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Toxicology of Cupric Salts on Honeybees. V. Gluconate and Sulfate Action on Gut Alkaline and Acid Phosphatases

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Some aspects of putative nontarget effects of cupric ions systemically fed to honeybees against their parasite mite *Varroa jacobsoni* have been investigated on the host phosphatases. The alkaline and acid forms extracted from the guts of worker bees exhibited substrate-inhibition features. Upon detailed kinetic analysis, cupric organic salts indicate activation effects at concentrations of about 1 m*M*. Concentrations up to 10 m*M* (alkaline form) and 25 m*M* (acid form) induced no important changes, except a partial quenching of the substrate-inhibition process, characterized by a wide increase in the constant of apparent inhibitory binding of substrate to the enzyme–substrate complex. Partial purification gave a single alkaline form with quite similar kinetic behavior in the absence of natural ions as in crude extracts. Cupric gluconate and sulfate demonstrated similar patterns, except an increase of the apparent Hill coefficient by sulfate only. The substrate constant of acid phosphatases was decreased at high cupric gluconate doses while its maximum velocity was biphasically increased (with observed maximum at 1 m*M*), resulting in a sustained activation. Chemiluminescence studies revealed that cupric ion activation is counteracted by oxygen radicals generated by cupric ions and also, *in vitro,* by the artificial substrate *para*-nitrophenylphosphate. The *para*-nitrophenol molecules released from the reaction are therefore responsible for biphasic effects selectively observed with gluconate salts. In apicultural practice, neither blockade of activity nor dramatic changes are to be expected at doses administered to bees against the parasite.

INTRODUCTION

Systemic treatments by feeding bees with cupric ions have been originally proposed by Popeskovic (1984) for the control of the ectoparasite mite *Varroa jacobsoni.* In effect, mites belong to the Chelicerate branch, characterized by poor tracheal development (regressed stigmata) and by the presence of hemocyanin pigments for oxygen distribution to cellular sites, in contrast to insects which are totally devoid of hemocyanins but have evolved a complete tracheal system (Grzimek, 1969–1971).

Since the activity of hemocyanin molecules involves copper ions as cofactors (Liao *et al.,* 1994), it was hypothesized that their functioning might be affected by an excess of copper. This would thus impair the mite respiratory metabolism in a rather specific manner, i.e., with lower effects on the insect host, the honeybee (Popeskovic and Bounias, 1986; Nectoux *et al.,* 1987).

Although cupric sulfate, first used as a laboratory model (Popeskovic, 1984), actually proved efficient in field trials (Guiraud *et al.,* 1989), organic cupric salts such as gluconate have proven to be as effective as a control (Bounias *et al.,* 1994) and to have far lower toxicity to bees, even including hormetic stimulations of the colonies (Bounias *et al.,* 1995). Since systemic treatments drive copper salts from the digestive tract to hemolymph, both gut and hemolymph enzymes come in contact with cupric ions. Such enzymes therefore represent possible targets for side effects of the treatments.

In a previous work (Nectoux *et al.,* 1995b), studies were made of the action of cupric salts on hemolymph trehalases, an important enzymatic class with respect to the control of carbohydrate fuel availability. Now, in the gut, the phosphatase class also potentially represents an interesting toxicological marker. First, these enzymes are widely involved in digestion processes and metabolite transport (Akai, 1969; Moldenke, 1976), and it has long been known that their catalytic activity involves the participation of divalent metal ions (Fernley and Walker, 1967). In addition, evidence has been provided that some alkaline forms can bind cupric ions at their active site (Banci *et al.,* 1988).

Consistent with these arguments, recent toxicological works have demonstrated that the responsiveness of hemolymph phosphatases of invertebrates to copper can be used as an indicator of copper pollution (Suresh *et al.,* 1993). Therefore, it could be hypothesized that a risk might exist of some nontarget effects of cupric treatment to honeybee phosphatases.

These considerations prompted the examination of the compared effects of cupric gluconate and sulfate on the ki-

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netic parameters of both acidic and alkaline phosphatases extracted from worker honeybee guts, where high enzyme activities had been previously found (Bounias, 1978). Although crude extracts are closer to actual toxicological conditions, comparison has been made of the major properties of the enzyme and the effects of cupric gluconate both on the native form and after elimination of the ions initially present in hemolymph, through partial purification. Since oxygen free radicals are known as potential enzyme inhibitors (Kruk, 1995), examination was made, using chemiluminescence studies, of the generation of such radicals by cupric salts, acting both *in vivo* and *in vitro,* and also by the artificial substrate specifically involved in *in vitro* studies.

MATERIALS AND METHODS

Animals and Sampling

Emerging worker honeybees (*Apis mellifera mellifera* L.) were collected in brood combs from still-operculated alveoli. They were weighed and grouped (7–10 individuals) in glass dishes placed in dim light, in a laboratory chamber at 31 ± 1°C and 60 ± 10% relative humidity. For each sample, the guts of 5 bees were extracted after removal of the stinging apparatus, then rinsed in distilled water, dried on filter paper, and weighed. They were then homogenized at 0–4°C in Potter Duall glass homogenizers in presence of 250 μl distilled water. The extracts were centrifuged for 15 min at 5000*g*, and the supernatant was kept at −25°C.

Biochemical Techniques

The original method using *para*-nitrophenylphosphate (pNPP) as chromogenic substrate was first proposed by Bessey *et al.* (1946) and further generalized to alkaline phosphatase studies by Tietz *et al.* (1967). Optimum responses were obtained at 20 μM magnesium chloride (final concentration) (Bounias, 1978; Shaker *et al.*, 1988), and 400 m*M* Tris–HCl buffer, pH 8.5. Enzymatic extracts were adjusted at 10 μl in a total reaction volume of 250 μl.

