THE EFFECT OF GERMICIDES ON THE VIABILITY AND ON THE RESPIRATORY ENZYME ACTIVITY OF GONOCOCCUS[1]

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The relationship between enzyme activity and destruction of bacteria by disinfectants has been the subject of numerous investigations in recent years. In 1927, Quastel and Wooldridge studied the effect of chemical and physical agents on various dehydrogenases of *Escherichia coli*. In several experiments, the treated organisms were subcultured to compare the viability of the organisms with their enzyme activity. When the dehydrogenases were completely inhibited (particularly those dehydrogenating the sugars), the organisms were found to be dead or greatly reduced in number. Casman and Rettger (1933), working with the effect of different temperatures on several species of the genus *Bacillus*, as well as some strict thermophiles, pointed out that the succinic dehydrogenase system of most of the organisms appeared to be distinctly inhibited at the maximum temperature of growth. Paraphenylenediamine oxidase and catalase activities varied considerably in this respect. Qualitative methods were employed to detect the enzyme activities. Similar studies were also made by Edwards and Rettger (1937) as well as by Wedberg and Rettger (1941) with a wider variety of organisms; essentially the same methods as those of Casman and Rettger were used.

Yudkin (1937) demonstrated that silver sulphate was lethal to suspensions of *E. coli* in concentrations much lower than those inhibiting the dehydrogenases (glucose, succinic, lactic and formic), hydrogenase and formic hydrogenlyase. Sykes (1939) studied the effect of alcohols and phenol derivatives on succinic dehydrogenase of *E. coli*. It was found that at concentrations of the germicides equal to those killing the organisms, the activity of the enzyme was considerably or completely inhibited.

Rahn and Barnes (1933) reported on the different criteria of death of yeast cells. When the organisms were subjected to the action of heat or mercuric chloride, the rate of loss of reproduction was about twice as rapid as the loss of fermentation, about three times that of the loss of semipermeability, and about forty times as large as the rate of coagulation of the protoplasm. Rahn and Schroeder (1941) investigated the problem of enzyme inactivation as the cause of death of bacteria. Contrary to the work of Edwards and Rettger (1937), these workers found that when half of the catalase and succinic dehydrogenase activities of *Bacillus cereus* were inhibited at 46° and 50°C., more than 99.9 per

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cent of the cells were found to be dead. The effect of solutions of mercuric bichloride, phenol (glycerinated) and trypaflavine on formic and lactic dehydrogenase of *Proteus* OX_{19} was studied by Braun and Vásárhelyi (1940). It was found that the dehydrogenases were inactivated by concentrations of the compounds which lie far below those necessary to kill the organisms.

Investigations on the effect of therapeutically active drugs employed in the treatment of gonococcal infections on the viability of *Neisseria gonorrhoeae* are numerous (Swartz and Davis, 1921; Thomas and Bayne-Jones, 1936; Thomas, 1939). However, the effect of these chemical agents on the respiratory enzymes of this pathogen is virtually unknown, although the gonococcus has been shown by Barron and Miller (1932) to have a relatively simple respiratory enzyme system. The purpose of the present investigation is to present a study of the relationship between the lethal concentration of drugs and those concentrations necessary to inactivate certain of the respiratory enzymes of the gonococcus. The degree of inhibition of the enzymes at the lethal concentration level of the gonococcides was also studied.

EXPERIMENTAL PROCEDURES

Test organism. The strain of *Neisseria gonorrhoeae* used in this investigation was isolated from the cervix of a patient with a clinical diagnosis of chronic gonococcal endocervicitis, according to the method described by Carpenter (1937). The culture is designated No. 1111. Its cultural, microscopical, tinctorial, and fermentative characteristics were found to be typical of the gonococcus.

Medium used. The organisms were cultivated routinely on beef-heart agar modified from the original method of Torrey and Buckell (1922) (medium B). The medium was enriched by the addition of 20 per cent egg digest prepared according to the method of Price (1935). Bacto proteose peptone No. 3 was substituted for Difco peptone. The agar concentration of the preparation was two per cent and the final pH was 7.2.

Suspending and diluting fluid. Since solutions of silver salts cannot be prepared in physiological sodium chloride and since distilled water cannot be used as a suspending fluid for gonococci, it was found necessary to use another suitable isotonic salt solution. This consisted of a 0.15 M sodium acetate solution ($NaC_2H_3O_2 \cdot 3H_2O$) in distilled water adjusted to pH 6.6 with acetic acid. Suspensions of organisms, as well as dilutions of the drugs, were always prepared with sterile acetate solution. All solutions and reagents were prepared in redistilled water from an all-glass still.

