0, whether initiated by levamisole (an pre-incubation of glyoxal with mercaptoethanol fo 20 min) or by glyoxal, was inhibited to almost 50 pe cent by 1 mM mercaptoethanol.

Characterisation of the reaction product(s). It i evident that the product formed is a thiol, since the thio reaction is one way to follow the process.

T.L.C of an incubate of 2mM each of levamisole an MG revealed, however, the presence of two majo spots. The R_F-values corresponded to those of authentic OMPI (0.53) and its hemimercaptal (0.43). Evidenc

Table 2. Effect of temperature on the initial velocity

t(°C)	V_i μmoles/min/l*	%
20	14.9	3
25	20.2	4
30	27.7	5
35	37.2	7
40	50.4	10

* Calculated from a continuous measurement of thio formation in an incubate of 1 mM levamisole and 10 mM MG.

Table 3. Specifity of the α-ketoaldehydes as catalysts

X	Formula	Relative activity *
Glyoxal	H– CO– CHO	1000
MG	CH_3 –CO– –CHO	78
Phenylglyoxal	C_6H_5– CO –CHO	31
Glyoxylic acid	HOOC –CHO	12
Formaldehyde	H –CHO	n.a.
Acetaldehyde	CH_3– –CHO	n.a.
Trifluoroacetaldehyde	CF_3– CHO	n.a.
Chloral	CCl_3—CHO	n.a.
Glycolaldehyde	$HOCH_2$—CHO	3
Glyceraldehyde	$HOCH_2$—CHOH CHO	5‡
Acroleine	CH_2=CH– –CHO	n.a.
Hexenal	CH_3–CH_2 –CH_2—CH=CH– –CHO	n.a.
Glutaraldehyde	OHC—CH_2—CH_2—CH_2– CHO	n.a.
Pyruvic acid	CH_3– –CO– –COOH	n.a.
Hydroxyacetone	CH_3—CO –CH_2OH	n.a.
Acetoine	CH_3– –CO– –CH$\begin{matrix}CH_3\\OH\end{matrix}$	n.a.
Diacetyl	CH_3– –CO– –CO– –CH_3	n.a.

* Conditions: 1mM levamisole, 1mM X kept at 25°– Method A.

† n.a. = no activity

‡ Becomes equipotent to MG when incubated for 24 hr at 30° in phosphate buffer pH 7.5 (glyceraldehyde-3-phosphate reacts similarly), confirming the known non-enzymatic transformation of glyceraldehyde into MG under certain conditions [7].

of the latter was obtained from t.l.c. of a mixture of OMPI and MG, kept for a few minutes at pH 7.5. The addition of a few drops of 1 N NaOH resulted in a disappearance of the hemimercaptal spot. A third minor spot was also present with a R_F-value identical to that of oxidized (= disulfide) OMPI (0.35).

Mass spectrometry of the same mixture showed the presence of 3 components: MG, OMPI and the hemimercaptal. Levamisole was no longer detectable nor any low molecular weight compound derived from MG.

U.v.-spectral analysis of the reaction mixture shows a shift of the 218 nm band of levamisole to a band at 207–208 nm with a decrease in absorbance. These changes parallel the thiol formation. The final spectrum completely corresponds to that of authentic OMPI under the same conditions (Fig. 3).

When ethylenediamine (EDA) is added to a mixture of levamisole and MG at pH 7.2, a red colour rapidly develops which fades to brownish-yellow. There is a direct relation between the EDA-reaction and the thiol formation in the incubate, when measured simultaneously. This reaction does not occur when MG is replaced by glyoxal in both the incubate and the reagent, but proceeds with glyoxal if MG is used in the reagent as described.

