TABLE IV—DISTRIBUTION OF THIAMINE HYDROCHLORIDE AND MONONITRATE BETWEEN WATER AND CHLOROFORM (AT VARIOUS pH VALUES) FOR THE DETERMINATION OF DISTRIBUTION COEFFICIENT[a]

pH	% Thiamine Salt in Chloroform[b]	% Thiamine Salt in Water (Total)	% Thiamine Salt in Water (Undiss.[c])	D
Thiamine HCl				
8	0.8	99.2	99.2	0.008
6	0.6	99.4	93.8	0.006
2	0.0	100.0	0.0	0.00
Thiamine Mononitrate				
8	1.2	98.8	98.8	0.012
6	0.8	99.2	93.6	0.008
2	0.0	100.0	0.0	0.00

[a] Each value is an average of three experiments. [b] Calculated by difference. [c] Calculated using Henderson-Hasselbach equation.

To determine the D for thiamine, 15 mg. of thiamine hydrochloride or thiamine mononitrate was dissolved in 25.0 ml. of the buffer solution of appropriate pH and shaken vigorously with 25.0 ml. of chloroform in a 125-ml. separator for 1 min. The phases were allowed to separate and the thiamine content in the aqueous phase was determined using the method described earlier (1). To determine the distribution coefficient, the concentration of undissociated thiamine in the aqueous phase was calculated using the Henderson-Hasselbach equation, and the concentration of thiamine in the chloroform phase was calculated by difference. Results are presented in Table IV.

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Keyphrases

Thiamine—bromothymol blue salt
Extraction—thiamine dye salt
Bromothymol blue concentration—salt extraction
pH effect—salt extraction
Equation—pH dependence of salt extraction

Structure and Activity Relationships in Molluscicides

By I. NABIH* and M. T. ELWASIMI

Noninfected snails of the *Biomphalari* and *Bulinus* types (intermediate hosts for *Schistosoma mansoni* and *Schistosoma hematobium*, respectively) can oxidize instantly and intensively *p*-phenylenediamine dihydrochloride and its derivatives to the corresponding colored trivalent nitrogen-free radicals. This reaction fails to occur with snails infected with miracedia of *Schistosomes*. The reaction is inhibited through inhibitors specific for enzymes of the protoporphyrin type, also through the potent molluscicide (bayluscide). Studies revealed the enzyme involved in the reaction is the peroxidase, along with the catalase. Both enzymes were found to be present in both types of snails.

BILHARZIASIS is a world health problem; it infects millions of people all over the world and causes a tremendous loss in economy and manpower.

To bring the disease under control different aspects would be encountered, mainly the irradication of snails, the intermediate vector of the parasite, through the application of molluscicides. The chemotherapy of the disease is still inadequate particularly in the areas where infection is widely distributed, due to the serious side effects the available agents exhibit when given either orally or by injection.

In the development and search for new molluscicides, extensive work that included investigations of thousands of compounds (mostly of phenolic type) was carried out (1–3). These investigations showed that activity of these phenolic compounds depends upon the nature of substituents on the aromatic ring. The most active were pentachlorophenol and 2,4-dinitro-6-

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cyclohexylphenol, whereas those bearing bulky groups on the ring were inactive, *e.g.*, 2,6-di-*t*-butylphenol. Also compounds of the quinoid structure such as benzoquinone and naphthoquinone showed significant activity. Of the aminophenols, 4-aminophenol was active while 2- and 3-aminophenols were inactive. The sample of 4-aminophenol used by the investigators was described to be colored, thus suggesting the transformation to quinoimine or benzoquinone. It had also been suggested that it may be worthwhile to consider the possibility of the conversion of those active phenols within the snails' body to quinoid-type substances with the toxic effect due to that type (3).

Chemically, many phenols can be oxidized to form a phenoxy radical, which may lead to a quinoid-type structure through dimerization or disproportionation. The transformation depends upon the nature of the substituents on the aromatic ring, *e.g.*, 4-substituted 2,6-di-*t*-butylphenoxy radical is a reasonably stable species in solution (4), also the dimerization of 2,4,6-tri-*t*-butylphenoxy radical is inhibited through the bulky *t*-butyl groups (5). This may explain the failure of those phenols bearing bulky groups to show any molluscicidal activity as the apparent biologically formed phenoxy radical, that may undergo the subsequent transformation to the quinoid structure, is prohibited by these bulky substituents on the ring.