Germicides employed. The drugs tested in this investigation were those which have been in common use for the treatment of gonococcal infections. They were: silver nitrate, protargol ("strong" silver protein compound), neo-silvol (colloidal silver iodide-protein compound), silver nucleinate ("mild" silver protein compound), argyrol ("mild" silver protein compound), potassium permanganate, merthiolate[2] (sodium ethyl mercuri thiosalicylate), and sulfanilamide. Stock

[2] Merthiolate powder was kindly donated by Eli Lilly and Company, Indianapolis, Indiana.

solutions of the drugs were prepared in doubly distilled water in concentrations up to 20 gm. per 100 ml. of solution. These stock solutions were found to be sterile with the exception of sulfanilamide. The sulfanilamide stock solutions (1.5 gm. per 100 ml. solution) were sterilized separately by each of three methods: (a) filtration through a Seitz filter, (b) autoclaving at 15 lb. for 20 minutes, (c) heating in the Arnold sterilizer at 100°C. for one hour on each of three successive days. The results obtained by any of the aforementioned methods were found to be identical. The serial dilutions from the stock solutions of the drugs were always prepared in sterile acetate solution.

General procedure followed for conducting germicidal and enzyme inhibition tests. The centrifuge method of Davis and Swartz (1920) for testing the effect of germicides on suspensions of gonococci was employed, with certain modifications. The suspensions of organisms prepared for the tests were standardized with the use of a photoelectric colorimeter to a turbidity equivalent to the No. 5 barium sulfate standard of McFarland (1907). This standard was prepared by adding 5 ml. of 0.048 M $BaCl_2$ to 95 ml. of 0.1 M H_2SO_4. The total count of this suspension was approximately three billion gonococci per ml. as determined in a Petroff-Hausser bacteria counter. From time to time in the course of this work, the standardized suspensions of organisms were checked by making total nitrogen determinations according to the method of Koch and McMeekin (1924). Values from 0.103 to 0.118 mg. total nitrogen per ml. of standardized suspension were obtained.

Kolle flasks were inoculated with a suspension prepared from 24-hour slants of gonococci. The flasks were incubated for a period of 48 hours in a moist incubator at 35° to 36°C. The growth was then suspended in sterile acetate solution, centrifuged for 20 minutes at approximately 3,700 r.p.m. and standardized in the photoelectric colorimeter. In the meantime, serial dilutions of the stock solutions of the drugs were prepared in sterile acetate solution in twice the final concentration desired. The germicide dilutions and the suspensions of organisms were used as soon as they were ready. Equal quantities of the drug dilutions and the gonococcus suspension were mixed and placed in a constant temperature water bath at 37°C. for exactly 20 minutes. The treated organisms were then sedimented in an angle type centrifuge for exactly 2.5 minutes at approximately 6,000 r.p.m. After the second washing, the organisms were finally resuspended in sterile acetate solution and restandardized to a turbidity equal to the No. 5 barium sulfate tube. The drug-treated organisms were tested for viability and enzyme activity. The enzyme actions investigated were: (a) lactic dehydrogenase activity, (b) glyceric dehydrogenase activity, (c) catalase activity, (d) peroxidase activity, and (e) indophenol oxidase activity.

The general procedure described was always followed for approximately the same length of time in order to make the various tests comparable. The viability and enzyme inhibition tests were first performed on organisms with tenfold dilutions of the stock solutions of the drugs. Once the endpoint zone was determined, intermediate dilutions of the chemical agents were tested. In the case of the viability tests, an untreated organism control was included. In the

enzyme inhibition tests, a blank without organisms, an untreated organism control, and a control containing organisms heated at 100°C. for a period of 15 minutes were included with each group of tests performed at one time.

The endpoint selected for the germicidal tests was the highest dilution of the chemical agents which completely inhibited the growth of the treated gonococcal suspensions as determined by subculture. The endpoint selected for the enzyme inhibition tests was the highest dilution of the drugs necessary to inhibit enzyme activity completely under the conditions specified.

Germicidal test. One ml. of the standardized drug-treated suspension was inoculated into each of 10 ml. quantities of 20 per cent hydrocele Torrey broth and 5 per cent egg-digest Torrey broth. The broth cultures were incubated in a moist chamber at 35° to 36°C. for a period of seven days. Subcultures from these tubes were made immediately and after 48, 96, and 168 hours' incubation on Douglas' "chocolate" agar plates (Carpenter, 1937). The inoculated plates were incubated at 35° to 36°C. for 48 hours in an atmosphere of 10 per cent carbon dioxide.

Dehydrogenase tests. The activity of lactic and glyceric dehydrogenases was studied by the Thunberg methylene-blue technique (Thunberg, 1917–18; 1930). In preliminary tests, it was found that a solution of proteose peptone No. 3 (2 per cent) and NaCl (0.5 per cent) in distilled water may be advantageously employed to accelerate enzymatic activity. Controls showed that organisms in the presence of this solution did not reduce methylene-blue when lactate and glycerate were omitted in the test. The tubes were incubated in a constant temperature water bath, and observations were made for a period of 120 minutes. The time required to produce 90 per cent leucomethylene blue, or the percentage reduction of the dye at the end of the experimental period, was recorded. The standards were prepared as follows: to Thunberg-type tubes were added one ml. of standard suspension of organisms, varying concentrations of stock solution of methylene-blue, and buffer to make a final volume of 3 ml. The amount of reduction in the tests was estimated by comparison in a colorimeter block before a daylight bulb.