Crude extracts (500 μl) were chromatographed on a glass column (11.5 cm height × 1.5 cm ϕ) filled with 1 g (8 ml) of Bio-Gel P-6DG (Bio-Rad, France) prepared in Tris buffer at pH 8.0. Upon elution in the same solvent at 35 ml/hr, fractions of 50 μl each were collected and the reaction velocities were determined in each one, together with the protein absorbance at 280 nm. The final reaction volumes were 200 μl in all cases. In enzyme-containing fractions, protein concentrations were determined by the method of Lowry *et al.* (1951).

For acidic phosphatases (Hudson *et al.*, 1947), 100 m*M* acetate–acetic acid buffer at pH 4.5 was used for incubation (Chung *et al.*, 1987). The absorbance of *para*-nitrophenol (pNP) released by the reaction was monitored in all cases at 400 nm, after adjusting the medium alkalinity at pH 13 by adding to the reaction medium 10 μl 5 *N* NaOH for reproducible maximum responses. The calibration curve prepared with pure pNP (Merck–Darmstadt, research grade) was linear up to 0.05 m*M*.

Chemiluminescence Methods

Chemiluminescence intensity (I_{CL}) was measured as follows: solutions were rapidly injected through a dark injection port, by means of an automatic syringe, into a cuvette mounted directly in the front of a M12FQC51 photomultiplier (PMT), with a S20 cathode sensitive in the 350–850 nm range. The detector was placed in a light-tight chamber. The high voltage of 1300 V for the PMT was obtained from a Polon ZWN 2.5 power supply. The anode current from the PMT was recorded with a Zeiss K-200 recorder.

Since light emissions from cupric salt solutions were weak, H_2O_2 was added to the investigated systems. The following radical scavengers were used as probes of the emissions: super oxide dismutase (SOD), for superoxide anion radical $O_2^{\cdot -}$ and thiourea and glutathione for hydroxyl radical $HO^{\cdot}$ (all reagents research grade from Sigma).

Kinetic Studies

Since the extracts all exhibited apparent substrate inhibition, the major kinetic parameters were calculated in the general case of non-Michaelian as well as Michaelian transitions, using a rigorous algebraic method available for such cases (Bounias, 1988).

Maximum velocities (V_M, a strictly theoretical parameter), Hill coefficients (h), regular substrate constants ($K_s = K_{50}^h$), and the constant of apparent substrate binding to enzyme–substrate complex (K_{ses}) and to free enzyme (K_{se}), were obtained from the basic equation

$$v_i = V_M \cdot S_i^h / [K_S + \alpha S_i^h + S_{i2}^h / K_{ses}], \quad (1)$$

with $\alpha = (1 + K_s/K_{se})$, here approximated to $\alpha = 1$.

The maximum observable response, v_m occurs at $S = S_m$, so that (Bounias, 1986)

$$S_m^{2h} = K_s \cdot K_{ses}. \quad (2)$$

Then, the particular case of velocity v_1 determined at $S_1 = 1$ m*M* and pairs of values (v_i, v_j) obtained for (S_i, S_j) chosen such that $S_i \times S_j = 1$ allow the determination of all four parameters. The regression slope of (v_i^{-1} versus v_j^{-1}), for $v_i > 1$, is $b = K_s \cdot K_{ses}$, which in turn gives

$$h = \log_n(b)/2 \cdot \log_n(S_m). \quad (3)$$

Maximum velocities can be obtained from the slope of a curve ($y_i = a + b \cdot x_i$) constructed with the following pairs:

$$\left.\begin{aligned}&[x_1 = v_i^{-1};\quad y_1 = (S_m^n + S_m^{-n})],\\&(x_2 = v_m^{-1};\quad y_1 = 2),\\&b = V_M\sqrt{K_{ses}\cdot K_s},\quad \text{and } a = \alpha\cdot\sqrt{K_{ses}\cdot K_s},\end{aligned}\right\} \quad (4)$$

which gives $V_M/\alpha \approx V_M$. Finally, K_{ses} and K_s are obtained from the Hill plot of equation (1):

$$\log_n[v/(V_M - v)] = h\cdot\log_n(S) - \log_n K_s[1 + S^h/K_{se} + (S/S_m)^{2h}], \quad (5)$$

characterized by two symmetrical asymptotic branches whose slopes are $(-h)$ and $(+h)$ and intercepting the vertical axis respectively at $y = -\log_n(K_s)$ and $y = +\log_n(K_{ses})$ (Bounias, 1988).

The intersection of these two asymptotes, at $x = \log_n(S_m)$ and $y = \log_n\sqrt{K_{ses}\cdot K_s}$, allows an improvement on the calculation of V_M. Finally, upon replotting the new values of the Hill coefficient and of V_M allowed iterative refinement of the calculation of the various parameters.

Statistics

Means ± SD (standard deviations) were calculated from the numbers of determinations (given between parentheses) obtained from independent batches of five to seven bees. Correlation coefficients (r) calculated from (n) pairs of data were given a significance using the distribution of Student's t variable:

$$t(r) = \sqrt{\nu}\cdot r^2/(1 - r^2),\ \nu = (n - 2). \quad (6)$$

Linear regression parameters ($y = a + [b \pm \sigma_b]\cdot x$) were calculated using the least-squares method, and the variance of the slopes (b) were given by the following relation:

$$\sigma_b^2 = [(b/r)^2 - b^2]/(n - 2). \quad (7)$$

TABLE 1

Average Fresh Weights of the Whole Body and of the Extracted Guts of the Honeybees Used in the Experiments on Various Forms of Phosphatases

Experiment	*N*	Whole body	Gut
Crude alk. form	(68)	116.6 ± 5.2	12.2 ± 2.7
Purified alk.	(15)	111.3 ± 1.2	11.8 ± 1.2
Crude acid form	(44)	105.5 ± 4.5	14.3 ± 2.0
Total	(127)	114.4 ± 6.1	12.7 ± 2.5

Note. Means (mg) ± SD are given for N individuals. No significant differences nor variance effects (ANOVA) were found between samples.