Neither the EDA-reaction, nor the DTNB reactivity of an incubate of levamisole and MG is affected by the addition of glyoxalase I, whereas in a mixture of GSH

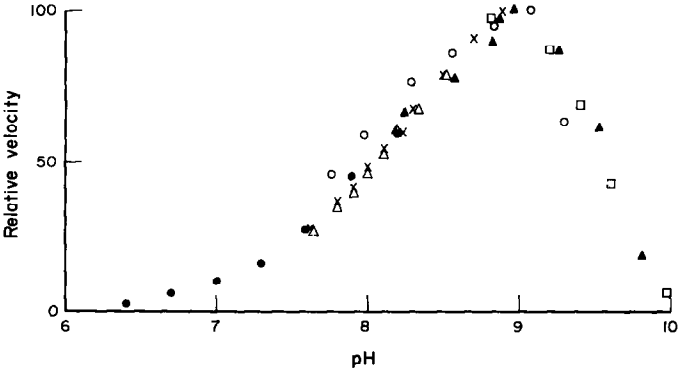

Fig. 2. Effect of pH on the rate of reaction between glyoxal and levamisole (1 mM)—Buffers: 50 mM.
● : phosphate.
× : phosphate–pyrophosphate–Method A.
△ : pyrophosphate.
▲ : pyrophosphate–carbonate–bicarbonate.
□ : bicarbonate–carbonate.
○ : methylglyoxal 1 mM-pyrophosphate.

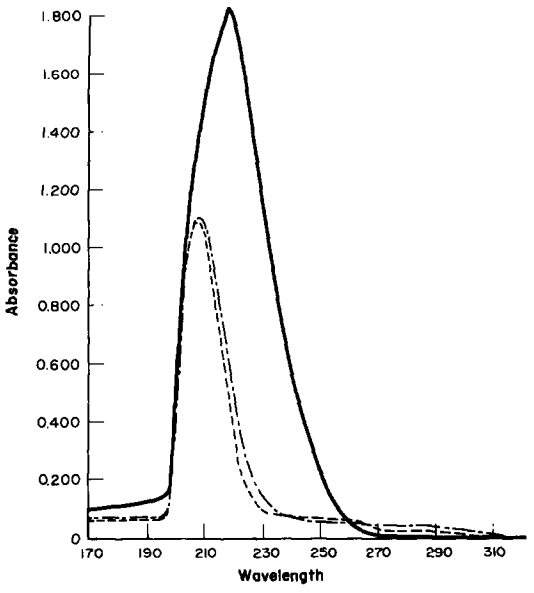

Fig. 3. U.v. spectra of levamisole (– –), an incubate of levamisole and 8 mM of MG after 7 hr (· · – · – ·) and authentic *dl*. OMPI in the presence of 8 mM MG (– – –).

and MG both reactions completely disappear under the same conditions.

When more concentrated solutions (> 2 mM) of levamisole and glyoxal are incubated they become turbid and a precipitate forms on standing. The final quantity of precipitate being similar, the rate of appearance is strongly dependent on the pH (8 min at pH 7.3 to less than 15 sec at pH 8.8 for a detectable turbidity). This is not seen with MG, even after standing for a very long period. The precipitate, after washing with cold water and lyophylisation is insoluble in water, ethylacetate, methanol, acetonitrile or chloroform, but soluble in acetic acid, dimethylsulfoxide or in water after alkalinisation to pH 12.0 with 1 N NaOH. The solubility and melting point (106°) are clearly different from OMPI (m.p. 86°). Upon t.l.c. the material shows up as a long band from the origin to a R_F-value of nearly 0.40. When it was suspended in water and a few drops of NaOH were added until complete dissolution, only one single spot, corresponding to OMPI, remained. Element-analysis ($C_{13}H_{16}N_2O_3S$), t.l.c. solubility, i.r., mass spectrometry and NMR, all suggest that the product is a polymer of the hemimercaptal from OMPI and glyoxal.

DISCUSSION

Refluxing a levamisole solution in alkali (1 N) for a few days results in a hydrolytic ring opening to OMPI, a thiol which is one of the major metabolites of the drug *in vivo*.

As shown in this paper, this hydrolysis is catalysed very specifically by α-ketoaldehydes, especially glyoxal. The reaction proceeds rapidly and is almost complete at an ambient temperature and a slightly alkaline pH.

After the reaction is completed between levamisole and MG, the incubate consists of three compounds. Levamisole is no longer detectable. MG is left unchanged as evidenced by mass spectrometry and by the fact that upon readdition of levamisole the reaction starts again.

