OXIDATION-REDUCTION POTENTIAL STUDIES ON THE RATE OF GERMICIDAL ACTIVITY OF QUATERNARY AMMONIUM COMPOUNDS

by

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INTRODUCTION

In the early days of bacteriology, research workers were primarily concerned with the identification of species and with the role of bacteria in nature. Gradually, a transition occurred wherein bacteriology shifted from the descriptive stage to those of function and morphology. Although these stages in the development of bacteriology continue, a new phase, which may be called quantitative bacteriology, is rapidly developing.

Researchers using bacteria as yardsticks for measuring reactions and activity of chemical agents, demand accurate methods of measurement. In assaying compounds for their germicidal activity, a number of methods for measuring bacterial kill are used. Generally, these methods include the standard plate and direct miscroscopic counts, optical density measurements of turbidity, gasometric analysis of metabolic products, and radioactive isotope tracer techniques.

Although electrode potential measurements have been used extensively in the field of chemistry for determining end points in neutralizations, oxidations, and precipitations, little information appears in the literature of bacteriology on this method as a quantitative technique for the determination of bacterial metabolism. Because electrical methods lend

themselves so well to automatic instrumentation, it is suggested that oxidation-reduction potential might be added to this list of methods.

Presently, it is the practice to correlate each quantitative procedure with a standard plate count. This practice is doubtfully justified, in view of the imposing evidence against plate count accuracy.

This paper is concerned with a measurement of bacterial metabolism unopposed, and altered by the influence of quaternary ammonium germicides. After these patterns have been established, the effect of some of the more common so-called quaternary neutralizers are studied. If electrode potential measurements offer a reliable means of estimating metabolic activity then when a suitable culture medium is inoculated with a suspension of living organisms, the potential should change with time until an equilibrium is established between the reducing power of the organisms and the oxidizing power of the air. As the death rate of the organisms exceeds the rate of cellular multiplication, in accordance with a growth curve obtained by standard plating procedure, the oxygen absorbing capacity of the culture medium exceeds the diminishing reducing capacity of the cells, and the electrode potential should return to some point at or near the original potential of the medium before inoculation. If this normal metabolic course is interrupted in such a way that the cells are no

longer able to survive, then the rate of return to the normal, initial equilibrium state should change accordingly. The slope of the time potential curve obtained under these conditions should be a function of the rate of germicidal activity of the interrupting substance. If a second substance is added which opposes the action of the first, the curve should again be changed in a manner which would indicate the efficiency of the antagonism.

This thesis will attempt to describe the development of a medium, and an apparatus for conducting oxidation-reduction potential studies, and apply these developments to an examination of the effects of quaternary ammonium germicides and their inhibitors on the normal time-potential curves of several test organisms.

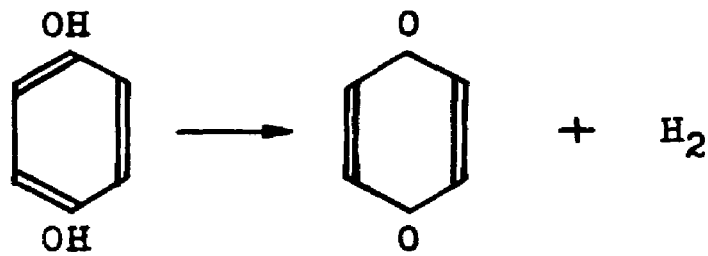
REVIEW OF THE LITERATURE

Before a presentation of some of the more important contributions to the field of biological oxidation reduction, a review of the basic concepts of these phenonomena is in order. Oxidation in its most obvious sense, implies the addition of oxygen to an oxidizable substance. When ferrous oxide is oxidized to ferric oxide,

$$2\ FeO + O \longrightarrow Fe_2O_3$$

it can be seen that this reaction is simply the addition of oxygen to the oxidizable substance, ferrous oxide. But in

the measurement of pH, one makes use of another kind of oxidation. When hydroquinone is converted to quinone,



it is said that hydroquinone is oxidized, yet there is the same number of oxygen atoms in both compounds. The definition must, therefore, take into consideration the removal of hydrogen. A third type of oxidation can be concieved which, unlike the first example, is not concerned with the addition of oxygen and, unlike the second, is not a case of hydrogen removal. Obviously, when ferrous chloride is transformed to ferric chloride,

$$FeCl_2 + Cl \longrightarrow FeCl_3$$

neither oxygen nor hydrogen enter into the reaction. This third example involves a transfer of one electron from ferric to ferrous iron,

$$Fe^{++} \longrightarrow Fe^{+++} + e$$

and it is shown that when ferrous iron has lost one electron it has been oxidized to the ferric state. Thus, we extend the definition of oxidation to include the electronic concept. Oxidation may be defined as a process in which a substance takes up positive or parts with negative charges. Conversely, reduction is a process in which a substance takes up negative or parts with positive charges.

Bancroft (1892) was probably the first to study oxidation-reduction potentials of reversible systems. A reversible system is one in which both the oxidized and reduced forms occur simultaneously. The system liberates an equivalent amount of energy in going from the reduced to the oxidized form as is required to reverse the reaction. The requisite of reversibility in a theoretical treatment and in an interpretation of electrode potentials is important. If a platinum or other inert electrode is immersed in a redox system, it will assume a potential which is a function of the ratio of the oxidized to the reduced forms. Oxidation-reduction potentials are usually designated by the symbol, Eh, which is the potential, in volts, that an inert electrode assumes when referred to the hydrogen electrode. By definition then,

$$Eh = Eo + \frac{RT}{nF} \ln \frac{[Ox]}{[Red]}$$

where Eh is the oxidation reduction potential referred to the hydrogen electrode; Eo is the observed potential; R is the gas constant, 8.315 joules; T is the absolute temperature; n is the number of electrous involved in the transfer; F is equal to 96,500 coulombs; $\ln x = 2.303 \log x$; and [Ox] and [Red] are the concentrations of the oxidized and reduced forms respectively.

Potter (1911) worked with several species of bacteria and noticed that, of the organisms studied, Escherichia coli

produced an electrode potential which was lower than that of the sterile medium. He noted a similar effect with Saccharomyces cerevisiae but failed to show the reducing property with Chromobacterium violaceum, Pseudomonas fluorescens and Sarcina lutea because his medium would not support the growth of these organisms. His study demonstrated that the reducing condition was influenced by the concentration of the nutrients, the size of inoculum, and the temperature. A determination of the reducing intensities during the growth of bacterial cultures was done by Gillespie (1920). Later, Clark (1923) began a series of studies on biological oxidation-reduction. In cooperation with Cannon, Cohen, and Clark (1926), he verified the findings of Gillespie. For the following decade, workers in both the fields of chemistry and bacteriology were very active in the pursuit of oxidation-reduction potential theory when applied to a living cell suspension. Ever since the observation by Rosakigyi (1887) that certain dyes were rendered colorless at some distance from the zone of bacterial growth, many have attempted to explain its cause. Cahen (1887) postulated that the effect was due to gaseous metabolic products which were easily oxidized as they left the cell. Conant (1926), while investigating several irreversible oxidation-reduction systems, added known amounts of reversible systems at known potentials. He came to the conclusion that bacterial reductions fall into the irreversible

class of reactions. This method of approach has provided an empirical approach to defining irreversible systems, but Kolthoff and Lingane (1941) are of the opinion that this method is without thermodynamic foundation. Included as a part of the historical development of this topic are the more important theories of biological oxidation. According to Weiland (1922) the process is based upon the activation and transfer of hydrogen atoms by enzymes elaborated by the cell. This is followed by a catalytic transfer from the enzyme to a hydrogen acceptor. Oxygen is the usual hydrogen acceptor but may be replaced by other easily reducible compounds. The enzymes responsible for this activation are the dehydrogenases. In contrast to the theory of Weiland, Warburg (1925) believes that bivalent metal complexes serve to transport oxygen to the cells, and in so doing, are oxidized to the trivalent state. The oxidized metal complex is then able to oxidize other materials in the substrate and by this action is itself again reduced to the original bivalent complex. The mechanism of biological oxidation seemed to be inevitably veiled in complexity, and the attention was again focussed on the accumulation of sufficient experimental evidence to clarify the process. Coulter (1928) determined the oxidation-reduction potential of sterile boullion. The following year Coulter and Isaacs (1929), working with aerobic cultures of Salmonella typhosa concluded that although bacterial reductions

are not of the reversible type, these investigators were able to observe potentials which varied with the concentration of the reduced form of the substances. Thorton and Hastings (1929) applied electrode potential measurements to a system of bacteria, methylene blue, and milk and demonstrated the poising effect of this dye. Young (1929) describes biologically dead cells that were still very chemically "alive" in an anaerabic system, as evidenced by continued carbohydrate oxidation. Michaelis (1930), Plotz and Geloso (1930), and Yudkin (1935) share the opinion that oxidation-reduction potentials can not be used to differentiate bacterial genera. This view was opposed by Hewitt (1930, 1, 2, 3, 4) and by Burrows and Jordan (1935) who have demonstrated characteristic time-potential curves for streptocci, C. diphtheriae, staphylococci, pneumococci, and the Salmonellae.