Quantitative catalase test. The catalase determinations were carried out with the drug-treated organisms according to the method of Kirchner and Nagell (1926). However, the total volume of the reaction mixture tested was smaller than that used by these investigators. The results were recorded as the quantity of standard potassium permanganate consumed in titrating the residual hydrogen peroxide.

Quantitative peroxidase test. The pyrogallol peroxidase test was also performed according to the method of Kirchner and Nagell (1926). As in the case of the catalase test, a smaller volume of the reaction mixture was also tested. The results were recorded as the quantity of purpurogallin formed.

Quantitative indophenol oxidase test. The oxidase test was developed after a number of preliminary tests were conducted to determine the optimal conditions for indophenol oxidase activity of gonococcus. Redistilled water from an all-glass still was particularly necessary for this determination since Wert-

heimer (1926) found that traces of metal impurities, such as copper and iron, accelerate the autoxidation of the Nadi reagent in air. The reagents were freshly prepared as follows: 0.144 per cent α-naphthol in 50 per cent ethyl alcohol; 0.108 per cent dimethyl-p-phenylenediamine HCl[3] in distilled water; 0.044 per cent sodium carbonate (anhydrous) in distilled water.

The conditions for the test will be found in the footnote to table 8. The final pH of the test mixture was 6.8. The test was conducted in a constant temperature water bath at 37°C. for a period of exactly 15 minutes, after which the reaction was arrested by the addition of a 2 per cent solution of unneutralized KCN. The dyestuff formed was extracted with a mixture consisting of equal parts of ethyl alcohol and chloroform. The amount of extracted dye was determined in a Duboscq type colorimeter using alpha-naphthol blue standards. Alpha-naphthol blue (also called indophenol blue and p-dimethylaminophenyl-α-naphthoquinone-imine) was prepared by the interaction of α-naphthol with dimethyl-p-phenylenediamine HCl (Rowe, 1924) using ferric chloride to accelerate the reaction (Guthrie, 1931). The dyestuff was purified by three precipitations from ethyl alcohol, dried *in vacuo*, and stored in an amber glass-stoppered bottle. The values obtained in the tests were recorded as the quantity of α-naphthol blue formed.

RESULTS

The germicidal and enzyme inhibition endpoints recorded in the tables represent averages of at least three separate experiments. The results obtained with the silver protein compounds were expressed as molar concentrations of silver. Silver determinations of these silver protein drugs were made according to the permanganate sulphuric-acid oxidation method of F. Lehmann (Hagers Handbuch, 1938).

Lethal effect of germicides on gonococci. From the results shown in table 1, it is seen that silver nitrate and merthiolate were the most active lethal compounds, whereas sulfanilamide was not lethal for the gonococcus under the conditions of the test.

In vitro effect of sulfanilamide on gonococcus. Tests were performed to determine whether the strain of gonococcus tested in this investigation was either resistant or sensitive to the action of sulfanilamide. Sulfanilamide broth tubes were inoculated with the standard suspension of organisms as well as graded dilutions; namely, 1–10 and 1–100 dilution of the standard suspension. This was done because the *in vitro* effect of sulfanilamide has been found to be dependent upon the size of the inoculum (e.g. Weld and Mitchell, 1939). The results are shown in table 2. Over a period of 20 minutes, sulfanilamide in a concentration of 1–133 (4.36×10^{-2} M) apparently did not affect the viability of the gonococcus. However, death of the organisms occurred at least after 24 hours' exposure to the drug.

Inhibition of gonococcal lactic dehydrogenase. It is seen from table 3 that silver

[3] A light colored product should be used so that the value of the blank remains low.

nitrate, potassium permanganate, and protargol were the most potent inhibitors of gonococcal lactic dehydrogenase activity among the substances tested.

Inhibition of gonococcal glyceric dehydrogenase. Table 4 shows that potassium permanganate, protargol, and silver nitrate were the most potent inhibitors

TABLE 1

*Minimal concentration of germicides lethal for the gonococcus**

COMPOUND	MIN. LETHAL CONCENTRATION (MOLAR)
Silver nitrate	7.35×10^{-6}
Protargol	3.78×10^{-6} (Ag)
Neo-silvol	1.63×10^{-5} (Ag)
Silver nucleinate	2.22×10^{-5} (Ag)
Argyrol	3.71×10^{-5} (Ag)
Potassium permanganate	6.32×10^{-5}
Merthiolate	2.73×10^{-6}
Sulfanilamide	Not lethal at 4.36×10^{-1}

* The standardized drug-treated suspensions were inoculated into egg-digest and hydrocele broth tubes. Exactly 0.2 ml. of the inoculated broths was plated on the surface of Douglas' chocolate agar plates at stated intervals of time. The inoculated broths and plates were incubated at 35° to 36°C. The broth tubes were examined for turbidity and the number of colonies appearing on the plates were enumerated and recorded.