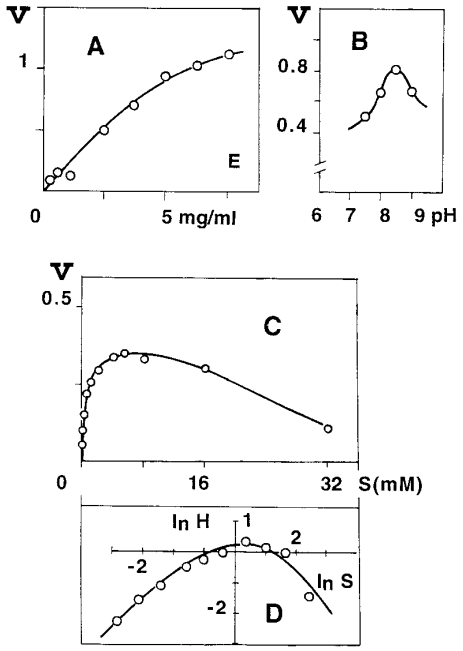

FIG. 1. (A) Variation of honeybee gut alkaline phosphatase activity (nmol/min) versus concentrations of gut extracts (E, mg/ml). (B) Velocity (nmol/min/mg gut) versus pH. (C) Variation of honeybee gut alkaline phosphatase velocity (v) with substrate concentrations (S, m*M* pNPP). Natural units. (D) Hill plot. H = $[v/(V_M - v)]$.

Finally, each parameter calculation, thus performed from n points, was independently replicated N-fold.

RESULTS

The average weights of the bees and of the guts extracted for the main three parts of the experiment are given in Table 1.

Alkaline Phosphatases

The dependence of enzyme activity on pH and enzymic concentrations in crude preparations are illustrated in Figs. 1A and 1B. The linearity was maintained up to 5 mg gut in 250 μl, and 10 μl extract at 250 mg gut/ml was used as a standard concentration for the whole experiment. Figure 1C demonstrates that the enzyme exhibits a behavior of the substrate-inhibition type, which was not apparently the case in former experiments, although in both cases the Hill coefficients were lower than unity (Bounias, 1978). However, this type of kinetic is not exceptional in phosphatases where in addition non-Michaelian kinetic behavior has been evidenced (Vargas *et al.*, 1990). The hypothesis of product inhibition could have been a plausible one, although only weakly influential at the beginning of the reaction, but this phenomenon does not give rise to a depression of enzyme activity at high substrate concentrations (Keleti, 1986).

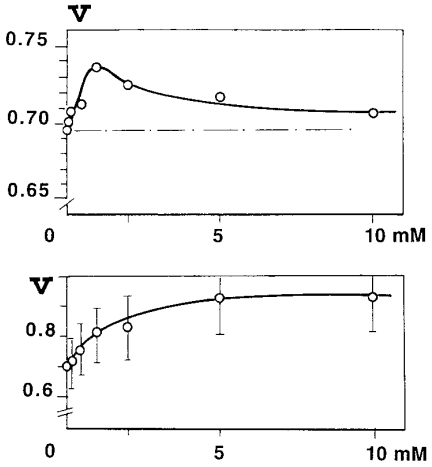

FIG. 2. Variation of honeybee gut alkaline phosphatase velocity (v, nmol/min/mg) at $S = 4$ mM versus concentrations of cupric gluconate (top) or sulfate (bottom).

The system has therefore been analyzed with respect to Eq. (1) (Bounias, 1986, 1988), and Fig. 1D presents the corresponding Hill plot.

The dose-related variations of alkaline phosphatase activity have been examined at fixed substrate concentration nearly giving the maximum observable velocity (v_m); that is, $S_m = 4$ mM. Cupric gluconate provides a typically biphasic response, with a maximum activation at 1 mM (Fig. 2A). In contrast, for cupric sulfate, only the activation phase was observed (Fig. 2B), with a 50% increase at $EC_{50} = 1.3 \pm 0.2$ mM, obtained

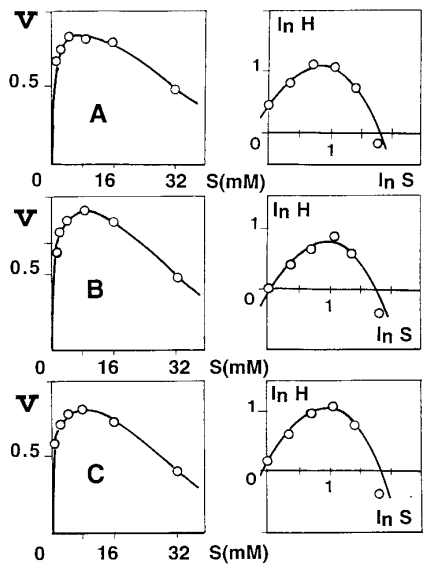

FIG. 3. Variation of honeybee gut alkaline phosphatase velocity (v, nmol/min/mg) versus substrate concentrations (S, mM) in presence of various concentrations of cupric gluconate: (A) 0.1 mM, (B) 1 mM, (C) 10 mM. Direct plots on left, and Hill plots on right ($H = [v/(V_M - v)]$).