In searching the literature for contributions leading to an understanding of bacterial oxidation-reduction, one cannot help but become aware of the complexity of this seemingly simple, and certainly often observed, activity which is characteristic of all actively metabolizing cultures. Very soon after the initial observation, numerous attempts have been made to explain its mechanism. To date, the author does not know of a single, universally accepted explanation. Continued publication of some very interesting side effects appear until 1936 or thereabout, but recently, very little study has been devoted to this aspect of bacteriology.

EXPERIMENTAL AND DISCUSSION

Choice of test organisms.

For most tests of germicidal activity of disinfectants, the choice of the organisms to be used is generally dictated by the kinds of bacteria to be encountered in the specific application at hand. However, when exploring the rates of activity and efficiency of antagonists, it is considered more desirable to use several organisms for graded response to the disinfectant. In the studies presented, the test organisms used are as follows:

Micrococcus pyogenes var. aureus, 209. This organism was obtained from the American Type Culture Collection under number 6538. It is a Gram (+) coccus of relatively low resistance to the action of quaternary ammonium germicides.

Salmonella typhosa (Hopkins strain), A.T.C.C. 6539, a Gram (-) rod and the standard test organism used by Ruehle and Brewer (1931) in the phenol coefficient method of testing disinfectants.

Psuedomonas aeruginosa, a laboratory strain originally isolated from river water, a Gram (-) motile rod of extremely high resistance to the action of quaternaries.

Escherichia coli, A.T.C.C. 9637 and a laboratory stock culture of Salmonella pullorum were occasionally used as test organisms.

Development of the redox medium

In general, it is not desirable to attempt a study of the unknown with more than one variable at a time. Likewise, it is no more desirable to study the effect of quaternaries on the oxidation-reduction potential of a culture growing on an unproven medium. Yet, preceeding this study, the author saw a need for a medium superior to F.D.A. broth for several reasons which outweigh the additional time required for its development.

Wright (1917) could not believe that the discrepancies in testing disinfectants could be attributed to the "personal equation", but blamed variation on the test medium. He ascribed much of this difficulty to the use of meat extract and to differences in pH. Reddish and Burlingame (1938) found great differences in germicidal activity which could be traced to the use of different lots and brands of peptone. In trying to alleviate these and similar discrepancies apparent in testing germicides, Wolf (1945) developed a medium which, in his hands, gave promising results. Wolf's casamino acids disinfectant test medium (Difco) has the following composition:

Casomino acids, tech., Difco.....5 g.

$K_2HPO_4 \cdot 3\ H_2O$.....3 g.

Uracil.....0.005 g.

Thiamin hydrochloride.....0.001 g.

Niacinamide.....0.001 g.

Salts solution*.....2.5 ml.

Distilled water q.s.....1000. ml.

The salts solution was prepared by dissolving $MgSO_4 \cdot 7\ H_2O$, 10 g.; $FeSO_4 \cdot 7\ H_2O$, 0.5 g.; $MnSO_4 \cdot 4\ H_2O$, 0.5 g. in 250 ml. distilled water. This medium offered excellent possibilities as a redox medium because it is not heavily poised (see Ward (1938) for a discussion of this point). Although Wolf reports successful daily transfers in this medium over long periods of time with both M. pyogenes var. aureus and Sal. typhosa, the author was unable to get growth of the latter organism. It was learned that by massive inoculation, this organism can become adapted to the medium, but since the adaptation might also be manifest by a change in resistance, the medium was used only as a basis for the redox medium to be described, and altered sufficiently to produce good growth of Sal. typhosa as well as M. pyogenes var. aureus.

In a study of the nutrition of Sal. typhosa, Burrows (1942) used a basal medium containing ammonium sulfate, 0.5%; sodium chloride, 0.05%; and potassium dihydrogen phosphate, 0.2%. He found that when glucose, 0.1% and tryptophane, 0.05%

were added to the basal medium, eight out of eleven strains produced growth.

The media of Wolf and Burrows were prepared and tubed in 10 ml. quantities. In addition, a mixture of purine bases containing adenine, guanine, uracil, and xanthine was prepared so that each was in a concentration of 50 micro grams per millilitre. A vitamin supplement containing thiamin hydrochloride, choline chloride, and inositol in concentration of 100 micro grams per ml.; pyridoxine hydrochloride, 250 micro grams; d-calcium pantothenate, riboflavin, nicotinamide and p-amino benzoic acid, 50 micro grams; and biotin, 0.05 micro grams was prepared. It was used in 0.5 ml. per 10 ml. of medium. A yeast extract solution was prepared in 20% concentration and used in 0.1 ml. per 10 ml. of medium. Table 1 shows the growth response of M. pyogenes var. aureus and Sal. typhosa in the media of Wolf and Burrows, when the specified additions were made. The figures in the table are recorded in percent transmission by turbidimetric examination after inoculation and incubation at 37^{0}C. for 24 hours. It can be seen from the growth responses shown in Table 1 that in 24 hours very little improvement could be made in Wolf's medium for the cultivation of M. pyogenes var. aureus. This medium is, however, entirely unsuited for Sal. typhosa. The medium of Burrows produced excellent response to Sal. typhosa when

Table 1. Growth responses of test organisms in Wolf's CADT medium and Burrows' medium.

Added Factors				Wolf's C.A.D.T.		Burrows	
Tube No.	Purines	Vit. Supp.	Yeast Extract	M. pyogenes var. aureus	Salmonella typhosa	M. pyogenes var. aureus	Salmonella typhosa
1	0	0	0	60	90.5	98	95
2	1	0	0	63	90	96.2	96.4
3	0	0.5	0	65.4	87.2	95	94.3
4	0	0	0.1	66.3	74	59.5	69.8
5	1	0.5	0	67.2	89.2	96	93.9
6	1	0	0.1	52	76	71.3	71
7	1	0.5	0.1	52.8	78	71	59.4
8	0	0.5	0.1	50	71.3	75.5	55.5

Note: Growth response is reported in percent transmission.

purine bases and yeast extract were added, but only fair growth of M. pyogenes var. aureus. On the assumption that a combination of the two media would provide for a satisfactory growth response of both test organisms, each medium was prepared in double strength, mixed in equal proportions, and tubed in 10 ml. quantities. When the combination was inoculated with these test organisms, good growth was obtained for both organisms. The results of the combination medium are presented in Table 2. Although the addition of vitamin supplement and yeast extract increased the growth response of these organisms in the combination medium, the author was interested in developing a medium in which the test organisms could initiate growth and yet be as simple as possible, to minimize poising effects. The combination medium enabled Sal. typhosa and M. pyogenes var. aureus to initiate growth and the response was later increased by increasing the amount of glucose in the formula. Ps. aeruginosa, Esch. coli, and Sal. pullorum were not included in this development because they would grow in Wolf's medium.

These exploratory media were initially adjusted to a pH of 7.0. Because of the insufficiently defined relationship of oxidation-reduction potential to pH (see Ward (1938)), it was considered logical to buffer the medium against appreciable changes in pH for at least 24 hours of growth.