TABLE 2

*The in vitro effect of sulfanilamide on the viability of the gonococcus**

TIME OF SUBCULTURE	STANDARD SUSPENSION (UNDILUTED)		STANDARD SUSPENSION (DILUTED 1-10)		STANDARD SUSPENSION (DILUTED 1-100)	
	Control†	Drug‡	Control†	Drug‡	Control†	Drug‡
20 minutes	++++	++++	++++	++++	++	++
24 hours	++++	0	++++	0	++++	0
48 hours	++++	0	++++	0	++++	0
168 hours	++++	0	++++	0	++++	0

* One ml. of organisms was inoculated into 10 ml. of broth with and without sulfanilamide, and incubated under aerobic conditions in a moist incubator. At the intervals stated, 0.2 ml. of the inoculated media was placed over the surface of Douglas' chocolate agar plates. The plates were incubated in an atmosphere of 10 per cent CO_2 at 35° to 36°C.

† Torrey "B" broth enriched with 20 per cent sterile hydrocoele fluid was used for the control broth tubes.

‡ The drug broth contained Torrey "B" broth enriched with 20 per cent hydrocoele fluid and 1-133 final concentration of sulfanilamide.

0 indicates no growth; ++ indicates from 201 to 500 colonies per plate; ++++ indicates over 1,000 gonococcus colonies per plate.

of gonococcal glyceric dehydrogenase activity. It may also be seen that gonococcal glyceric dehydrogenase was inhibited by smaller concentrations of the drugs than was lactic dehydrogenase. Sulfanilamide is a possible exception, since with this compound inhibition of lactic dehydrogenase was slightly greater.

Inhibition of gonococcal catalase. As is shown in table 5, potassium permanganate was found to be the most powerful inhibitor of gonococcal catalase among the compounds tested. Silver nitrate and protargol were also very potent inhibitors, but the other compounds affected the enzyme little or not at all.

Inhibition of gonococcal peroxidase. Silver nitrate, potassium permanganate, and protargol in high dilutions, as shown in table 6, completely inhibited gonococcal peroxidase whereas with the other germicides, much higher concentrations were necessary. Sulfanilamide, in the concentration used, had no appreciable effect on peroxidase.

TABLE 3

*Effect of germicides on gonococcal lactic dehydrogenase**

DRUG	MOLAR CONCENTRATION	PER CENT INHIBITION
Silver nitrate	9.77×10^{-5}	100
Protargol	1.89×10^{-4} (Ag)	100
Neo-silvol	8.17×10^{-3} (Ag)	100
Silver nucleinate	1.79×10^{-1} (Ag)	94
Argyrol	1.85×10^{-1} (Ag)	92
Potassium permanganate	6.32×10^{-4}	100
Merthiolate	2.46×10^{-1}	85
Sulfanilamide	4.36×10^{-2}	26

* Each Thunberg-type tube contained 0.5 ml. of 0.0005 M methylene blue chloride, 0.5 ml. of 0.555 M lactic acid neutralized to pH 7.4 with NaOH, 0.1 ml. of peptone-NaCl solution, 1.9 ml. of M/20 phosphate buffer (Clark and Lubs) pH 7.4, and 1.0 ml. of drug-treated suspension of organisms. The test was conducted at 37°C. Observations were made for a period of 120 minutes.

TABLE 4

*Effect of germicides on gonococcal glyceric dehydrogenase**

DRUG	MOLAR CONCENTRATION	PER CENT INHIBITION
Silver nitrate	1.17×10^{-5}	100
Protargol	1.49×10^{-5} (Ag)	100
Neo-silvol	4.09×10^{-3} (Ag)	100
Silver nucleinate	2.55×10^{-4} (Ag)	100
Argyrol	4.65×10^{-3} (Ag)	100
Potassium permanganate	7.90×10^{-5}	100
Merthiolate	2.46×10^{-1}	100
Sulfanilamide	4.36×10^{-2}	2

* The conditions for this series of experiments were the same as those given in the footnote to table 3, with the exception that 0.471 M glyceric acid neutralized to pH 7.4 was used as the substrate instead of lactic acid.

Observations on the relation of gonococcal catalase to peroxidase. It should be noted that the substrate used to test for peroxidase activity consisted of pyrogallol as well as hydrogen peroxide. Since the gonococcus has a strong catalase

activity, the possibility arose that the presence of catalase may interfere with the peroxidase test. Experiments were therefore carried out to determine the effect of gonococcal catalase on the quantitative peroxidase test. A large num-

TABLE 5

*Effect of germicides on gonococcal catalase**

DRUG	MOLAR CONCENTRATION	PER CENT INHIBITION
Silver nitrate	2.94×10^{-3}	100
Protargol	3.78×10^{-3} (Ag)	100
Neo-silvol	8.17×10^{-2} (Ag)	3
Silver nucleinate	1.79×10^{-1} (Ag)	0
Argyrol	1.85×10^{-1} (Ag)	8
Potassium permanganate	6.32×10^{-4}	100
Merthiolate	2.46×10^{-1}	5
Sulfanilamide	4.36×10^{-2}	0

* The catalase test was performed in 125 ml. Erlenmeyer flasks containing 26.0 ml. of M/150 phosphate buffer (pH 6.5), 3.0 ml. of N/1 H_2O_2 (diluted from superoxol) and 1.0 ml. of drug-treated acetate suspension of organisms. The test mixture was placed in an ice bath (1°–2°C.) for one hour, after which 2.0 ml. of 25 per cent H_2SO_4 was added to arrest enzyme activity. The entire contents of the flask was then titrated with standard N/10 $KMnO_4$.