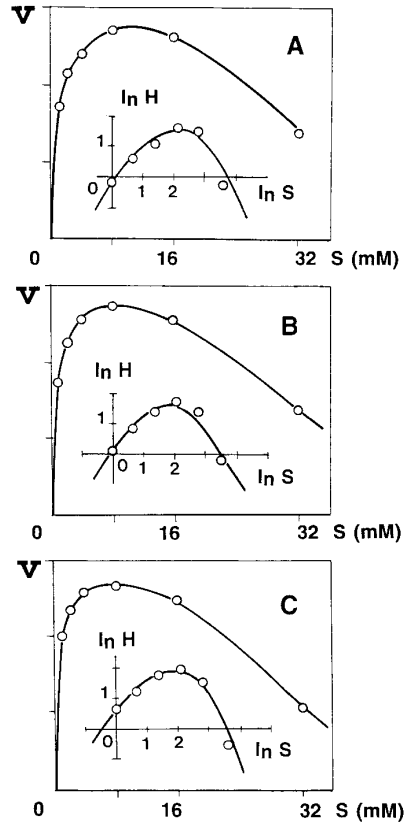

FIG. 4. Variation of honeybee gut alkaline phosphatase velocity (v, nmol/min/mg) versus substrate concentrations (S, mM) in presence of various concentrations of cupric sulfate: (A) 0.1 mM, (B) 1 mM, (C) 10 mM. Outer graphs give direct plots and insets the Hill plots ($H = [v/(V_M - v)]$).

from classical Hill plots (Bounias, 1994). An asymptotic maximum, $V_M = 0.95 \pm 0.1$ nmol/min/mg gut, was calculated, and the Hill coefficient was $h = 1.44 \pm 0.12$.

These observations deserved more detailed analysis, and the influence of cupric salts has been examined on the saturation curves (Figs. 3 and 4) at the lower, optimum, and maximum concentrations; that is, $C = 0.1$, 1, and 10 mM, and the whole set of parameters has been calculated.

The data presented in Figs. 3 and 4 illustrate the direct curves and their Hill plots, and the calculated values of apparent kinetic parameters are indicated in Table 2.

Changes are observed as follows for the various parameters. S_m is increased, although weakly significantly, by higher gluconate and lower sulfate values, while h is increased at 1 mM sulfate. These discrepancies would favor the hypothesis of an influence of the moiety rather than cupric ion effects. In contrast, maximum velocities are increased by both compounds at 1 mM consistent with dose–effect relationships plotted in Fig. 2 and discussed below.

Values of K_s exhibit only minor changes, while K_{ses} increases with increasing concentrations of cupric salts, and in

TABLE 2
Apparent Kinetic Parameters Determined in Alkaline Phosphatases from Crude Extracts of Honeybee Guts

	S_m	h	V_M	K_S	K_{ses}
Controls	3.9 ± 0.4 (5)	0.8 ± 0.1 (4)	0.6 ± 0.03 (2)	0.7 ± 0.3 (3)	5.4 ± 1.0 (2)
Gluconate					
0.1 m*M*	4.7 ± 0.3 (2)	0.64 ± 0.24 (5)	1.0 ± 0.1 (3)[b]	0.5 ± 0.1 (4)	15.8 ± 8.3 (2)
1 m*M*	5.2 ± 2.2 (4)	0.74 ± 0.2 (3)	1.33 ± 0.32 (3)[d]	0.95 ± 0.48 (6)	9.1 ± 11.6 (2)
10 m*M*	6.2 ± 1.0 (3)[b]	0.84 ± 0.20 (4)	1.05 ± 0.12 (4)	0.76 ± 0.18 (2)	37.4 ± 10.4 (2)[a]
Sulfate					
0.1 m*M*	7.7 ± 2.3 (2)[a]	1.0 ± 0.3 (4)	1.54 ± 0.14 (3)[c]	0.82 ± 0.13 (4)	84 ± 66 (2)
1 m*M*	6.2 ± 0.9 (2)[b]	1.25 ± 0.18 (3)[b]	1.58 ± 0.08 (3)[d]	0.83 ± 0.06 (2)	128 ± 37 (2)[b]
10 m*M*	5.9 ± 1.1 (2)[a]	1.17 ± 0.4 (5)	1.50 ± 0.07 (3)[d]	0.47 ± 0.05 (2)	447 ± 142 (2)[b]

Note. S_m, K_s, and K_{ses} are in m*M*, (h) is dimensionless, V_M is in nmol/min/mg gut. Means ± SD for N independent determinations with $n = 6$ points each, i.e., $N \times n$ measurements. Comparison with control values (t test): [a]$0.05 < P < 0.01$; [b]$0.01 < P < 0.05$; [c]$0.001 < P < 0.01$; [d]$P < 0.001$.

a more marked way with sulfate. This indicates a progressive quenching of the substrate inhibition.

Figure 5 presents the chromatographic profiles, with a final fivefold rate of purification, and one major molecular species, corresponding well to the single electrophoretic form (not provided) as previously isolated (Bounias, 1978). The kinetic analysis of the native molecule gave quite similar patterns as did crude extracts, indicating that the enzyme

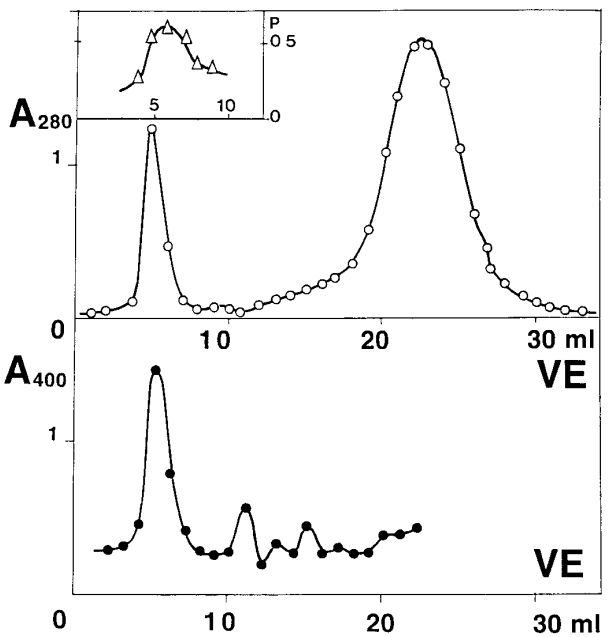

FIG. 5. Elution profiles (VE, volume of elution in ml) of honeybee gut alkaline phosphatase activity and protein concentrations on Bio-Gel P-6-DG. (●) Enzymatic activity (A_{400}, absorbance of released pNP at 400 nm). (○) Protein absorbance (A_{280}) at 280 nm. (△) Protein concentrations (mg per tube) determined from Lowry *et al.* (1951).