Table 2. Growth response of test organisms in a medium composed of equal portions of Wolf's and Burrows' Media.

Added Factors				Combination Medium	
Tube No.	Purines	Vit. Supp.	Yeast Supp.	*M. pyogenes* var. *aureus*	*Salmonella typhosa*
1	0	0	0	34.0	69.8
2	1	0	0	34.9	63.7
3	0	0.5	0	28.5	61.2
4	0	0	0.1	31.2	56.8
5	1	0.5	0	37.5	59.0
6	1	0	0.1	33.8	61.0
7	1	0.5	0.1	32.0	55.6
8	0	0.5	0.1	25.5	50.4

Note: Growth response is reported in percent transmission.

Although Hewitt (1950) in his monograph on oxidation-reduction potentials says that "---the attempt to fix the pH of culture media by buffering the broth, is full of pitfalls," the choice of fixing this important but unknown variable is a case of choosing the lesser of two unavoidable pitfalls.

Eliminating the original 0.2% potassium dihydrogen phosphate in Burrows medium, the combination was made up containing 1x, 2x, and 3x the ratio of 1.5 g. potassium dihydrogen phosphate and 4.0 g. dipotassium hydrogen phosphate per liter which resulted in an initial pH of 7.0. These changes in the amount of a fixed ratio does not alter the initial pH but simply doubles or triples the buffer capacity of the medium. Table 3 shows the change in pH after 24 hours of incubation at 37°C after inoculation of the test organisms.

These data show that while 1.5 g. potassium dihydrogen phosphate and 4.0 g. dipotassium hydrogen phosphate is the proper ratio to produce an initial pH of 7.0, this ratio permitted changes in pH varying from - 0.58 to + 0.2 pH units below and above neutrality. Twice the amount of buffer restricted changes to - 0.2 to + 0.16 units below and above neutrality, while three times this amount, although providing adequate pH control, inhibited the growth of Sal. typhosa.

On the basis of these preliminary findings it was decided to adopt the following formula for the medium used in this study which will be known as "redox" medium:

Table 3. The effect of increasing the buffer capacity of the combination medium.

Buffer Capacity	Initial pH	*M. pyogenes* var. *aureus* 24 hr.	*Sal. typhosa* 24 hr.	*Ps. aeruginosa* 24 hr.
1x	7.0	6.57	6.42	7.20
2x	7.0	6.80	6.78	7.16
3x	7.0	7.0	No growth	7.10

Wolf's C.A.D.T. medium, Difco.....	10 g.
Ammonium sulfate, C.P.....	5
Dextrose.....	1
Potassium dihydrogen phosphate.....	3
Dipotassium hydrogen phosphate.....	8
Distilled water, q.s.....	1000 ml.

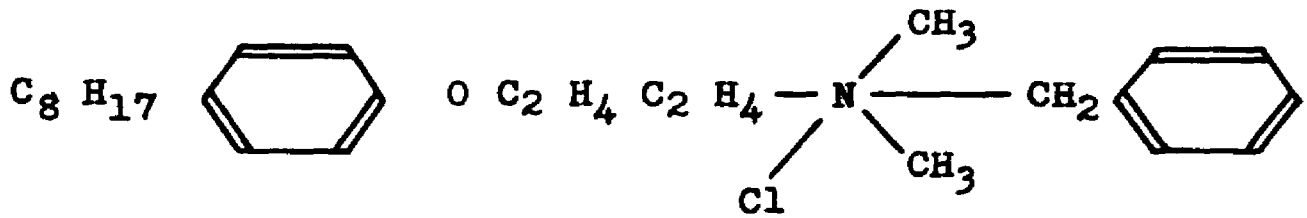
This medium has a final pH of 7.0 after autoclaving at 10 pounds pressure (116°C) for 10 minutes. In the presence of this large amount of buffer, the medium is light amber in color. Excessive heating causes additional browning to take place.

Choice of the germicide.

The most important quaternary ammonium germicides, from the commercial standpoint, are those which are prepared from vegetable or animal oils. By hydrolysis, saponification or hydrogenation, the corresponding mixture of alcohols is produced. This mixture is then treated with hydrochloric acid and the addition product of alkyl chloride and dimethyl amine is reacted with benzyl chloride to form a quaternary, alkyl dimethyl benzyl ammonium chloride. If coconut oil is used for the primary raw material, then the quaternary will be a mixture of dodecyl, 45%; tetradecyl, 18%; and hexadecyl, 10% compounds. The mixture has a consistancy of heavy oil,

difficult to free from water. Separation of this mixture is costly and not done commercially.

In order to provide for a reproducible disinfectant it is necessary to (1) have a compound in the powder form which will (2) be 100% active ingredient, and (3) be a single compound rather than a mixture of homologs. The compound, paratertiaryactylphenoxyethoxyethyldimethylbenzyl ammonium chloride satisfies these requirements and has the structure,



Commercially, this compound can be obtained under the names of phemeral, pfansteil 20, or hyamine 1622, but of these, hyamine 1622 is the only one which is 100% active ingredient. This compound is a stable, amorphous, white powder, easily soluble in water forming a clear, colorless solution, having a phenol coefficient of 300 for M. pyogenes var. aureus and 257 for Sal. typhosa at 37°C.

INSTRUMENTATION

The electrode vessel.

A vessel for bacterial culture must lend itself to easy assembly, must withstand repeated sterilization, and must be

designed with an accessible opening through which samples may be withdrawn. Because of the surface active properties of quaternary ammonium compounds, the absorption from solution to the glass surface of the container must be kept at a minimum. Effects of adding reagents or removing aliquot portions from the vessel must be reduced to insignificance. These things can be accomplished by the use of rather large cultures. An electrolytic beaker without a pouring lip and of 180 ml. capacity forms the container for the electrode vessel used in this study. The beaker was fitted with a four-hole #11 rubber stopper, previously treated with alkali to remove sulfur. One hole is 18 mm. in diameter to accomodate a short section of 18 mm. glass tubing for sampling purposes. The protruding end of this tubing is covered with an aluminum cap. The other three holes are bored to 6 mm. in diameter to accomodate a salt bridge, an electrode, and a mixing pipette.

The salt bridges are fashioned from 6 mm. capillary tubing having a 1 mm. bore. The electrodes were procured from the Leeds and Northrup Company under stock number 7705. These electrodes are 1 sq. cm. platinum plates, spot welded to a short platinum wire, sealed in soft glass tubing of 6 mm. outside diameter. A terminal at the top is sealed into the glass and connected to the short length of platinum wire at the bottom by means of a copper wire.

The reference electrode is of the high temperature calomel type and is manufactured by National Technical Laboratories under catalog number 8970-90. It is designed for use in any constant temperature system operating at any temperature between 0 and 100 degrees C. At 37°C. this electrode has a potential of 0.237 volts with respect to the hydrogen electrode, but its potential for any temperature may be calculated by the relation,

$$E_{SAT.\,KCl} = 0.246 - 0.00076\,(t - 25)$$

where t is the operating temperature in degrees centigrade.

The mixing pipettes are modified 10 ml. volumetric pipettes on which the bottom tube is flared and notched to prevent constriction if it should accidentally rest on the bottom of the electrode vessel. A photograph of the assembled electrode vessels is shown in Fig. A.

The mixing device.

The large electrode vessel adequately compensates for unwanted glass surface absorption and provides a large system for withdrawing samples or adding reagents with minimum effect. But unfortunately, the depth of the medium contributes to electrode potential stratification or differences in potential from the surface to the bottom of the medium. These differences become appreciable when growing a test organism like Ps. aeruginosa which is aerophilic and tends to form a pellicle on the surface of the medium.