This correlation between the molluscicidal activity of those types of phenols bearing nonbulky groups and the chemical oxidation of related types to quinones *via* free radical suggested the present work, to study the possibility of the biological formation of free radicals by snails and to detect the biological system involved in this process.

p-Phenylenediamine and its derivatives were chosen as substrates as they can be chemically oxidized to the colored trivalent nitrogen-free radicals that include the so-called "Wurster's salts." The color formation renders a possible spectrophotometric means to detect and follow the different effects on the reaction. These radicals are formed, chemically, through the oxidation of *p*-phenylenediamine derivatives, *e.g.*, with bromine (6).

Two representatives of this group, namely *p*-phenylenediamine dihydrochloride (I) and *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride (II) were chosen. Both are readily soluble in water and can be prepared in a high state of purity by methods described by Nabih (7) and Michaelis (6), respectively.

Both compounds were submitted independently in appropriate dilutions to the biological action of two types of freshly collected water snails acting as the intermediate hosts for *Schistosoma mansoni* and *Schistosoma hematobium*, namely *Biomphalaria alexandrina* and *Bulinus truncatus*, respectively.

Both types can oxidize I instantly in the typical transformation pathway that usually follows when it is chemically oxidized with bromine, a transient yellow color forms, turning to green. Spectroscopic measurements showed the product to be identical with that obtained through the chemical oxidation, having absorption maxima at 462 mμ and 479 mμ (8).

Also, both types of snails were able to oxidize II to an intensive red color. Spectroscopic measurements showed the product to be identical with the corresponding free radical obtained when II was submitted to the chemical oxidation with one equivalent of bromine. It showed absorption maxima at wavelengths of 515 and 550 mμ (8).

When an excess of snails was used in the reaction, then the color of the solution faded and gradually disappeared. The same disintegration of color occurred when its solution was treated with a second equivalent of bromine to change it to the corresponding *p*-quinonediimine form (9) (Fig. 1).

When these experiments were applied on snails of the *Biomphalaria* type infected with *miracidia* of *Schistosome* using II as the substrate, the characteristic red color of the free radical either developed in a slow and faint pattern or did not at all (Fig. 2). This technique offered an easy and rapid way to differentiate between infected and noninfected snails when collected in patches from the field.

These findings suggested the presence of the enzyme peroxidase in the snail's body that may be involved in these transformation processes.

Peroxidase is an enzyme that contains a ferriprotoporphyrin hematin prosthetic group. It acts on hydrogen peroxide to cause catalytic oxi-

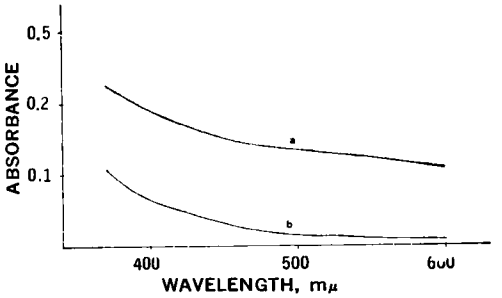

Fig. 1—Further oxidative effect on a solution of II. Key: a, Biomphalaria *snail;* b, *a 2nd equivalent of bromine.*

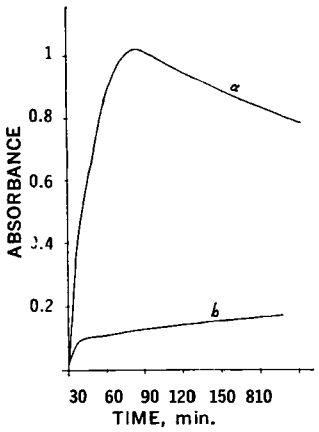

Fig. 2—Rate of color development in a solution of II. Key: a, *noninfected* Biomphalaria *snail;* b, *infected* Biomphalaria *snail.*