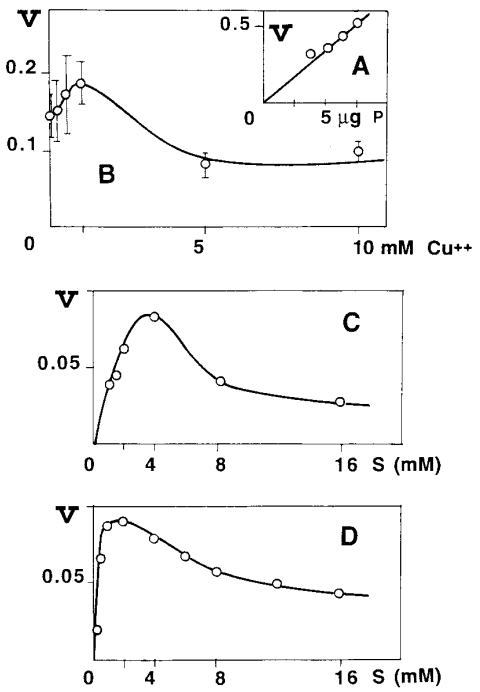

FIG. 6. (A–B) Variation of the velocity (v, μmol/min/mg of proteins) of partly purified honeybee gut alkaline phosphatases versus: (A) concentrations of protein extracts (μg) and (B) cupric gluconate concentrations at S = 4 m*M*. (C–D) Variation of the velocity of partly purified honeybee gut alkaline phosphatases versus substrate concentrations (S, m*M*): (C) controls and (D) in presence of 1 m*M* cupric gluconate.

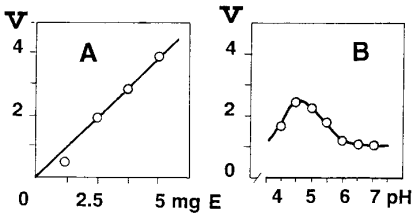

FIG. 7. (A) Variation of honeybee gut acid phosphatase activity (nmol/min) versus concentrations of gut extract (E, mg/ml). (B) Velocity (nmol/min/mg gut) versus pH.

activity was not altered by other ionic species that could eventually have been present in crude hemolymph.

The reaction was fairly linear with enzyme concentrations (Fig. 6A) and exhibited a markedly biphasic response to cupric gluconate (Fig. 6B) with a maximum activation at 1 m*M* by a factor $f = 1.4$. The kinetic parameters calculated from saturation curves of controls (Fig. 6C) and in presence of 1 m*M* cupric gluconate (Fig. 6D) gave similar values and variations with cupric ions for both purified and crude enzyme, and confirmed the observed increase of the Hill coefficient and constant K_{ses}.

Acid Phosphatases

Figure 7 presents the dependence of enzymatic activity on extract concentrations (Fig. 7A), with linearity up to 5 mg per 250 μl, and on pH values (Fig. 7B), with the maximum velocity attained at pH = 4.5. The kinetic study of controls (see Fig. 9A) gave the final data indicated in Table 3.

Dose-related variations of acid phosphatase activity at $S = 4$ m*M* (Fig. 8A) demonstrate a monotonic increasing curve, while at $S = 8$ m*M* the response was biphasic (Fig. 8B). All parameters were thus studied at 1, 10, and 25 m*M* cupric gluconate. The experimental data are depicted in Fig. 9, and the calculated values are indicated in Table 4. By comparison with the alkaline form, acid phosphatases exhibited higher S_m values, without changes except a drop at 25 m*M* cupric gluconate.

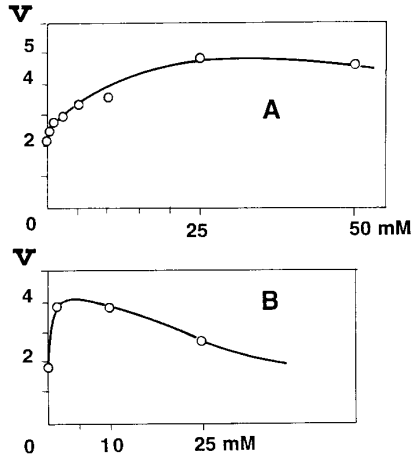

FIG. 8. Variation of honeybee gut acid phosphatase velocity (v, nmol/min/mg) versus concentrations of cupric gluconate at substrate concentrations: (A) $S = 4$ m*M* or (B) $S = 8$ m*M*.

This latter dose also significantly depressed K_s, while an increase of V_M at 1 m*M* was the only other major change, associated with an increase of K_{ses}. This means that, again, the maximum activation was obtained upon partial suppression of the substrate-inhibition process, and that an improvement occurred on the rate of transformation of substrate at the catalytic site level. The remaining activation effect observed at 25 m*M* was due to another mechanism involving a decrease of apparent K_s; that is, an improvement of the binding of substrate to the free enzyme, whose consequences are however attenuated by the lowering of S_m.

Based on defined structural properties, dose–effect relationships finally exhibit a biphasic curve for V_M and a monotonic decreasing function for K_s, as shown by Fig. 10.