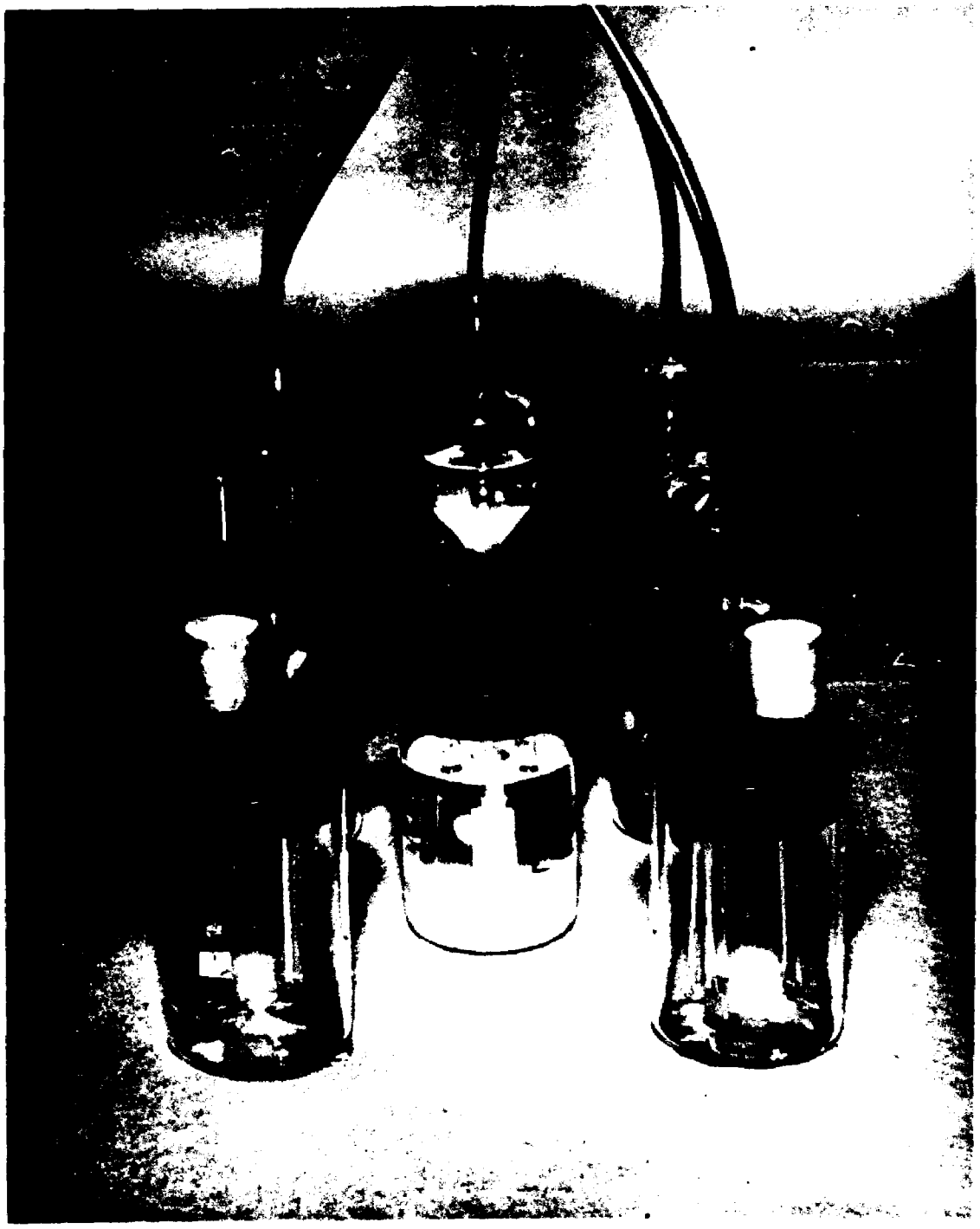
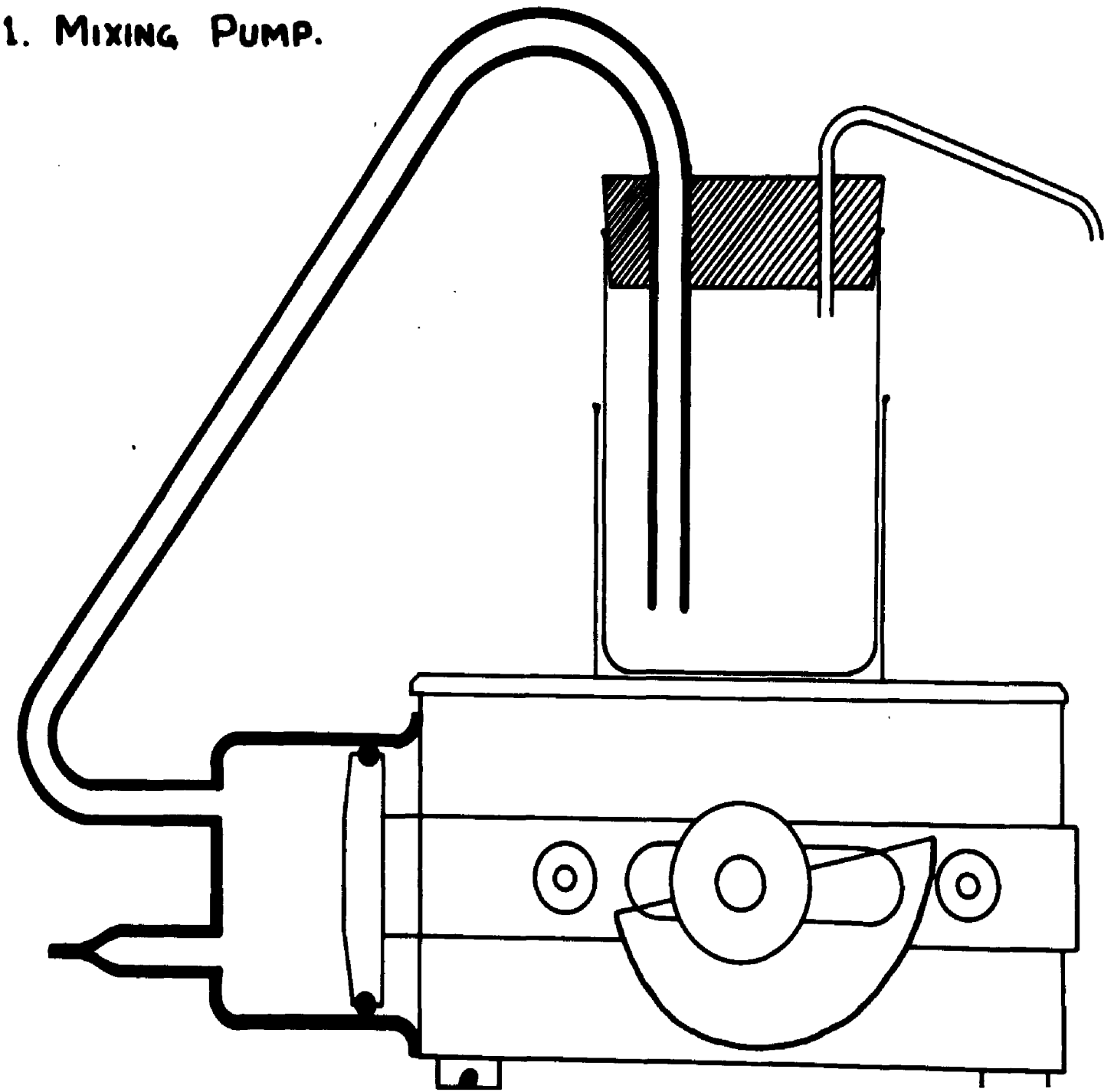


Fig. A. Assembled Electrode Vessels.

Duplication of results has always plagued those working with oxidation-reduction potential measurements in bacteriology. Mudge and Smith (1933) have made a study of the effects of agitating a bacterial suspension. Ward (1938) found that stirring eliminates poising and zoning effects and also prevents the indicator electrodes from becoming sluggish by coating with cells. Mechanical stirring is one solution to effective agitation but unless this method is used under anaerobic conditions with tight mercury seals around the impeller shaft, aeration is apt to result. Ridley (1928) devised a rather ingenious device around the principle of a recycling siphon, operating by water power, which he used for studying "dynamic incubation" of bacteria. However, this apparatus provides aeration in addition to agitation.