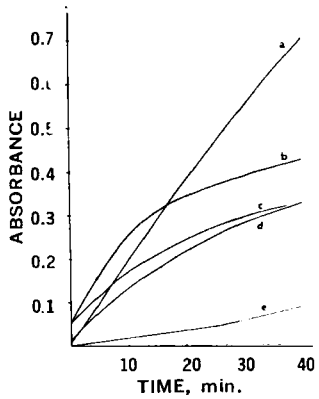

Fig. 3—Rate of color development in a solution of II by Biomphalaria *snails in presence of different inhibitors. Key:* a, *without;* b, *azide;* c, *bayluscide;* d, *cyanide;* e, *sulfide.*

dation of many organic compounds of the amine type, *e.g.*, *p*-phenylenediamine (10), phenolic type (11), and hydroquinones (12, 13).

It catalyzes generally the reaction:

$$AH_2 + H_2O_2 \rightarrow 2H_2O + A$$

The proposed mechanism of action goes through a free radical intermediate (14).

The authors' studies showed that cyanide, sulfide, and azide, inhibitors specific for enzymes of the protoporphyrin type, could inhibit the foregoing reactions when applied under the same experimental conditions on solutions of II (Fig. 3).

They also tried the inhibitory effect of the potent molluscicide, 5,2′-dichloro-4-nitrosalicylanilide (bayluscide) which showed an inhibitory effect on the reaction.

Further, when freshly collected snails of both types were placed in a hydrogen peroxide solution of an appropriate dilution ($1 \times 10^{-3}M$) evolution of gaseous oxygen took place. This suggested the presence of the enzyme catalase, beside the peroxidase. Both enzymes are closely related and are referred to as hydroperoxidases as both use hydrogen peroxide as a common substrate. Snails infected with *Schistosoma mansoni* did not show the same decomposing effect on hydrogen peroxide as the noninfected snails (Fig. 4).

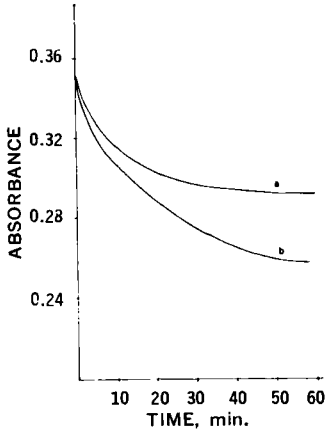

Fig. 4—Rate of decomposition of hydrogen peroxide. Key: a, *infected* Biomphalaria *snail;* b, *noninfected* Biomphalaria *snail.*

The inhibitory effect of some agents that can block the catalytic decomposition of hydrogen peroxide to its components (oxygen and water) have been investigated in connection with the snails' reaction, including cyanide, azide, sulfide, fluoride, hydroxylamine, and bayluscide.

Estimations of the rate of decomposition were made from titrimetric and spectrophotometric measurements for the residual peroxide.

These studies suggest the presence of both enzymes, peroxidase and catalase, in the snail's body, which are the specific catalysts for the foregoing reactions. The former is apparently involved in the biological oxidation of compounds of phenolic and amine types to quinones through the initial formation of free radicals within the snail's body. The quantitative assays for each in some different species of snails are now under determination and will be reported later.

MATERIALS AND METHODS

Preparation of Materials

p-Phenylenediamine Dihydrochloride (I)—Prepared in a high state of purity either by the method described in (7) or through the direct passage of dry HCl gas in absolute ethanol solution of pure *p*-phenylenediamine, then purified and recrystallized (6).

N,N-Dimethyl p-Phenylenediamine Dihydrochloride (II)—It could be obtained in a pure form by the passage of dry HCl gas in dry ether solution of

the free base, followed by purification and recrystallization (6).

The solutions of I and II used in all experiments were freshly prepared in bidistilled water solution.

All spectrophotometric measurements were run on a Unicam Sp. 500 spectrophotometer and cells with a light path of 1 cm.

The snails used were freshly collected from the field.

