Structure and Activity in Molluscicides II. Activity Assay of Catalase and Peroxidase in Snails

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Catalase and peroxidase, protoporphyrin enzymes present in snails and affected by molluscicidal agents, were studied in some species of snails of biological importance (*Biomphalaria* and *Bulinus* types, intermediate hosts for schistosomiasis, intestinal and renal, respectively). The activity values of these enzymes in snails infected with *miracidia* of *Schistosomes* were less than those for the noninfected ones. Also, determinations were run for snails of *Lymnaea* (intermediate host for cattle liver fluke, *Fasciola hepatica*) and *Physa* types.

SCHISTOSOMIASIS is a world health problem, and after malaria, it is the most widespread disease in tropical and subtropical regions. It infects about 200,000,000 (1) of the world inhabitants causing tremendous losses in the areas it prevails. Unfortunately, the disease is still out of control.

One of the major approaches in the combat of the disease is eradication by molluscicides of snails, the intermediate vector of the parasite.

In spite of the extensive work with molluscicides, no approach has yet been made in the establishment of a structure-activity relationship.

In a previous communication (2) the authors reported the relation between the specificity in structure of molluscicides of phenolic type and the activity of both protoporphyrin enzymes, peroxidase and catalase, within the snail's body. The former enzyme is involved in the biological formation of free radicals from substrates of aromatic amines and phenols.

The color developed, due to the formation of trivalent nitrogen-free radicals by the uninfected snails, from *p*-phenylenediamine dehydrochloride and its derivatives led to a new quick method for identification of infected and noninfected snails (the infected snail does not develop the color or only faintly, depending on the intensity and age of infection).

The activity in phenolic molluscicides depends mainly on the nature of substituents on the ring. This may be explained due to the initial formation of a phenoxy radical through the snails' body enzymes. The subsequent transformation of the radical to the snail toxic quinoid structure is similarly dependent on that nature of substituents on the ring.

In the present work, quantitative microassays for the activity of both enzymes, peroxidase and catalase, were run in different species of snails of biological importance.

For these studies, fresh water snails of *Biomphalaria alexandrina* and *Bulinus truncatus* types were selected. Both types are prevalent in this country and act as intermediate hosts for *Schistosoma mansoni* and *Schistosoma hematobium*, respectively.

Also, the studies were run on snails of *Lymnaea caillaudi* (intermediate host for the cattle liver fluke, *Fasciola hepatica*) and the *Physa acuta* types.

The method used for activity assay of peroxidase, present in snails' whole extract, depended on spectrophotometric measurement for the rate of oxidation of *p*-phenylenediamine as a substrate at the wavelength 485 mμ in a buffered solution at pH 7 (3, 4).

For catalase, the activity assay depended on the spectrophotometric measurements for the rate of decomposition of hydrogen peroxide through the enzyme present in the snails' extract at wavelength 240 mμ in a buffered mixture at pH 7 (5–7).

Determinations were run on snails at two different seasons (summer and winter). Under both conditions the snails, after collection from the field, were directly submitted to assay determinations. The temperature ranged between 33° in summer and 15° in winter.

The results reveal that the activity differs from season to season. In summer the activity is higher than in winter. Also, it differs according to the species in the same season.

These findings agree with the observed changes in the rate of the oxygen consumption by fresh water molluscs which depends largely on the temperature, season, and species. Species studied showed increase in the oxygen consumption with increasing temperature, but the precise relationship varies from species to species and at different seasons within a species (8–10).

Also, activity determinations were run on snails of the *Biomphalaria* type infected with the *miracidia* of *Schistosoma mansoni*. It had been observed that the activity for both enzymes is less in the infected snails than the noninfected ones. These findings may be rationalized since the *Schistosomes* live anaerobically (11). They habitate the liver in the snail's body, and may tend to destroy, as a defense mechanism, any enzyme or biological factor that would furnish free oxygen in their surroundings as this would interfere with their anaerobic metabolism.

Also, it can be due to damage in function of organs in the snail's body, due to infection, involved in the biogenesis of both enzymes.

The studies for the end transformation products with their intermediate for each of the important molluscicides *in vivo* and *in vitro* by both enzymes are now under investigation and will be reported later.

EXPERIMENTAL

All spectrophotometric measurements were run on a Unicam SP 500 spectrophotometer using cells with a 1-cm. light path. Snails were freshly collected from the field.

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Extract Preparation

A known weight of snails of the same type was homogenized in a blender with 0.006 *M* phosphate buffer, pH 7 (Solution I diluted 1:10) at 1–4°, then centrifuged (3,500 T/min., for 10 min.). The sediment was stirred with cold phosphate buffer 0.006 *M* and allowed to stand in the cold with occasional shaking. The extraction process was repeated three times. The whole extract was cleaned through centrifugation several times. The final volume of the extract was recorded. The extraction did not take longer than 24 hr.