In this work, the author wanted to thoroughly mix the cultures under examination and yet not provide them with any additional air that they would not otherwise obtain naturally from surface exposure. This requirement dictates the use of a force which can act under the surface but not through it. The idea of the recycling siphon was appealing but a partial vacuum, created by lowering a liquid surface, was broken by the admittance of outside iar. The author modified a piston type pump of 32 ml. per stroke capacity, operating through a step-down gear train at a speed of 10 strokes per minute. This pump was obtained from the Lyon Industries, Inc. under part number AS 754. A diagram of the modified pump is shown

FIG. 1. MIXING PUMP.



in Fig. 1. The inlet check valve was removed and the outlet was sealed off. This pump was designed with a neoprene piston ring which requires lubrication. This is provided for by making the pump recycle hydraulic brake fluid into a reservoir mounted on top of the pump frame. A rubber stopper in the reservoir is provided with a 10 mm. glass tube to the piston and four 5 mm. tubes which connect the mixing device to the mixing pipettes in the electrode vessels. Under these conditions, the system is closed to outside air and displaces 32/4 or 8 ml. of culture medium in each electrode vessel, by means of an air transformer, at the rate of 10 times per minute.

A portion of a recording, showing the reoxidation curves of Sal. typhosa, M. pyogenes var. aureus, and an uninoculated control, is reproduced in Fig. 2. It was noted that the electrod responses in both of the inoculated media were so erratic during the period of reoxidation that, from the standpoint of determining the rate of reoxidation, an interpretation of these results was impossible. Although the uninoculated medium responded with little variation during this time, it must be remembered that no metabolic changes are present in the absence of an inoculum. This figure is presented at this time to emphasize the instability of electrode response when a germicide is added to an actively metabolizing culture under static incubation. If, however, the suspensions are agitated, the

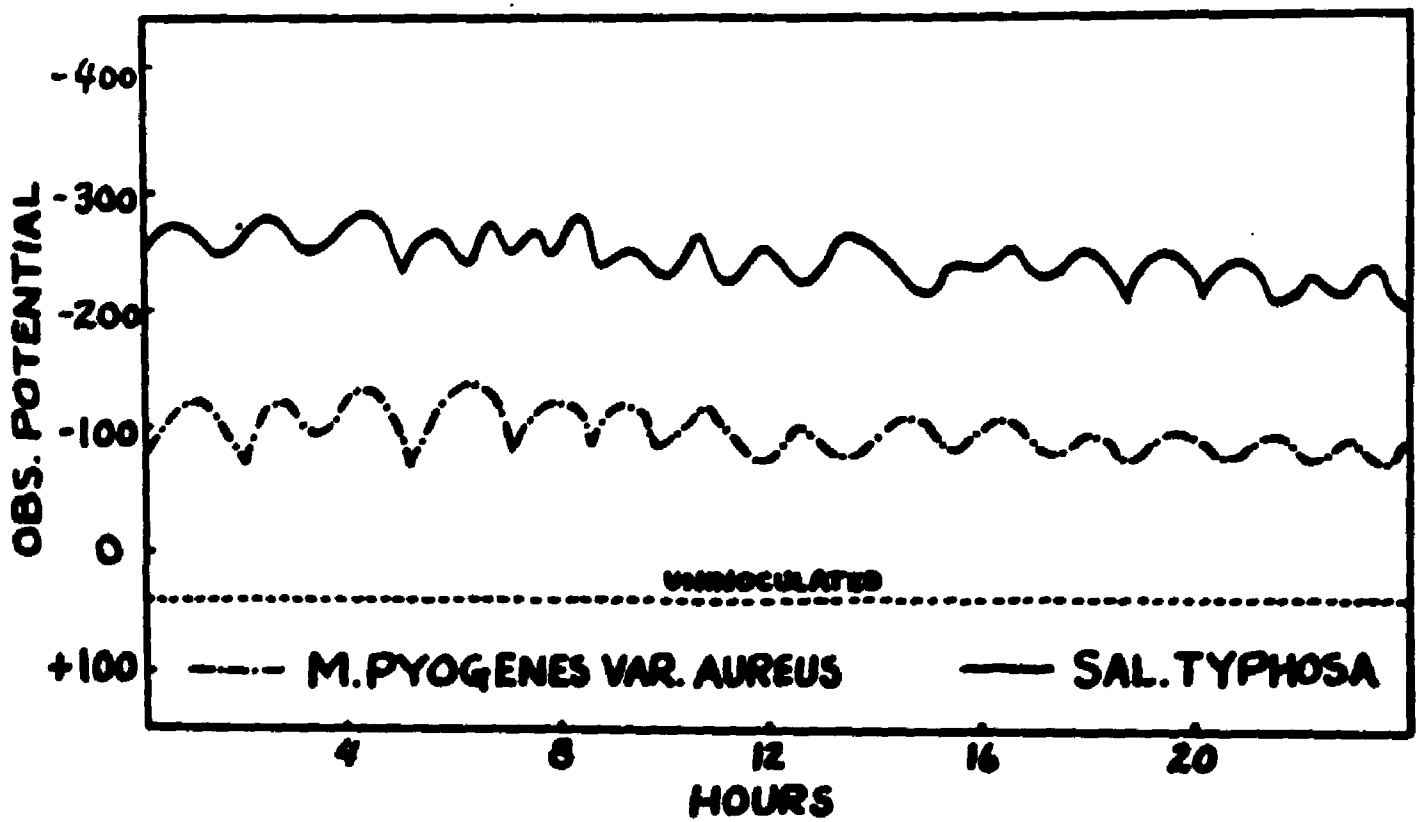


FIG. 2. REOXIDATION CURVES OF ORGANISMS GROWN UNDER STATIC INCUBATION.

germicide is rapidly distributed throughout the suspension and the erratic electrode responses are eliminated. Thus, from the standpoints of reproducibility and the interpretation of reoxidation curves, the use of a mixing device is essential, and was employed in producing all of the curves that will be described.

The series-bridge balanced isolation amplifier.

A bacterial culture may be considered as an electrical half-cell, according to Cohen (1931). But as a half-cell, the bacterial culture is of very limited capacity. Obviously, if it is necessary to increase its capacity, this can be done by increasing the total amount of oxidizable material in the system. This is one way to overcome the effect of electrode polarization. However, increasing the cell capacity is similar to, if not identical with intentionally poising the system at a given potential. Another way to eliminate low readings due to low cell capacity and electrode polarization is to measure the electrode potential with a classical potentiometer, except that before each reading is taken, the operator must make an accurate estimate of a potential which is constantly changing. The futility of this approach is apparent. The logical way is to insert a device between the electrodes which will give an accurate indication of the potential difference without drawing appreciable current through the circuit. Allyn and Baldwin (1932) devised a circuit for this purpose in which