TABLE 6

*Effect of germicides on gonococcal peroxidase**

DRUG	MOLAR CONCENTRATION	PER CENT INHIBITION
Silver nitrate	1.96×10^{-3}	100
Protargol	3.78×10^{-3} (Ag)	100
Neo-silvol	8.17×10^{-2} (Ag)	72
Silver nucleinate	1.79×10^{-1} (Ag)	49
Argyrol	1.85×10^{-1} (Ag)	46
Potassium permanganate	6.32×10^{-4}	100
Merthiolate	2.46×10^{-1}	78
Sulfanilamide	4.36×10^{-2}	2

* The peroxidase test was performed in 125 ml. Erlenmeyer flasks containing 21.0 ml. of M/150 phosphate buffer (pH 7.2), 3.0 ml. of N/1 H_2O_2 (diluted from superoxol), 5.0 ml. of 1.56 per cent pyrogallol solution in pH 7.2 M/150 phosphate buffer, and 1.0 ml. of drug-treated organisms. The enzyme reaction was carried out in a 20°C. constant temperature water bath for exactly 15 minutes. Two ml. of a 25 per cent solution of H_2SO_4 were added, the purpurogallin formed was extracted with purified ethyl ether after standing at room temperature for five minutes. The concentration of the dyestuff was determined in a Duboscq type colorimeter compared with a purpurogallin standard prepared by the method of Graebe (1914).

ber of catalase inhibitors mentioned in the literature were tested. The volumetric methods for determining catalase activity advocated by Bailey (1917) and by Morgulis (1921) were employed, since it was found that inhibitors such as

hydroxylamine and formalin interfered with the permanganate titration method. No inhibitor was found which completely inhibited gonococcal catalase without also affecting gonococcal peroxidase activity to a large extent. However, hydroxylamine hydrochloride in a concentration of 9.97×10^{-4} M was selected for the purpose since catalase was found to be completely inhibited whereas peroxidase was inhibited by about 60 per cent (table 7).

Standard suspensions were first subjected to the action of silver nitrate and potassium permanganate in the usual manner so that graded concentrations of the drugs effected the inhibition of peroxidase activity at the endpoint zones. The respective drug-treated suspensions were then tested for peroxidase (pyrogallol) activity with the addition of 9.97×10^{-4} M $H_2NOH \cdot HCl$ to the test mixture. In parallel experiments, comparisons were also made with similar drug-treated suspensions without the addition of hydroxylamine to the reaction mixture. It was found that the same endpoints were obtained with both drugs whether hydroxylamine was added to the test mixture or not.

TABLE 7

*The effect of various concentrations of hydroxylamine hydrochloride on gonococcal catalase and peroxidase**

FINAL CONCENTRATION OF $H_2NOH \cdot HCl$ (M)	INHIBITION OF CATALASE	INHIBITION OF PEROXIDASE
	per cent	*per cent*
9.97×10^{-5}	32	2
9.97×10^{-4}	100	56
9.97×10^{-3}	100	100

* The conditions for the catalase test are given in the footnote to table 5, with the modification that the volumetric method was used. The conditions for the peroxidase test are given in the footnote to table 6. The H_2NOH solutions were adjusted to pH 7.2. Both catalase and peroxidase tests were conducted at pH 7.2 at a temperature of 20°C. for exactly 15 minutes.

It seemed possible that part of the purpurogallin formed might not have been produced peroxidatively, but instead by the independent oxidative action of gonococci on pyrogallol by some system which did not require hydrogen peroxide. Experiments were carried out to determine how much purpurogallin was formed when pyrogallol was used without the presence of hydrogen peroxide in the enzyme test. The results indicated that under these conditions a negligible quantity of purpurogallin was produced by the action of gonococci on pyrogallol.

Callow (1926) stated that, except for streptococci, the peroxidase system of all bacteria was heat-stable when benzidine or guaiac with hydrogen peroxide were used as substrates. *N. gonorrhoeae* was not included in her studies. In the present study, the heated enzyme controls in the pyrogallol peroxidase tests showed the enzyme to be heat-labile. Following the general methods employed by Callow, it was found that a weak blue color was obtained when both aqueous and acetate suspensions of gonococci were tested with the benzidine-hydrogen peroxide reagent. In both instances, the colors faded within a short period of

time. However, when the suspensions were heated at boiling temperature for a period of 15 minutes, the qualitative benzidine peroxidase test gave a stronger and more lasting color reaction. As rated above, the qualitative pyrogallol peroxidase test was found to be negative when suspensions were heated in the same way. While the addition of KCN to the qualitative pyrogallol peroxidase test using unheated suspensions of gonococci was negative, inconclusive results were obtained when varying concentrations of KCN were added to the gonococcal benzidine peroxidase test mixture. Further work is necessary to establish the true nature of gonococcal peroxidase.