Oxidative Effect of Biomphalaria Snails on I

One snail of the *Biomphalaria* type (diameter 9 mm.) was immersed in 20 ml. (1×10^{-3} *M*) solution of I. The color developed instantly; after 2 min. the snail was removed and the spectroscopic measurements were run.

These measurements showed peaks of maximum absorption at 462 and 479 mμ (6).

The same peaks appeared when the measurements were run on the formed colored solution when a *Bulinus* snail (7 mm. diameter) was treated as above, with *Biomphalaria*, using I ($1 \times 10^{-3}M$) solution, as substrate.

Oxidative Effect of Biomphalaria Snails on II

One snail of the *Biomphalaria* type (diameter 7 mm.) was immersed in 20 ml. ($1 \times 10^{-3}M$) solution of II; after 2 min., the snail was removed and the absorption spectrum for the developed color was determined. Two peaks showed at 515 and 550 mμ (6).

The same peaks appeared when a snail of the *Bulinus* type (7 mm. diameter) was similarly treated as above using II in solution as substrate.

Further Oxidative Effect of Biomphalaria Snails on II—Fifty snails of nearly equal diameters (mean diameter 1 cm.) immersed in 20 ml. ($1 \times 10^{-3}M$ solution) of II. After 10 hr. the solution became colorless and spectrophotometric measurements were run (Fig. 1-*a*). Parallel, a bromine water solution was added to 10 ml. (1×10^{-3} *M* solution) of II till the red color disappeared, then spectrophotometric measurements were run (Fig. 1-*b*).

Oxidative Effect of Noninfected and Infected Biomphalaria Snails on II—A noninfected snail (8 mm. diameter) was immersed in 10 ml. ($1 \times 10^{-3}M$ solution) of II; the variation in absorbance was measured against time at wavelength 515 mμ (Fig. 2-*a*). The same was run using an infected snail (8 mm. diameter) (Fig. 2-*b*). Similar results were obtained with noninfected and infected snails of the *Bulinus* type.

The Inhibitory Effect of Some Agents on Color Development with II Using Biomphalaria Snails—The color developed by II using *Biomphalaria* snails is compared in five experiments using one snail (1 mm. diameter each) for each experiment:

a, 5 ml. of II ($1 \times 10^{-3}M$) + 5 ml. bidistilled water
b, 5 ml. of II ($1 \times 10^{-3}M$) + 5 ml. sodium azide ($1 \times 10^{-2}M$)
c, 5 ml. of II ($1 \times 10^{-3}M$) + 5 ml. *bayluscide* ($1 \times 10^{-3}M$)
d, 5 ml. of II ($1 \times 10^{-3}M$) + 5 ml. KCN ($1 \times 10^{-4}M$)
e, 5 ml. of II ($1 \times 10^{-3}M$) + 5 ml. Na_2S ($2 \times 10^{-4}M$)

Spectrophotometric measurements were run every 10 min. (Fig. 3) at a wavelength of 515 mμ.

Decomposing Effect of Noninfected and Infected Snails of Biomphalaria Type on H_2O_2 Solution—A noninfected snail (8 mm. diameter) was immersed in 10 ml. ($1 \times 10^{-3}M$ solution) of H_2O_2. An infected snail (8 mm. diameter) was immersed in 10 ml. ($1 \times 10^{-3}M$ solution) of H_2O_2. Spectrophotometric measurements, at a wavelength of 240 mμ, were carried out every 5 min. for both solutions. The results show that the rate of decomposition with the infected snail is slower than with the noninfected one (Fig. 4).

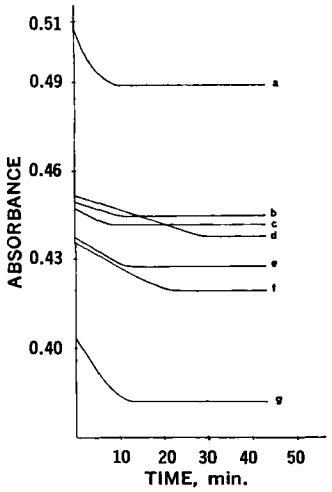

Fig. 5—Rate of decomposition of hydrogen peroxide by Biomphalaria *snails in presence of different inhibitors. Key:* a, *sulfide;* b, *azide;* c, *bayluscide;* d, *fluoride;* e, *hydroxylamine;* f, *cyanide;* g, *without.*