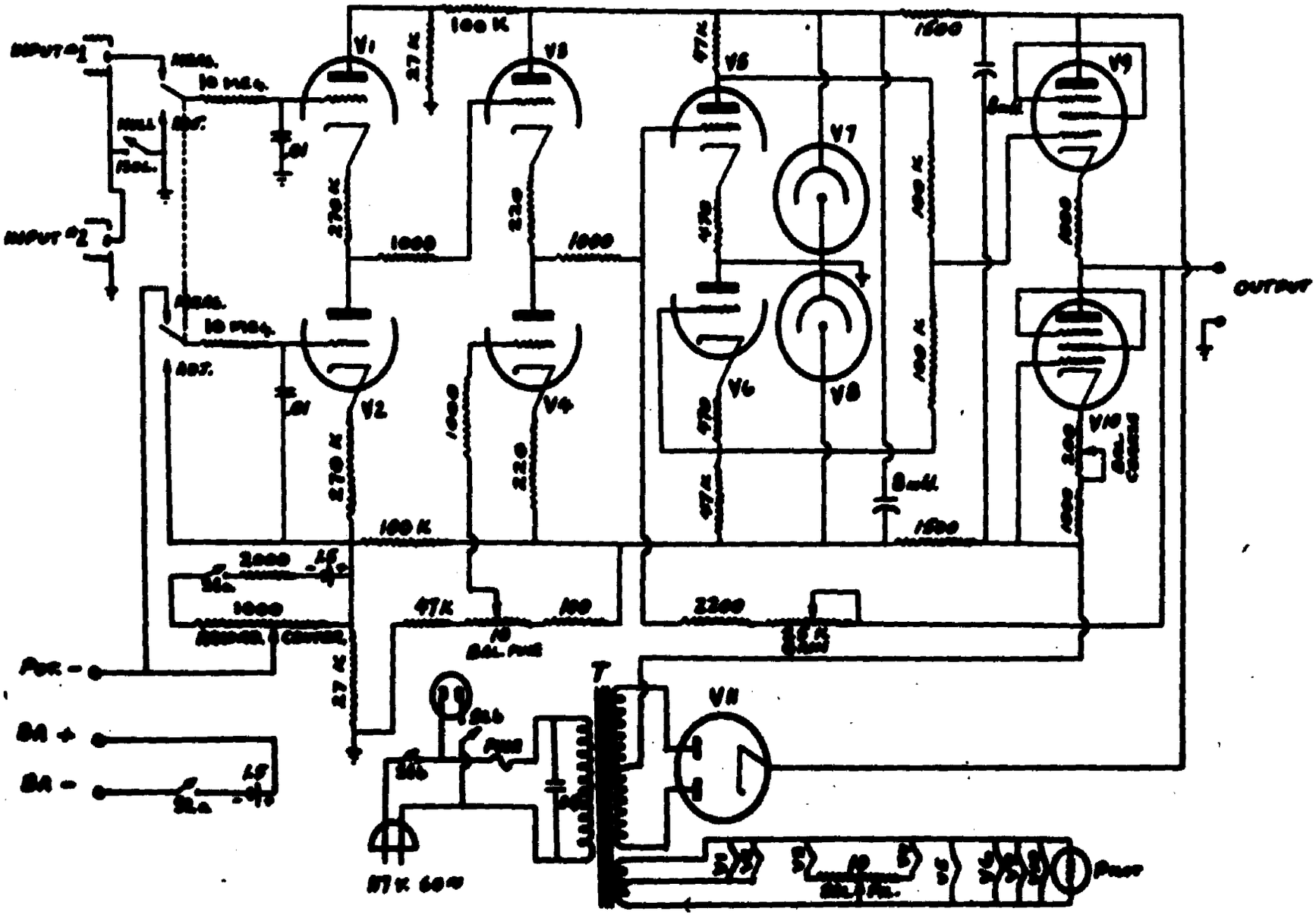
they incorporated a vacuum tube with an input impedance of 7 megohms as the isolating element and connected a student-type potentiometer in series with the reference cell and the cathode return of the tube. The measuring electrode was connected directly to the tube grid. Such a system is entirely satisfactory but is of the null reading type, that is, all electrode potentials are equaled by a potential provided by an external battery. Under this condition no current flows in the circuit and the battery potential is measured with the potentiometer.

When studying the effect of quaternary ammonium germicides on electrode potentials, the investigator is never sure of the time to take manual measurements. Therefore, a continuous strip-chart recording is justified. Coulter and Isaacs (1929) used a recording potentiometer with an input impedance of 2,500 ohms. The author has chosen to incorporate a series-bridge balanced direct current amplifier with an input impedance of 10,000,000 ohms input impedance to reduce polarization of the measuring electrodes to a minimum. The output from this amplifier is used to drive a recorder.

The bridge principle in direct current amplification makes use of even numbers of tubes connected in opposition to each other so that at balance, their output equals zero. This principle compensates for variation in power supply but does not compensate for tube aging effects. As an electron

tube becomes old, its cathode emission decreases and the tube characteristics change. To compensate for this type drift, the series method of Artzt (1945) was employed. If two tubes of the same type are connected in series so that a given change in characteristics of one tube will cause positive drift, the same change in characteristics in the other tube will cause a negative drift. Thus, the method nullifies tube aging effects. By using a series-bridge balanced amplifier, it is possible to eliminate two of the most troublesome properties of D.C. amplifiers: power supply variation and tube aging effects. A third difficulty lies in grid current flow in the input tubes. The circuit of Sulzer (1950) has excellent characteristics in this respect in that the input grid circuit is limited to 10 microamperes at zero input, and is obtained by reducing the filament from the normal 6.3 to 3.0 volts. In addition, the grid is operated at ground potential and the plate voltage is reduced to 20 volts. The amplifier shown in Fig. 3 was designed and built by the author and incorporates a regulated plate and filament power supply, degenerative feedback for increased stability, and a series-bridge balanced cathode follower output stage to match the impedance of a recorder. The amplifier is provided with fine and coarse balancing adjustments, a gain control varying from 0.5 to 2.5 times the input, and a recorder centering bias control, all accessible from the front panel. A function switch is included which, when depressed, places the

FIG. 3. SERIES-BRIDGE BALANCED AMPLIFIER.



input grids at zero potential for balancing. When released, this switch connects the input terminals to the grids of the tubes so that this potential is duplicated at the output terminals. The bias control positions the zero point on the recorder scale so that no attention is required as the indicator electrodes go from plus to minus with respect to the reference calomel cell.

The recorder.

The recorder used in this study was a Leeds and Northrup strip-chart, 4-point apparatus. The range of the instrument was modified to 0 to 600 millivolts in 5 mv. divisions. The chart is 60 line uniform paper driven at a speed of 1.5 inches per hour. A circuit diagram of the modified recorder is shown in Fig. 4. Electrical modifications in addition to the range change are: the line coming from the commutator minus terminal was opened and connected to the amplifier input minus terminal. The other end of this lead to the recorder standardization switch is connected to the amplifier output minus terminal. The commutator positive line leading to the slidewire contact was opened and connected to the amplifier input positive terminal while the slidewire contact was connected to the amplifier output positive terminal. These changes are shown in the diagram.