The Fate of Biphasic Responses

Biphasic dose–velocity curves were observed first for either crude (Fig. 2) or prepurified extracts (Fig. 6) of alkaline

TABLE 3
Apparent Kinetic Parameters Determined in Alkaline Phosphatases from Purified Extracts of Honeybee Guts

	S_m	h	V_M	K_S	K_{ses}
Controls	3.0 ± 0.8	0.70 ± 0.05	0.11 ± 0.04	1.5 ± 0.1	3.1 ± 0.5
	(3)	(7)	(4)	(3)	(3)
Cupric gluconate, 1 m*M*	1.5 ± 0.02	1.54 ± 0.48	0.15 ± 0.07	0.80 ± 0.67	19.5 ± 13.2
	(2)	(4)	(4)	(3)	(4)
Student's test t (ν)	3.1 (3)	3.2 (9)	0.68 (6)	1.68 (4)	2.37 (5)
P (t)	0.05	0.01	0.52 (NS)	0.17 (NS)	0.06

Note. S_m, K_s, and K_{ses} are in m*M*, h is dimensionless, V_M is in μmol/min/mg proteins. Means ± SD for N independent determinations with $n = 6$ points each, i.e., $N \times n$ measurements. NS, not significant.

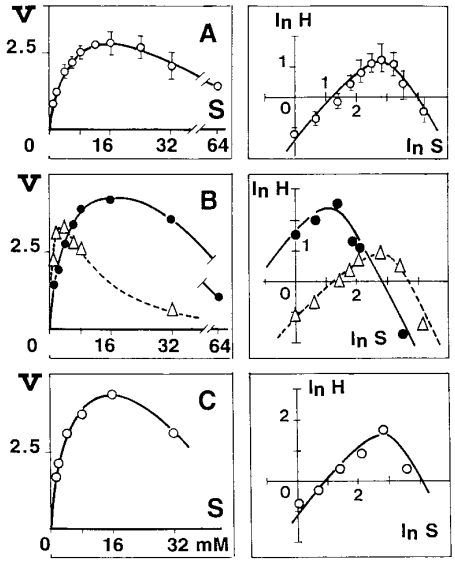

FIG. 9. Variation of honeybee gut acid phosphatase velocity (v, nmol/min/mg) versus substrate concentrations (*S*, m*M*) in presence of various concentrations of cupric gluconate: (A) controls, (B) Cu = 1 m*M* (●) or 10 m*M* (△); (C) Cu = 25 m*M*. Direct plots on left, and Hill plots on right (H = $[v/(V_M - v)]$).

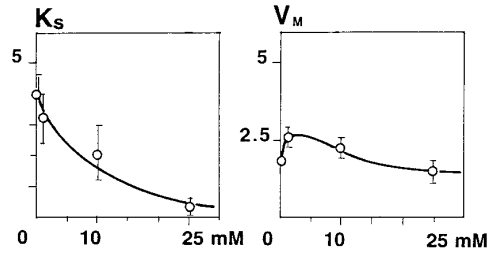

FIG. 10. Dependence of the calculated parameters for acid phosphatase activity. (Left) Apparent substrate constant (K_s, m*M*) and (right) theoretical maximum velocity (V_M, nmol/min/mg) versus concentrations of cupric gluconate (m*M*).

phosphatases with cupric gluconate only, while sulfate only gave an activation (Fig. 2). On the other hand, acid phosphatases exhibited either monotonic or biphasic activation, according to substrate concentrations used in the reaction medium. These discrepancies suggest that it is unlikely that, in the present cases, the biphasic mechanism is a homotropic one, i.e., involving one single species as the effector (Bounias, 1990). Although the activation phases may well result from direct action of cupric ions, the inhibition further appearing at higher cupric ion concentrations could indirectly originate from oxygen free radicals.

The inhibitory action of such radicals on enzymes is now well documented, and the various steps likely involved in the mechanism (see Kruk, 1995, for review) will be presented below.

Figure 11 first presents the chemiluminescence (CL) recorded in presence of cupric sulfate and gluconate, either alone or with pNPP. The cupric sulfate system emits somewhat stronger and with a different shape than gluconate, but the emissions are very low, although the addition of pNPP markedly increased the CL yield. The involved emitter is likely ($^1O_2$), which can be confirmed by using scavengers of ($HO^\bullet$) and ($O_2^{\bullet -}$) radicals as follows.

Figure 12 then indicates that in both of the investigated systems, the CL intensity is well decreased by the ($HO^\bullet$) scavengers, whereas more weakly by SOD. The quenching effects were more marked with the cupric sulfate system.

Interestingly, *p*-nitrophenol, the product of the reaction, strongly quenched the CL intensity obtained with cupric sulfate, whereas it increased the light emission associated with cupric gluconate. The mechanisms can be hypothesized as follows.

Let R−SH a reducing thiol. Then, the oscillations between the alternative valences of cupric ions allow a sequence of reactions to occur:

TABLE 4
Apparent Kinetic Parameters Determined in Acid Phosphatases from Crude Extracts of Honeybee Guts

	S_m	*h*	V_M	K_S	K_{ses}
Controls	15.0 ± 3.4	0.99 ± 0.26	3.75 ± 0.41	4.0 ± 0.7	70 ± 17
	(5)	(5)	(5)	(4)	(3)
Cupric gluconate					
1 m*M*	14 ± 1	0.86 ± 0.11	5.1 ± 0.5	3.25 ± 0.9	20.1 ± 3.5
	(2)	(2)	(2)[a]	(2)	(2)[b]
10 m*M*	15 ± 1	0.85 ± 0.24	4.5 ± 0.7	2.13 ± 1.06	35.9 ± 8.6
	(2)	(3)	(2)	(3)[a]	(2)
25 m*M*	3.25 ± 0.25	1.25 ± 0.54	3.04 ± 0.77	0.25 ± 0.05	97.7 ± 13.4
	(2)[c]	(2)	(3)	(2)[c]	(2)

Note. S_m, K_S, and K_{ses} are in m*M*, *h* is dimensionless, V_M is nmol/min/mg gut. Means ± SD for *N* independent determinations with $n = 6$ points each, i.e., $N \times n$ measurements. Comparison with control values (*t* test): [a] $0.05 < P < 0.01$; [b] $0.01 < P < 0.05$; [c] $P < 0.001$.