Inhibition of gonococcal indophenol oxidase. The data presented in table 8 show that silver nitrate and protargol are potent inhibitors of indophenol oxidase. Much higher concentrations of merthiolate were required to effect complete

TABLE 8

*Effect of germicides on gonococcal indophenol oxidase**

DRUG	MOLAR CONCENTRATION	PER CENT INHIBITION
Silver nitrate	2.94×10^{-3}	100
Protargol	7.76×10^{-3} (Ag)	100
Neo-silvol	8.17×10^{-2} (Ag)	79
Silver nucleinate	1.79×10^{-1} (Ag)	51
Argyrol	1.85×10^{-1} (Ag)	50
Potassium permanganate	†	†
Merthiolate	2.46×10^{-1}	100
Sulfanilamide	4.36×10^{-2}	55

* The quantitative indophenol oxidase tests were performed in 25 × 100 mm. pyrex test tubes containing 5.0 ml. of the diamine-naphthol reagent, 5.0 ml. of M/20 phosphate buffer of pH 6.6 and 1.0 ml. of drug-treated acetate suspension of organisms. Tests were carried out in a 37°C. water bath for a period of exactly 15 minutes, after which 2.0 ml. of a 2 per cent solution of unadjusted KCN were added. The dyestuff was extracted with 1:1 ethyl alcohol-chloroform mixture and compared in a Duboscq type colorimeter with alpha-naphthol blue standards.

† Could not be determined.

inhibition; with the other drugs, the highest concentration used caused only partial inhibition. The effect of potassium permanganate on gonococcal indophenol oxidase could not be determined accurately since supplementary experiments showed that enough permanganate was present in the washed cells to oxidize the substrate independent of the enzyme activity of the bacteria.

General consideration of the results of the germicidal and enzyme inhibition tests. In table 9, a compilation of the results of the various tests is presented. With the exception of silver nitrate and potassium permanganate, there was no obvious correlation between the concentration of a drug which was lethal for gonococcus and that causing complete inhibition of the various enzymes tested. However, sulfanilamide is a notable exception. Within the limits of solubility of this drug, no apparent effect on viability could be observed during the test period. In line with this finding, there was relatively little effect on the activity

TABLE 9

*Compilation of the results of the germicidal and enzyme inhibition tests on gonococcus**

COMPOUND	VIABILITY TEST	DEHYDROGENASES		CATALASE	PEROXIDASE	INDOPHENOL OXIDASE
		Lactic	Glyceric			
Silver nitrate						
Per cent inhibition	Lethal	100	100	100	100	100
Concentration of drug†	1–800,000	1–60,000	1–500,000	1–2,000	1–3,000	1–2,000
Molarity‡	7.35×10^{-6}	9.77×10^{-5}	1.17×10^{-5}	2.94×10^{-3}	1.96×10^{-3}	2.94×10^{-3}
Protargol						
Per cent inhibition	Lethal	100	100	100	100	100
Concentration of drug†	1–200,000	1–4,000	1–50,000	1–200	1–200	1–100
Molarity‡	3.78×10^{-6}	1.89×10^{-4}	1.49×10^{-5}	3.78×10^{-3}	3.78×10^{-3}	7.76×10^{-3}
Neo-silvol						
Per cent inhibition	Lethal	100	100	3	72	79
Concentration of drug†	1–50,000	1–100	1–200	1–10	1–10	1–10
Molarity‡	1.63×10^{-5}	8.17×10^{-3}	4.09×10^{-3}	8.17×10^{-2}	8.17×10^{-2}	8.17×10^{-2}
Silver nucleinate						
Per cent inhibition	Lethal	94	100	0	49	51
Concentration of drug†	1–80,000	1–10	1–7,000	1–10	1–10	1–10
Molarity‡	2.22×10^{-5}	1.79×10^{-1}	2.55×10^{-4}	1.79×10^{-1}	1.79×10^{-1}	1.79×10^{-1}
Argyrol						
Per cent inhibition	Lethal	92	100	8	46	50
Concentration of drug†	1–50,000	1–10	1–400	1–10	1–10	1–10
Molarity‡	3.71×10^{-5}	1.85×10^{-1}	4.65×10^{-3}	1.85×10^{-1}	1.85×10^{-1}	1.85×10^{-1}
Potassium permanganate						
Per cent inhibition	Lethal	100	100	100	100	§
Concentration of drug†	1–100,000	1–10,000	1–80,000	1–10,000	1–10,000	§
Molarity‡	6.32×10^{-5}	6.32×10^{-4}	7.90×10^{-5}	6.32×10^{-4}	6.32×10^{-4}	§
Merthiolate						
Per cent inhibition	Lethal	85	100	5	78	100
Concentration of drug†	1–900,000	1–10	1–10	1–10	1–10	1–10
Molarity‡	2.73×10^{-6}	2.46×10^{-1}	2.46×10^{-1}	2.46×10^{-1}	2.46×10^{-1}	2.46×10^{-1}
Sulfanilamide						
Per cent inhibition	Not lethal	26	2	0	2	55
Concentration of drug†	1–133	1–133	1–133	1–133	1–133	1–133
Molarity‡	4.36×10^{-2}	4.36×10^{-2}	4.36×10^{-2}	4.36×10^{-2}	4.36×10^{-2}	4.36×10^{-2}