The second reaction product is free OMPI as shown by DTNB reactivity, u.v. spectra, mass spectrometry and t.l.c. in comparison with OMPI, prepared chemically. The identification of the third compound as the hemimercaptal was based on several findings. Thus, t.l.c. of an equimolar mixture of authentic OMPI and MG, kept at pH 7.5, shows 2 spots. The addition of a few drops of 1 N NaOH leads to complete disappearance of the lower spot in favour of the one corresponding to OMPI. Exactly the same occurs when a mixture of levamisole and MG is analyzed by t.l.c. It is known that hemimercaptals readily dissociate at a higher pH, to free thiol and the aldehyde [3]. The EDA reaction is claimed not to be catalyzed by the thiol but by the hemimercaptal [4]. The finding, that a mixture of levamisole and MG shows this reaction indicates that OMPI and MG can be combined to the corresponding hemimercaptal. The hemimercaptal was also identified by mass spectrometry of the incubate. Its presence is not surprising because of the well known ready addition of the thiols to the nucleophylic carbon of the carbonyl group.

The presence of unchanged MG, and the fact that the reaction reaches completion even at substoechiometric concentrations of the keto-aldehyde (ratio 4 to 1 – data not shown) led us to conclude that we were dealing with a true catalytic process in which the reaction product (OMPI) has an affinity for the catalyst (hemimercaptal formation). The general reaction then can be described as

$$\text{LMS} + \text{K} \xrightarrow[\text{H}_2\text{O}]{} \text{OMPI} + \text{K=HM}$$

where LMS = levamisole, K = the α-ketoaldehyde and HM = the hemimercaptal.

This is a first-order reaction in each of the components when the initial concentrations are concerned but not on a concentration-time base. That necessarily indicates a complex mechanism [5].

The experimental concentration-time curve reveals that the reaction proceeds as a pseudo first order reaction at the beginning (up to 20 per cent conversion). Apparently, the concentration of free ketoaldehyde (K) remains almost constant. It is only thereafter that the aldehyde concentration decreases, due to hemimercaptal formation, with subsequent deviation from first-order kinetics. That also indicates that free OMPI is the true reaction product.

A detailed mathematical analysis of the kinetics is complex because we were unable to differentiate free OMPI and its hemimercaptal without disturbing the equilibrium.

Although the exact mechanism of the hydrolytic ring opening is not entirely understood, our experiments provide evidence of the scheme, shown in Fig. 4.

The optimum pH found at or above the pK (8.03) of levamisole indicates that the deprotonated form is the reactive species. (The sharp fall in reactivity above pH 9.0 might be due to a nucleophylic attack of the OH-ions inactivating the aldehyde function.) Since levamisole produces a strong red colour with a solution of 2, 3-dichloro-5, 6-dicyano-*p*-benzoquinone in acetonitrile, it can act as an electron donor. The α-ketoalde-

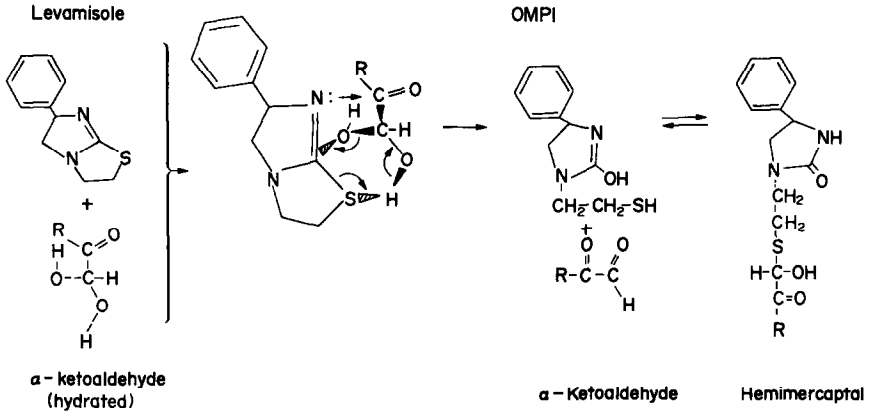

Fig. 4. Illustration of the reaction mechanism proposed for the hydrolyte cleavage of levamisole as catalyzed by α-ketoaldehydes.

hydes, known as electron acceptors [4], could thus combine with levamisole through a CT-complex. The failure to detect any absorption band, characterizing the complex, could be due, among other causes, to the subsequent (rapid) hydrolysis. However, no thiol formation could be detected with dichloro-dicyano-benzoquinone under conditions where the reaction with glyoxal proceeds very rapidly. Apparently, a CT complex alone is not sufficient for ring opening but it could create conformational conditions favouring a subsequent concerted mechanism. This involves a synchronous attack of both OH-groups of the hydrated α-ketoaldehyde on the C–S bond, one behaving as an acid and the other as a base, which results in a hydrolytic cleavage.