Rate of Decomposition of Hydrogen Peroxide by Fresh Snails and its Inhibition by Different Inhibitors—Decomposition was spectrophotometrically measured through changes in the absorbance at 240 mμ, using for each measurement one snail of *Biomphalaria* type (8 mm. diameter) + 5 ml. H_2O_2 ($1 \times 10^{-2}M$) + 5 ml. of the inhibitor of the following concentrations (Fig. 5):

a, Na_2S ($2 \times 10^{-5}M$)
b, Sodium azide ($2 \times 10^{-3}M$)
c, Bayluscide ($2 \times 10^{-4}M$)
d, Sodium fluoride ($2 \times 10^{-3}M$)
e, Hydroxylamine hydrochloride ($2 \times 10^{-3}M$)
f, Potassium cyanide ($2 \times 10^{-5}M$)
g, Bidistilled water as control.

The results show that the rate of decomposition of the peroxide is depressed through the presence of the inhibitors.

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Keyphrases

Molluscicides—structure-activity relationship
Snails—species of *Biomphalaria, Bulinus*
Schistosoma—infecting organism
P-Phenylenediamine—color reagent
Colorimetric analysis—spectrophotometry

Salt Effects in the Spectrophotometric Examination of a Complex of *N'*-Methylnicotinamide Cation

By DANA BROOKE* and DAVID E. GUTTMAN†

The extent of complex formation, as determined spectrally, between the *N'*-methylnicotinamide cation and the electron donor, 8-chlorotheophyllinate anion, was found to vary greatly when different salts were used to adjust ionic strength. The degree of complex formation when sodium perchlorate was used was found to be much smaller than when sodium acetate was used. An examination of these salt effects indicated that the observed behavior was explicable on the basis that ion pair formation occurred between the pyridinium cation and perchlorate anion.

THE NECESSITY of maintaining a constant ionic strength in studying the behavior of ionic species in solution is widely recognized. It is not, perhaps, so widely appreciated that a so-called inert salt introduced for this purpose can exert an effect quite different from that anticipated solely on the basis of ionic strength variation. Recent studies in this laboratory involving the interaction of *N'*-methylnicotinamide cation (NMN) with 8-chlorotheophyllinate anion (CT) have demonstrated quite dramatically that neutral salts can influence observed behavior independently of their contribution to the maintenance of ionic strength. As will be seen, the extent of interaction of NMN with CT, as detected spectrally, was found to vary considerably depending on whether sodium acetate or sodium perchlorate was used to maintain a constant ionic strength. The observed behavior can be reasonably explained on the basis that extensive ion pair formation occurred between perchlorate anion and NMN.

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Ion pairing of quaternary nitrogen compounds is well documented. For example, Azzari and Kraus (1) reported the formation of ion pairs between the tetrabutyl ammonium cation and such anions as iodide, bromide, and nitrate in water at 25°. Fuoss (2) showed that ionic associations could be treated by mass law considerations, and he and his co-workers (3) reported the ion pair formation constant for the tetraethyl ammonium-picrate ion pair to be 0.8 L. mole^{-1} in water. Kosower (4), in a description of charge transfer interactions, asserted that the major contributing form to the ground state complex between 1-methyl-pyridinium cation and iodide anion was the ion pair.

EXPERIMENTAL

Materials—The chloride salt of NMN was prepared by the method of Huff and Perlzweig (7). Nicotinamide was reacted with an excess of methyl iodide in alcohol to yield NMN as the iodide salt, m.p. 204° uncorrected [reported, 205.5–206.5° (7)]. The iodide salt was converted to the chloride salt by reacting the NMN iodide with an aqueous slurry of silver chloride. The resulting aqueous solution of NMN chloride was filtered and concentrated to yield white crystals of NMN chloride. The chloride salt was recrystallized from hot alcohol, and gave a m.p. of 241° uncorrected [reported, 237–238° (7)].

8-Chlorotheophylline was purchased from the Aldrich Chemical Co. and used without further puri-