* In the case of neo-silvol, merthiolate, silver nucleinate and argyrol, the drugs could not be used in a concentration higher than 1–10 for reasons of solubility. Similarly, concentrations of sulfanilamide above 1–133 (4.36×10^{-2}M) could not be prepared.

† Expressed in terms of dilution of the drugs.

‡ Expressed in terms of moles of germicide causing death or inhibition as indicated with the exception of the silver-protein compounds. In the case of the silver-protein compounds the molarity is calculated on the basis of the silver content.

§ Could not be determined accurately.

TABLE 10

The activity of the enzymes at the lethal concentration level of the drugs

DRUGS	LETHAL CONCENTRATION (M)	PER CENT INHIBITION AT LETHAL CONCENTRATION				
		Dehydrogenases		Catalase	Peroxidase	Indophenol Oxidase
		Lactic	Glyceric			
Silver nitrate	7.35×10^{-6}	12	62	0	1	4
Protargol	3.78×10^{-6} (Ag)	23	43	0	0	0
Neo-silvol	1.63×10^{-5} (Ag)	34	12	0	0	0
Silver nucleinate	2.22×10^{-5} (Ag)	0	20	0	0	0
Argyrol	3.71×10^{-5} (Ag)	43	36	0	0	0
Potassium permanganate	6.32×10^{-5}	65	63	29	40	*
Merthiolate	2.73×10^{-6}	25	0	0	0	0

* Could not be calculated accurately.

of the enzymes, although it should be pointed out that partial inhibition of indophenol oxidase (55 per cent) and of lactic dehydrogenase (26 per cent) occurred in the presence of the highest concentration of sulfanilamide tested.

Degree of inhibition of enzymes at the lethal concentration of drugs. Thus far, the relationship between the minimal lethal concentration and the smallest amount of drug causing total inhibition of enzyme activity has been considered. It is of value to know what the activity of each enzyme was at the minimal lethal concentration of the drugs. For this purpose, the results of the effect of progressive tenfold dilutions of each drug on the enzymes were plotted. The activity of the enzymes was then calculated graphically at the lethal concentration level of each drug. Table 10 presents the results of these calculations.

It is noted that at the lethal concentration level of the drugs, the dehydrogenases were generally inhibited to a greater extent than were the other enzymes tested. Potassium permanganate was found to produce the most marked effect on the enzymes (indophenol oxidase activity was not calculated because it could not be determined accurately). In contrast to the total enzyme inhibition studies, lactic dehydrogenase activity was inhibited to a greater degree by the action of some of the drugs tested at the lethal concentration level than glyceric dehydrogenase activity at that concentration. The enzymes inhibited most markedly at the lethal concentration of the drugs were lactic dehydrogenase by potassium permanganate, and glyceric dehydrogenase by silver nitrate, as well as by potassium permanganate.

GENERAL DISCUSSION

Certain limitations in an investigation of this nature should be recognized. For example, there was found to be a lack of coincidence between the lethal and the enzyme inhibition endpoints (table 9). This is particularly apparent from a study of table 10, where the degree of inhibition of the enzymes at the lethal concentration of the chemical agents is shown. Several contributing factors may be considered to explain why total inhibition of the enzymes studied does not take place at the lethal concentration of the drugs.

There exists the possibility that the various germicides tested cause the death of gonococcus by inhibiting an enzyme system for which no test was made in the present study. Other fundamental processes, such as the phosphorylating and glycolytic systems, may be limiting factors whose inhibition affects more directly the viability of gonococcus.

Manometric studies have shown a close relationship to exist between the effect of chemical agents on the respiration and on the viability of bacteria (Ely, 1939; Greig and Hoogerheide, 1941). In fact, Bronfenbrenner, Hershey and Doubly (1939) have concluded that bacteriostatic dilutions of germicides depress the oxygen uptake of bacteria by only about 10 per cent, whereas effective germicidal concentrations do so in excess of 80 per cent. It should be noted that the manometric results were expressed as depression of oxygen uptake of the organisms by the action of the chemical agents tested. This really represents the sum total effect of the drugs on the function of several enzymes

in a system necessary to oxidize a particular substrate. In this instance, glucose and horse serum were used in the reaction mixture.

Various mechanisms, whether accessory to the function of the respiratory enzymes or not, may operate and complicate the correlation of enzyme activity with the death of the cell. Fildes (1940), for instance, has come to the conclusion that mercury combines with the sulfhydryl compounds present in *E. coli*; this effect is reversed by the addition of —SH compounds to the suspension. Casman and Rettger (1933) have shown that catalase and succinic dehydrogenase in certain organisms tested may actually inhibit peroxidase activity (qualitative benzidine test).