Activity Measurements

Catalase—*Reagents*—potassium dihydrogen phosphate, A.R., KH_2PO_4; disodium hydrogen phosphate, A.R., $Na_2HPO_4.2H_2O$; hydrogen peroxide, *ca.* 30% (w/v).

Preparation of Solutions—Solution I: phosphate buffer (0.06 *M*, pH 7): 3.522 g. KH_2PO_4 and 7.268 g. $Na_2HPO_4.2H_2O$, were dissolved in doubly distilled water and made up to 1,000 ml. Solution II: hydrogen peroxide–phosphate buffer (0.06 *M* phosphate, *ca.* 1.25×10^{-2} *M* H_2O_2 pH 7). 0.16 ml. H_2O_2 30% (w/v) was diluted to 100 ml. with buffer (Solution I). The absorbance of this solution should be about 0.500 at 240 mμ with a 1-cm. light-path cell. This solution should be freshly prepared.

Procedure—Measurements were carried at wavelength 240 mμ in silica cells of 1-cm. light path, the final volume was about 3 ml. against a control (cell containing H_2O_2-free phosphate buffer, Solution I).

In the experimental cell, 3 ml. hydrogen peroxide–phosphate buffer (Solution II) was placed with about 0.05 ml. extract. The time at (Δ) required for a decrease in the absorbance from 0.450 to 0.400 was recorded; if it was longer than 60 sec. the measurement was repeated with a more concentrated solution of the sample.

Calculations (*cf. Reference 12*)—The activity = $17/\Delta t$ units per assay mixture in the cell, where one unit is defined as "the amount of enzyme which liberates half the peroxide oxygen from a hydrogen proxide solution of any concentration in 100 seconds at 25°C. (7)."

Table I illustrates results obtained for catalase activity[1] in different species of snails.

Peroxidase—*Reagents*—Hydrogen peroxide about 30% (w/v); *p*-phenylenediamine; potassium dihydrogen phosphate, A.R., KH_2PO_4; disodium hydrogen phosphate, A.R., $Na_2HPO_4 \cdot 2H_2O$.

Preparation of Solutions—All solutions were prepared in bidistilled water. (*a*) Hydrogen peroxide (3×10^{-3} *M*). Stock solution: 0.66 ml. of hydrogen peroxide (30% w/v) was diluted to 200 ml. Before use this stock solution was diluted 1:10. (*b*) *p*-Phenylenediamine (1% w/v), 1 g. of *p*-phenylenediamine was dissolved in about 60 ml. hot bidistilled water, then filtered and the filter was washed with about 25 ml. The total filtrate was quickly cooled and diluted to 100 ml. (*c*) Phosphate buffer (0.067 *M* pH 7), 3.522 g. KH_2PO_4 and 7.268 g. $Na_2HPO_4.2H_2O$ were dissolved in water and made up to 100 ml.

Table II illustrates results obtained for catalase activity in different species of snails.

Spectrophotometric Measurements—Wavelength 485 mμ, 1-cm. light path, final volume in the cell *ca.* 3.5 ml. Control cell as for experimental cell but containing bidistilled water instead of hydrogen peroxide. One-milliliter sample, 2 ml. phosphate buffer (III), 0.1 ml. H_2O_2 (I), and 0.1 ml. *p*-phenylenediamine (II) were pipeted successively into the cell and thoroughly mixed. Using a stopwatch, the absorbance after 30 sec., 15 sec., and 3 min. was recorded.

Calculation (*cf. Reference 13*)—*K''* activity per 1-g. snails

$$\frac{\Delta E \times \text{volume of enzyme extract}}{\Delta t \quad \text{weight of snails}}$$

where ΔE = change in absorbance during limited period of time (ΔE) and Δt = measured time reaction.

[1] The activity is in units (7) per g. snails.

TABLE I—CATALASE ACTIVITY IN DIFFERENT SPECIES OF SNAILS[a]

Type	Activity[b]	Temperature, °C.
Biomphalaria alexandrina	25.87	33
	24.207	20
(Infected)	13.26	21.5
	11.53	20
Bulinus truncatus	58.43	33
	36.33	30
	27.67	15
Lymnaea caillaudi	29.27	33
	21.67	27
	19.174	20
Physa acuta	44.35	33
	30.78	30
	28.019	20
	27.58	18

[a] Results are mean values for duplicate determinations of duplicate samples. [b] These units can be converted to Kat. f. if multiplied by 10.8 (12).