That the hydrated aldehyde is involved can be assumed because it is known that this is the main species present in water at concentrations below 1 M. [3]. Further evidence is provided by the inhibition from mercaptoeoethanol which is the same (50 per cent) whether the reaction is initiated by glyoxal or by levamisole after preincubation of glyoxal with mercaptoethanol. If the unhydrated aldehyde was the true catalyst, its removal by preincubation with mercaptoethanol, would have resulted in an almost complete inhibition of the reaction with levamisole (see also [6]).

The lack of effect of trifluoroacetaldehyde could mean that the concerted mechanism, as proposed, is by itself unable to hydrolyze levamisole.

A Dreiding model of the molecules involved reveals that the approach of levamisole to the α-carbonyl may result in a configuration of the molecules whereby a concerted mechanism can occur. These models also showed that phenyl and even CH_3-substitution as in phenyl- and methylglyoxal sterically hinder the approach of both molecules in the way we described. That explains the poor catalytic effect of phenylglyoxal whose electron-acceptor properties are better than glyoxal as judged from the calculated lowest empty molecular orbital (LEMO) values.

It is the sequence of both phenomena, charge transfer and a concerted mechanism, which may explain the high specificity of the reaction for the α-ketoaldehydes.

In a recent survey, Szent-Györgyi [4] stresses the role which MG or some related α-ketoaldehyde may play in cell growth and proliferation through hemimercaptal formation with GSH. Strong arguments are the facts that (1) metabolic pathways do exist for the formation of MG (apart from the nonenzymatic formation from dihydroxyacetone-7- or from glyceraldehyde and glyceraldehyde-3-phosphate—Table 4) and γ, δ-dioxovalerate [8] and (2) the abundant and universal presence of the glyoxalase system in living cells (except tumours for glyoxalase II [9]).

If the hemimercaptal from the α-ketoaldehyde(s) and GSH plays the important role suggested by Szent-Györgyi [4], its activity is limited in time by the presence of the glyoxalase system. The hemimercaptal of OMPI and MG, being almost as effective in the EDA reaction as the GSH-hemimercaptals, is however not destroyed by glyoxalase I. If the EDA-reaction is an expression of the physiological role, then it can be assumed that the OMPI-hemimercaptal will act for much longer periods than the (natural) GSH derivative.

If the role of the glyoxalase system is merely to destroy the highly reactive (noxious?) α-ketoaldehydes, levamisole could simulate, at least to some extent, the enzymatic reaction, by trapping the aldehyde. This action, being of minor importance in normal cells, could be beneficial for those cells lacking the glyoxalase activity.

A third, and very intriguing consequence of the reaction described, is the release in the cell of a thiol, OMPI. Preliminary experiments in the laboratory, revealed several peculiar characteristics of this thiol when compared with other thiols such as GSH, cysteine, dithiothreitol, mercaptoethanol, cysteamine, penicillamine and CoASH. Thus it reduces Brilliant Green, dichlorophenol indophenol and ferricyanide faster and at lower concentrations. Shortly after incubation with cytochrome *c*, the reduction by OMPI is fairly weak but becomes by far the most pronounced after 48 hr of contact. It is also less susceptible to autoxidation and to oxidation by hydrogen peroxide than GSH, dithiothreitol or mercaptoethanol at pH 7.4.

The importance of thiols for cellular metabolism and a variety of cellular functions is well documented. However, when administered *in vivo*, thiols, because of their high reactivity, do not easily reach the cell interior and the target. Due to the reaction described, levami-

sole may act as a pro-drug, releasing a thiol right there where it could play an important role. In view of this, it is worth mentioning that common effects have been described [10] on some components of the immune system of levamisole, *d*-penicillamine and 5-mercapto-pyridoxine. Recently, the hypothesis has been raised that the immunomodulating properties of levamisole are at least partly due to the enhancement by OMPI of microtubule integrity and function in leukocytes [11].

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