The present conception of cellular respiration is essentially a transfer of electrons from foodstuffs (substrates) to molecular oxygen by various respiratory enzymes (Barron, 1939). The dehydrogenases occupy a primary position in the respiratory chain of electron transport while cytochrome (indophenol) oxidase plays the terminal role in the series of respiratory enzymes and intermediate carriers. Despite the accumulation of knowledge in regard to the biochemistry of catalase and peroxidase, the real physiological function of these two enzymes is still considered obscure (Lipton, Arnold and Berger, 1939; Green, 1940). These heme catalysts are sometimes regarded as "secondary respiratory enzymes" because of their incidental function in the respiratory chain (Werkman, 1939).

When the effect of drugs on the enzymes is considered, the present investigation shows that the dehydrogenases are the most labile enzymes, while oxidase, catalase and peroxidase are found to be comparatively more resistant to the action of these compounds. It may also be recalled that Casman and Rettger (1933) found succinic dehydrogenase to be distinctly inhibited at the maximum temperature of growth, whereas catalase and oxidase varied considerably in this respect. The work of Haas, Harrer and Hogness (1942) is of interest in this connection. These workers tested the effect of 1×10^{-3} M 2,4-dinitro-o-cyclohexylphenol upon isolated components of the respiratory system of yeast. This chemical agent inhibited *Zwischenferment* (hexosemonophosphoric dehydrogenase) and the total respiration of yeast almost completely, while cytochrome *c* reductase (an intermediary flavoprotein functioning between hexosemonophosphate and cytochrome *c*) was inhibited by 70 per cent. Cytochrome oxidase was not affected at all by this compound. In general, the respiratory enzymes functioning at the beginning of the electron transport chain appear to be affected most readily by the action of the chemical compounds tested.

In the present investigation, the gonococcus was shown to contain both heat-labile pyrogallol and heat-stable benzidine peroxidase activity. The general property of thermostability of bacterial peroxidases was pointed out by Stapp (1924) and Callow (1926). They used the guaiac and benzidine tests. In addition, Stapp found bacterial peroxidases to be resistant to the action of acid, alkali, salts and narcotics. This led Oppenheimer (1926) to conclude that Stapp was working with a purely chemical oxidation system and not with an enzyme. It has been pointed out by Green (1940) that practically all Fe porphyrins can

act as heat-stable peroxidases. Stephenson (1939) has suggested that many peroxidases reported in bacteria are probably of this type.

SUMMARY AND CONCLUSION

The effect of eight germicides on *Neisseria gonorrhoeae* has been studied in an attempt to compare the loss of viability with the inhibition of five respiratory enzymes. The drugs tested were silver nitrate, protargol, neo-silvol, silver nucleinate, argyrol, merthiolate, potassium permanganate and sulfanilamide. The enzymes selected for the inhibition studies were lactic and glyceric dehydrogenases, catalase, peroxidase and indophenol oxidase. Within the limits of the experiments, the following conclusions seem warranted:

1. Death of the cells occurred before significant enzyme inhibition took place. A possible exception to this observation was found in the case of silver nitrate and potassium permanganate. With both of these compounds, the minimal concentration causing complete inhibition of glyceric dehydrogenase corresponded very closely to the lethal concentration levels of these drugs. However, at the lethal concentration of these two chemical agents, approximately 65 per cent inhibition of glyceric dehydrogenase activity was found.

2. Of the eight germicides studied, merthiolate, silver nitrate, protargol and potassium permanganate showed the highest lethal activity on gonococcal suspensions.

3. On the basis of total inhibition, silver nitrate, potassium permanganate and protargol were the most effective enzyme inhibitors. At the lethal concentration of the compounds, potassium permanganate and silver nitrate were found to be the most active enzyme inhibitors. Glyceric and lactic dehydrogenase activities were generally inhibited by chemical agents in smaller concentrations than those of the other enzymes tested.

4. The effect of sulfanilamide on gonococci places this drug in a different category from the other compounds tested. No apparent effect on viability was observed within the 20-minute test period, although exposure of the drug to the organisms for a period of 24 hours caused the death of the cells. Furthermore, the inhibition of respiratory enzymes by sulfanilamide was *nil* with the exception of indophenol oxidase and lactic dehydrogenase. These enzymes were inhibited by 55 per cent and 26 per cent respectively at the highest concentration of the drug tested.

5. The presence of gonococcal catalase has been shown not to interfere with the results obtained in tests for peroxidase activity of gonococci.

6. When pyrogallol with hydrogen peroxide was used as the substrate, a heat-labile peroxidase could be demonstrated with gonococcus. However, the use of benzidine and hydrogen peroxide revealed heat-stable peroxidase-like activity.

7. A quantitative, colorimetric, indophenol oxidase test has been developed for use in studies with the gonococcus.

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