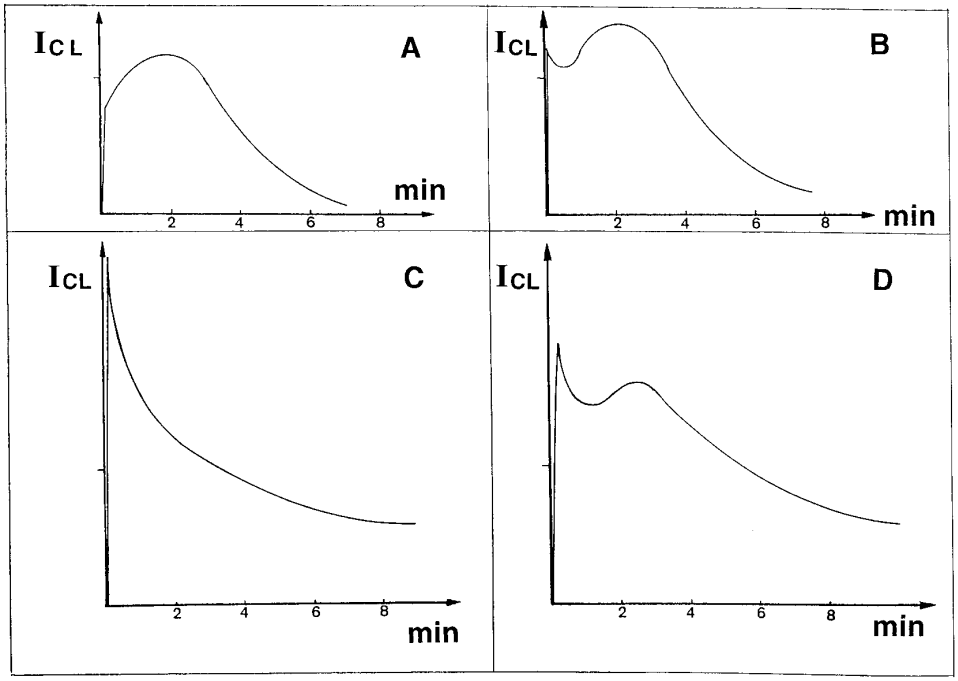

FIG. 11. Kinetic study of I_{CL} (here expressed in arbitrary units) induced by the addition of 1 m*M* H_2O_2 to a 10 m*M* solution of: (A) cupric gluconate in carbonate buffer, pH 9.2, (B) buffered cupric gluconate + 4 m*M* *p*-nitrophenylphosphate, (C) buffered cupric sulfate, or (D) buffered cupric sulfate + 4 m*M* *p*-nitrophenylphosphate. Temperature 310 K.

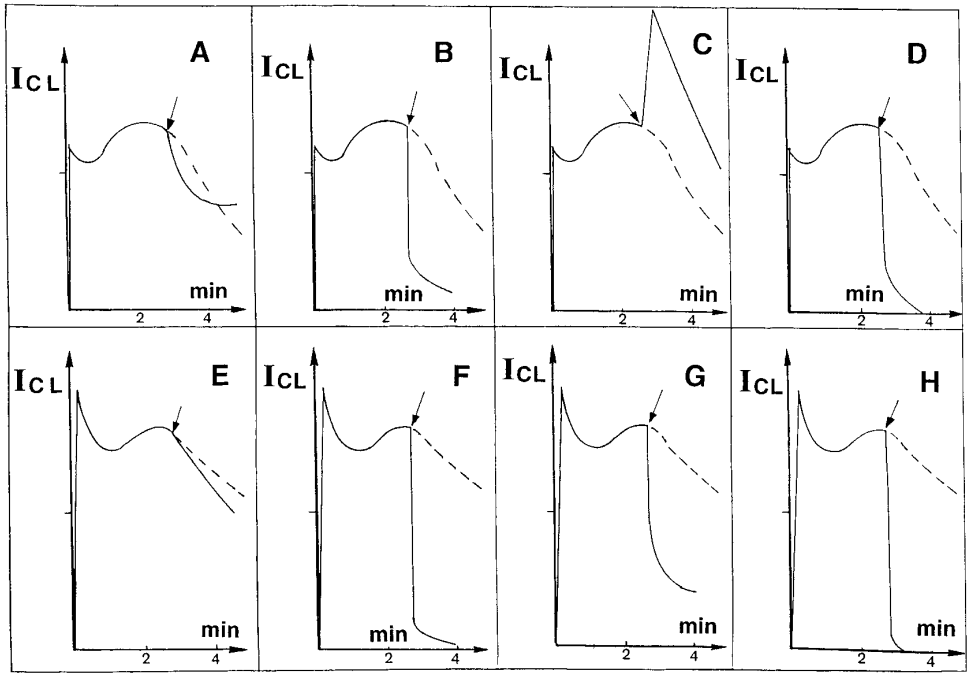

FIG. 12. The effects of HO˙ radical inhibitors of *p*-nitrophenol and of SOD on the I_{CL} (in arbitrary units) recorded from the following system: {10 m*M* cupric gluconate + 4 m*M* *p*-nitrophenylphosphate + buffer, pH 9.2 + 1 m*M* H_2O_2}. (A) SOD 100 μg/ml, (B) thiourea 0.5 m*M*, (C) *p*-nitrophenol 1 m*M*, and (D) glutathione 5 m*M*. Temperature 310 K. (E, F, G, H) The effects of the same inhibitors on the alternative system prepared with cupric sulfate. Arrows indicate the addition of quenchers.