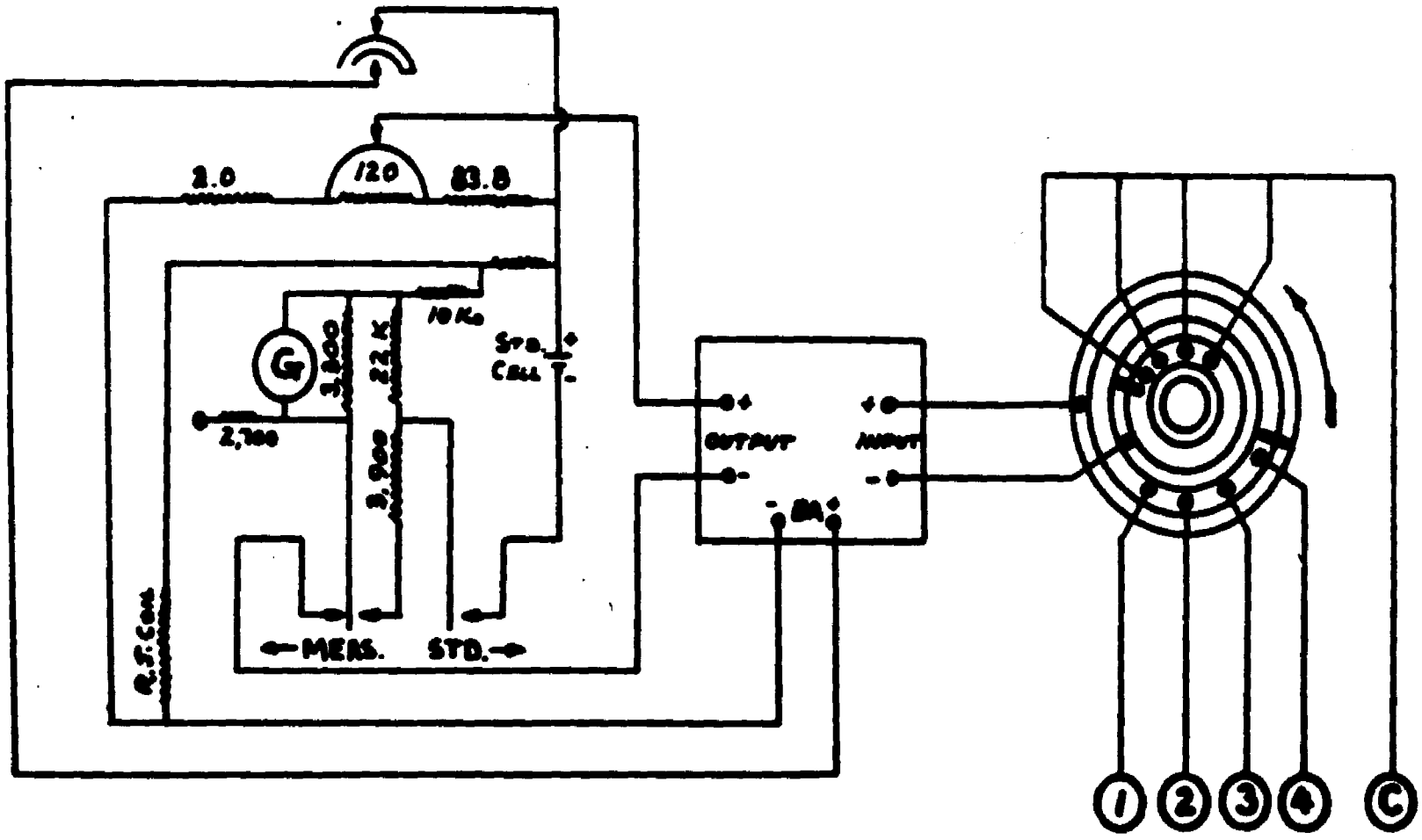


FIG. 4. DIAGRAM OF MODIFIED RECORDER.

EXPERIMENTAL PROCEDURE

Instructions for the operation of apparatus.

The procedure for setting up the apparatus may best be given in stepwise form for clarity and ease of presentation.

1. Prepare 400 ml. of redox medium by dissolving 4 g. Wolf's casamino acid disinfectant test medium, Difco; 2 g. ammonium sulfate; 0.4 g. glucose; 1.2 g. potassium dihydrogen phosphate; 3.2 g. dipotassium hydrogen phosphate, in 400 ml. distilled water. These constituents are brought to a boil, cooled, and filtered.

2. Clean platinum electrodes with hot nitric acid, and rinse in distilled water.

3. Scrub electrolytic beakers in an anionic detergent solution, immerse in chromic acid cleaning solution, rinse in distilled water, and invert to air dry. Clean mixing pipettes in same manner.

4. Attach 1/8 inch neoprene tubing to bulb end of mixing pipette and install a short length of 4 mm. glass tubing into the other end of the tubing. Plug the open end of the glass tubing with cotton.

5. Assemble the electrode vessels by inserting electrodes, mixing pipettes, and temporarily plug the salt bridge holes with a short length of capillary tubing sealed off at one end.

6. Dispense 98 ml. of redox medium into each cell through the sampling tube. Cover this tube with an aluminum cap.

7. Check the distance of the electrodes and mixing pipettes from the bottom of the electrode vessel. This distance should be 1/2 inch.

8. Take two salt bridges at a time and place the long arms together. Dip these ends into an 18 mm. test tube. Place the short ends into another 18 mm. test tube. Support these tubes in a test tube rack to hold them upright. Pour previously melted saturated potassium chloride agar into the tubes up to the top. Repeat for the other two salt bridges.

9. Autoclave the four salt bridges and the assembled electrode vessels at 10 pounds pressure for 10 minutes.

10. Remove the electrode vessels to a 37°C. water bath.

11. Inspect salt bridges for minute bubbles in the capillary. If these bubbles are present, they must be worked out while the agar is still hot by tilting the arm of the bridge closest to the bubble into a downward position. After salt bridges are worked until free of air bubbles, place in refrigerator to solidify.

12. With solidified, sterile salt bridges nearby, remove the temporary glass plug from the stopper in the sterilized

electrode vessels and quickly insert the long arm of the bridge into the stopper hole to a depth of 1/2 inch from the bottom. The short arm need not be handled aseptically since it is going to be immersed in the saturated potassium chloride reservoir. Repeat the installation of the salt bridges on the other three electrode vessels.

13. Position the mixing pump piston so that its first stroke will raise the medium into the bulb of the mixing pipette. This prevents an initial bubbling of air through the medium. Remove the cotton plugs from the glass couplings and insert these couplings into the gum rubber tubing on pump reservoir.

14. Short all electrode leads together.

15. Depress function switch, located in the center of the amplifier panel, to "Adjust" position. Set "Recorder" switch, located at the right of the pilot light on amplifier panel, to the "On" position to start the recording motor. Observe the position of the indicator on the recorder. It should be on zero scale. If not, rotate recorder centering bias control to a full counter clockwise position. If the recorder is still not on zero, balance the amplifier by means of "Coarse" and "Fine" balance controls.

16. Lift function switch to "Measure" position and rotate "Recorder Centering" control until recorder indicator is at the desired point on the scale. Most curves were prepared with this adjustment set at 450 mv.

17. Return function switch to "Adjust" position and connect red spring clip to cell #1 (left front); black spring clip to cell #2 (left rear); green spring clip to cell #3 (right rear); and blue spring clip to cell #4 (right front); and attach the short, small diameter wire to the saturated calomel reference cell (center). These electrode leads are color coded to match the inks found on the strip charts.

18. Lift function switch to "Measure" position and start water bath stirring motor and electrode vessel mixing motor by turning a speed control rheostat in a clockwise direction until the desired speed is attained.

19. Inoculate electrode vessels as follows:

Cell #1. Test organism, 24-hr. susp., 1 ml.

Cell #2. Uninoculated control.

Cell #3. Test organism, 24-hr. susp., 1 ml.

Cell #4. Test organism, 24-hr. susp., 1 ml.

It is assumed that the apparatus is assembled in accordance with the foregoing diagrams. A photograph of the complete apparatus is shown in Fig. B. When the isolation amplifier was first placed in service, the author noted an initial drift of approximately 100 mv. in the first 24-hour period. This drift was later attributed to uneven heating of components within the amplifier. To eliminate drift, the amplifier has been kept on ever since. Should it be necessary to turn the amplifier off for repair or tube replacement, it is suggested that it be allowed 24 hours after starting to regain its thermal equilibrium.