TABLE II—PEROXIDASE ACTIVITY IN DIFFERENT TYPES OF SNAILS[a]

Type	Activity[b] per g. Snails	Temperature, °C.
Biomphalaria alexandrina	754×10^{-5}	28
	682×10^{-5}	18
(Infected)	546×10^{-5}	21.5
	462×10^{-5}	18
Bulinus truncatus	513×10^{-5}	20
	452×10^{-5}	15
Lymnaea caillaudi	451×10^{-5}	27
	422×10^{-5}	20
Physa acuta	264×10^{-5}	20
	245×10^{-5}	18

[a] Results are mean values for duplicate determinations of duplicate samples. [b] The peroxidase activity unit is defined as the amount of purpurogallin (mg.) which is formed in 5 min. at 25° from 1.25 g. pyrogallol in 500 ml. solution containing 12.5 mg. H_2O_2 (13).

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Keyphrases

Molluscicides—structure, activity relationship
Snails, *Schistosoma masoni* infection—analysis
Peroxidase activity—*p*-phenylenediamine oxidation
Colorimetric analysis—spectrophotometer
Catalase activity—hydrogen peroxide decomposition
UV spectrophotometry—analysis

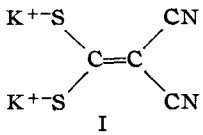
Antiradiation Compounds X. Derivatives of 3,3-Dimercaptoacrylonitrile

By WILLIAM O. FOYE and JOEL M. KAUFFMAN

The dipotassium salt of 2-cyano-3,3-dimercaptoacrylonitrile was found to give fair protection to mice *versus* X-rays. Derivatives prepared for further radioprotective evaluation include dialkyl thioethers, metal complexes, and fused heterocyclic dithioles. A series of 3,3-dimercaptoacrylonitriles bearing aromatic and heterocyclic rings was also obtained from the condensation of arylacetonitriles with carbon disulfide in the presence of alkoxide ion. Of these various derivatives, the zinc complex and the benzoyl derivative showed some radioprotective properties.

Condensations of carbon disulfide with certain amines and mercaptans have given dithiocarbamates (1, 2) and trithiocarbonates (3) with radioprotective activity. In order to determine whether nonbasic compounds, unrelated to the 2-mercaptoethylamine structure, having a dithio acid group may have radioprotective properties, condensations of a number of active methylene compounds with carbon disulfide have been carried out. Some of the products obtained have been examined for radiation protection in mice. Radiation protection of animals by dithio acids has not been previously reported.

Reaction of malononitrile with carbon disulfide is known to give the dipotassium salt of 2-cyano-3,3-dimercaptoacrylonitrile (4) (I). Brown's procedure was repeated, utilizing potassium hydroxide in 95% ethanol,



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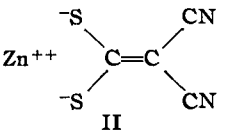

II

to give a product melting at 313–316° (dec) (m.p. previously listed as > 250°) with loss of water below 300°. Analysis indicated a monohydrate, and the infrared absorption spectrum showed the presence of water and C≡N, C=C, and C—S bonds (960 and 872 cm.$^{-1}$) (5). A trihydrate of the sodium salt of this compound has been reported by Soderback (6). Antiradiation tests carried out at the Walter Reed Army Institute of Research[1] showed this compound to give fair protection to mice *versus* 825 r (X-rays) at a dose level of 350–700 mg./kg. Accordingly, derivatives of this compound have been prepared as well as analogs in which one of the cyano groups was replaced, primarily by aromatic rings.

A zinc complex (II) was obtained as the hemihydrate which was much less water soluble than the potassium salt. The infrared spectrum was similar to that of the potassium salt, except that the absorption due to CS_2 appeared as a wide doublet centered at 880 cm.$^{-1}$. A cupric complex was also obtained as the hemihydrate, extremely insoluble in water, but a ferrous complex was too rapidly oxidized to be characterized.

The dimethylthioether and diethylthioether are known compounds (7) and were prepared for testing, but the monomethyl- or monoethyl-thioether could not be isolated. The dibutylthioether could not be crystallized or purified as well. Condensations of I with 2,3-dichloropyrazine and 2,3-dichloroquinoxaline were successful, however, giving pyrazino-[2,3-*d*]-1,3-dithiole-$\Delta^{2,\alpha}$-malononitrile (III) and quinoxalino[2,3-*d*]-1,3-dithiole-$\Delta^{2,\alpha}$-malononitrile.

Carbon disulfide condensation with cyanoacetamide to give IV*a* has been reported (4), using the hydroxide–ethanol method. An attempt to repeat

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