$$R{-}SH + Cu(II) \rightarrow R{-}S^{\bullet} + Cu(I) + H^{+} \quad (8\text{-}1)$$

$$Cu(I) + O_2 \rightarrow CuO_2 \rightarrow Cu(II) + O_2^{\bullet -} \quad (8\text{-}2)$$

$$Cu(II) + O_2^{\bullet -} \rightarrow Cu(I) + O_2 \quad (8\text{-}3)$$

$$Cu(I) + O_2^{\bullet -} \rightarrow Cu(II) + O_2^{2-} \quad (8\text{-}4)$$

$$O_2^{2-} + 2H^{+} \rightarrow H_2O_2 \quad (8\text{-}5)$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + HO^{\bullet} + HO^{-}. \quad (8\text{-}6)$$

In addition, the role of H_2O_2 in the generation of singlet oxygen can be explained through a recycling reaction in three steps:

$$O_2^{\bullet -} + H_2O_2 \rightarrow HO^{\bullet} + HO^{-} + {}^1O_2 \quad (9\text{-}1)$$

$$O_2^{\bullet -} + HO^{\bullet} \rightarrow HO^{-} + {}^1O_2 \quad (9\text{-}2)$$

$$2O_2^{\bullet -} + 2H^{+} \rightarrow H_2O_2 + {}^1O_2. \quad (9\text{-}3)$$

The first two steps [(9-1) and (9-2)] are the most important $^1O_2$ generators, while $O_2^{\bullet -}$ and $HO^{\bullet}$ are released at relatively high concentrations, although differently in presence of either sulfate or gluconate.

These reactions clearly explain the differences observed for gluconate and sulfate (Fig. 2), with the biphasic effect on alkaline phosphatase specifically occurring for gluconate, due to the further release of oxygen radicals by pNP at higher substrate concentrations. The same explanation holds for acid phosphatase, since in Fig. 8 it clearly appears that a biphasic effect of cupric gluconate is seen at the higher substrate concentration, i.e., at higher rates of *p*-nitrophenol release.

The *in vivo* effects can now be discerned, mainly as a cupric ion stimulation of the enzymes, from the *in vitro* action, where this stimulation is first balanced by pNPP-generated radicals for both cupric sulfate and gluconate. Then, these oxygen-radical emissions are specifically quenched in the presence of cupric sulfate, whereas they are enhanced in presence of cupric gluconate.

DISCUSSION

Two major points are worthy to be raised from these results. First, owing to the purely biochemical viewpoint, the observed copper system-induced changes in the kinetic mechanism of honeybee phosphatases provide further support to their possible influence at active site: interactions with substrate could explain the observed changes in substrate-inhibition features, although K_s is only poorly altered for alkaline forms.

It would be important, indeed, to check for all kinetic parameters in the presence of oxygen-radical scavengers, in order to more accurately determine which of the observed changes reflects the action of a direct binding of copper or indirect effects via radical action. However, such a purpose deserves a thorough preliminary work, in order to select scavengers strictly devoid of any action on the enzyme kinetic parameters. Then, the mechanisms of observed biphasic responses would be better established after the kinetic contribution of oxygen radicals during the inhibition phases has been thus clarified. It is noteworthy, however, that an increase of the Hill coefficient rather seems to be associated with the presence of a sulfate moiety and likely concerns secondary sites, as suggested by the findings of Bertini *et al.* (1989).

A similar case of biphasic effects has been recently reported for fish hepatic alkaline phosphatases: a stimulation at lower copper doses is followed by a further inhibition at excess amounts of metal (Lan *et al.*, 1993). Here, again, the question might be raised of what are the respective contributions of cupric ions, as *in vivo* effector, and of analytical reagents, as *in vitro* modifiers.

Finally, phosphatase activation consistently appears as the major *in vivo* effect that can be expected from field treatments, as already seen in other arthropods by Suresh *et al.* (1993). That copper is able to enhance phosphatase activity suggests its possible involvement in the mode of action of the catalytic site. This hypothesis is strengthened by recent evidence that copper does behave as an effective catalyst of phosphoesterase activities, based on artificial enzyme studies (Kim and Suh, 1994).

The second remark is more closely relevant to field toxicology. That a substrate inhibition appeared during the past decade might reflect a genetic adaptation of bees to the presence of organophosphate pollutants such as pesticides, consistent with the findings of Hoy (1987). On the other hand, although product inhibition (either by phosphate or by pNP-induced radicals) has been demonstrated to be involved in this class of enzymes by Fernley and Walker (1967), Williams and Naylor (1971), and Moran *et al.* (1989), this would not be sufficient to explain the kinetic behavior of the honeybee gut phosphatases.

CONCLUSION

Which effects can actually be expected from field treatments needs to be examined with respect to the doses actually ingested by workerbees in the hives. When 0.44 to 1.1 m*M* solutions are administered in syrup, a bee can absorb about 22 to 55 nmol of cupric derivative (Bounias *et al.*, 1995a). Assuming that the totality of this copper is vehicled by hemolymph, representing about 40 μl in a young worker, the body concentration that will reach cellular sites would be about 1.4 m*M*, which is an activating dose, without blockade to be expected.

In addition, this expected increase in activity during field treatments might be beneficial to honeybee colonies, which are currently subjected to pesticide pollution, via cross-detoxication of phosphatase-susceptible organophosphorous

residues. These remarks, added to the allowance of gluconate as a safe food additive (U.S. Pharmacopeia and Codex, 1981a,b), suggest that cupric gluconate could be classified as one of the safest ways of control of *V. jacobsoni*. It is worthy to add that, as a long-acting treatment, it can be easily used in complement with shock treatments by synthetic miticides. Then, since the latter could thus be used over shorter periods, rather as indicators of parasite population levels, this would finally result in an additional lowering of the risks of toxic side effects of these pesticides.

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