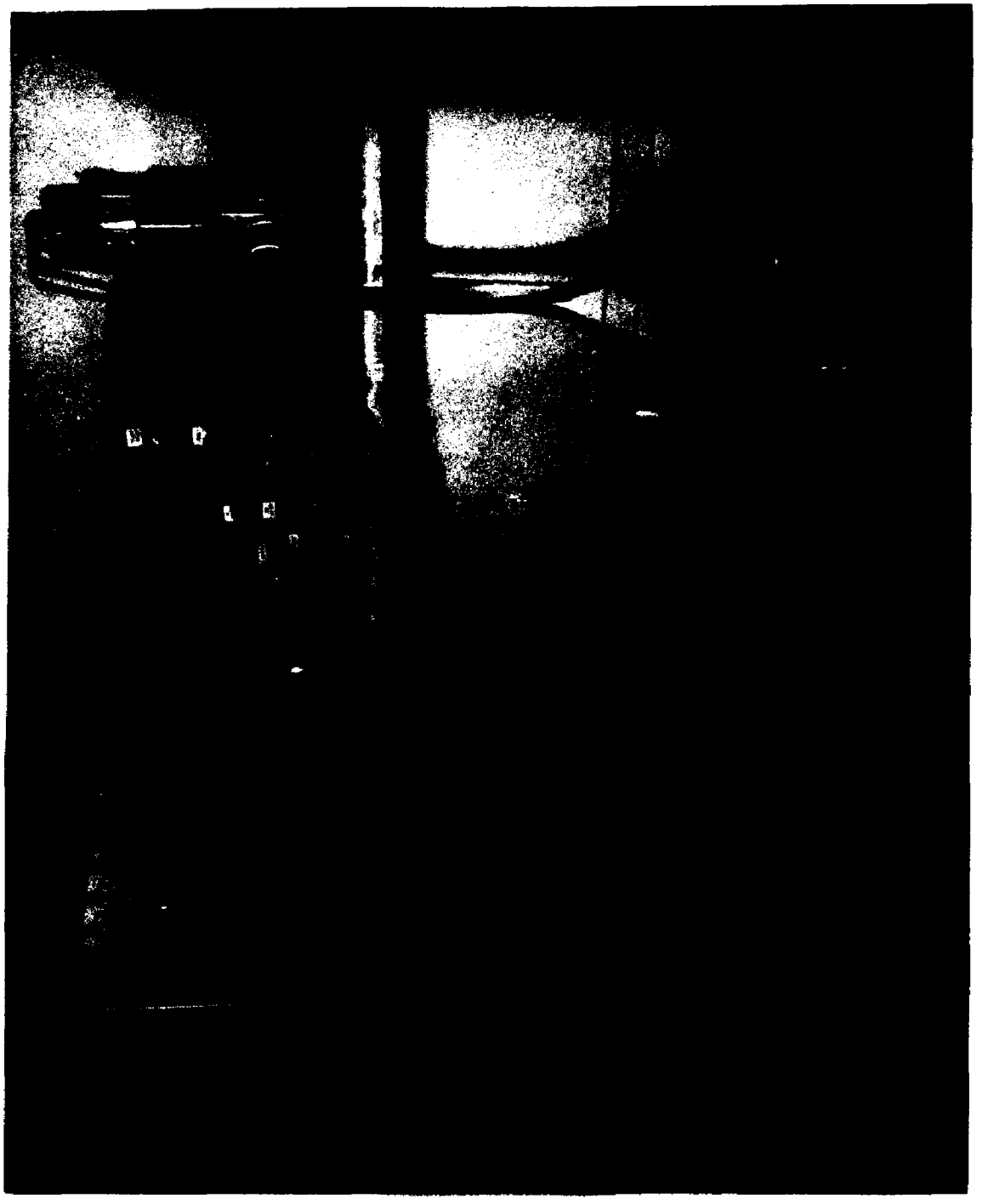


Fig. B. The Complete Apparatus.

Potentiometric measurement of Eh.

When the recorder centering bias is adjusted to 450 mv., as it was in the electrode potential studies to follow, there are three scales to keep in mind. The first of these scales is the numerical scale on the strip-chart recorder. This scale runs from 0 to 600 mv., full scale. The second scale is the observed potential, Eo scale. Since the recorder is biased at 450 mv., this means that at zero input the recorder will read 450 mv. This was done so that no attention would be required as the indicator electrodes went from positive to negative with respect to the reference cell. The second scale, Eo may be calculated from recorder scale readings by subtracting 450 mv. The third scale is that of oxidation-reduction potential, Eh. In most studies, it is customary to refer the potential of the indicator electrode to a hydrogen electrode which is arbitrarily assigned the value of 0.000. Therefore, to convert from Eo to Eh, one must use the relation,

$$Eh = Eo + 237$$

where Eh is the oxidation-reduction potential in millivolts, and Eo is the observed potential in the same units, and 237 is the potential in millivolts of the reference calomel half-cell in saturated potassium chloride, operating at 37°C. The conversion from one scale to another has been calculated throughout the range of the recorder and is given in Table 4.

Table 4. Table of conversion from recorder scale readings to observed potential and oxidation-reduction potential.

Recorder Scale	Observed* Potential	Oxidation-Red. Potential
0	-450	-213
50	-400	-163
100	-350	-113
150	-300	- 63
200	-250	13
250	-200	37
300	-150	87
350	-100	137
400	- 50	187
450	0	237
500	50	280
550	100	337
600	150	387

*Recorder centering bias control set at 450 mv.

In this study of rates of germicidal activity, little concern need be given to Eh as such, although this value may be calculated at any time by the relationship given in the preceeding paragraph. Rather, attention should be directed to a change in potential with respect to time. Therefore, the time-potential curves are calibrated in terms of the observed potential for obvious reasons of simplicity which will provide for even units on the millivolt scale.

Normal time-potential curves.

The time-potential curves to be described were prepared by inoculating 1 ml. of a 24-hour redox medium culture of the specified test organism into 98 ml. of redox medium in an electrode vessel. The recorder and mixing pump were started prior to inoculation so that the potential of the uninoculated medium could be determined. The uninoculated medium ranges from 45 to 50 mv.

Fig. 5 through 9 are presented to show the normal time-potential curves of the five test organisms. These organisms were inoculated into individual cells and continuous recordings were made of their uninhibited metabolic activity for 24 hours. These graphs were reproduced from the original recordings, with the transient responses due to live voltage fluctuations removed. Inasmuch as the original recordings were 36 inches long for a 24-hour period, it was considered more

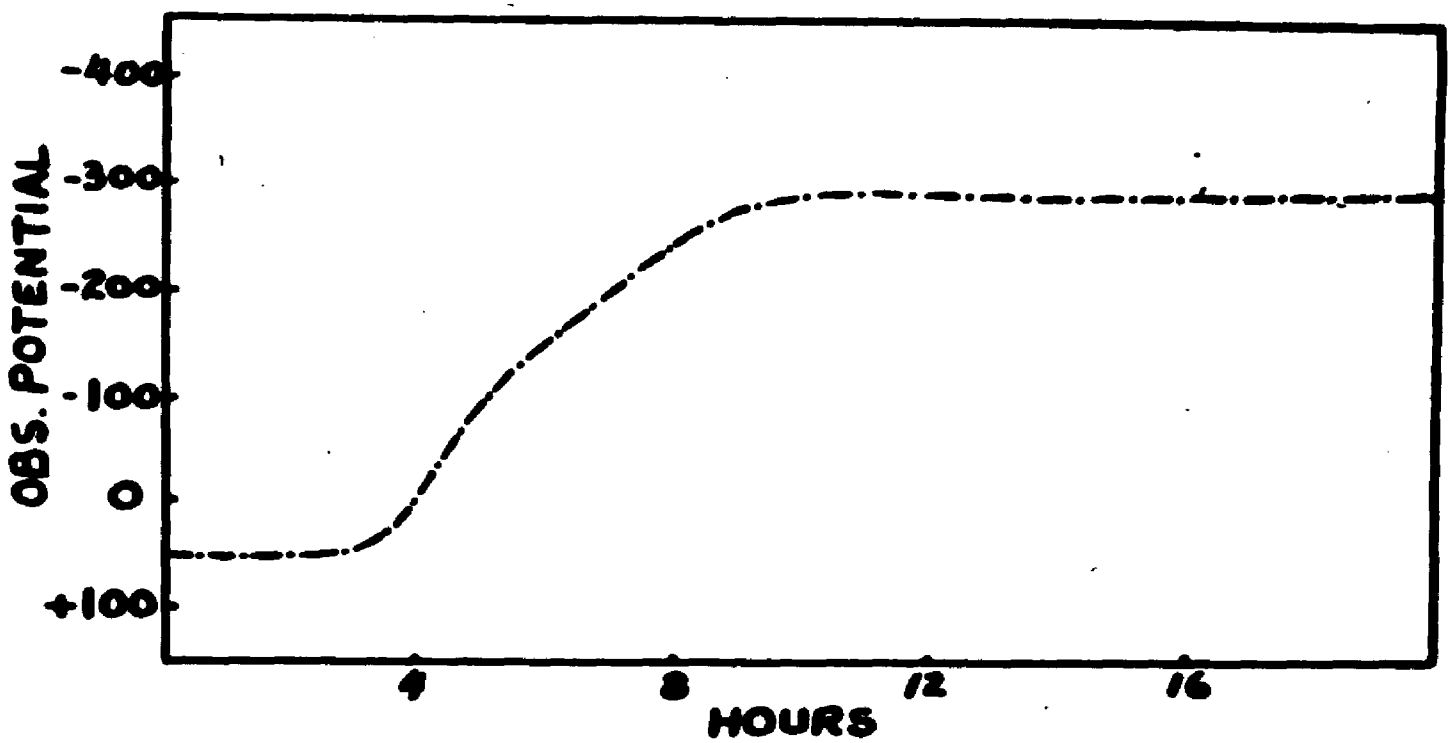


FIG. 5. GROWTH RESPONSE OF M. PYOGENES VAR. AUREUS.

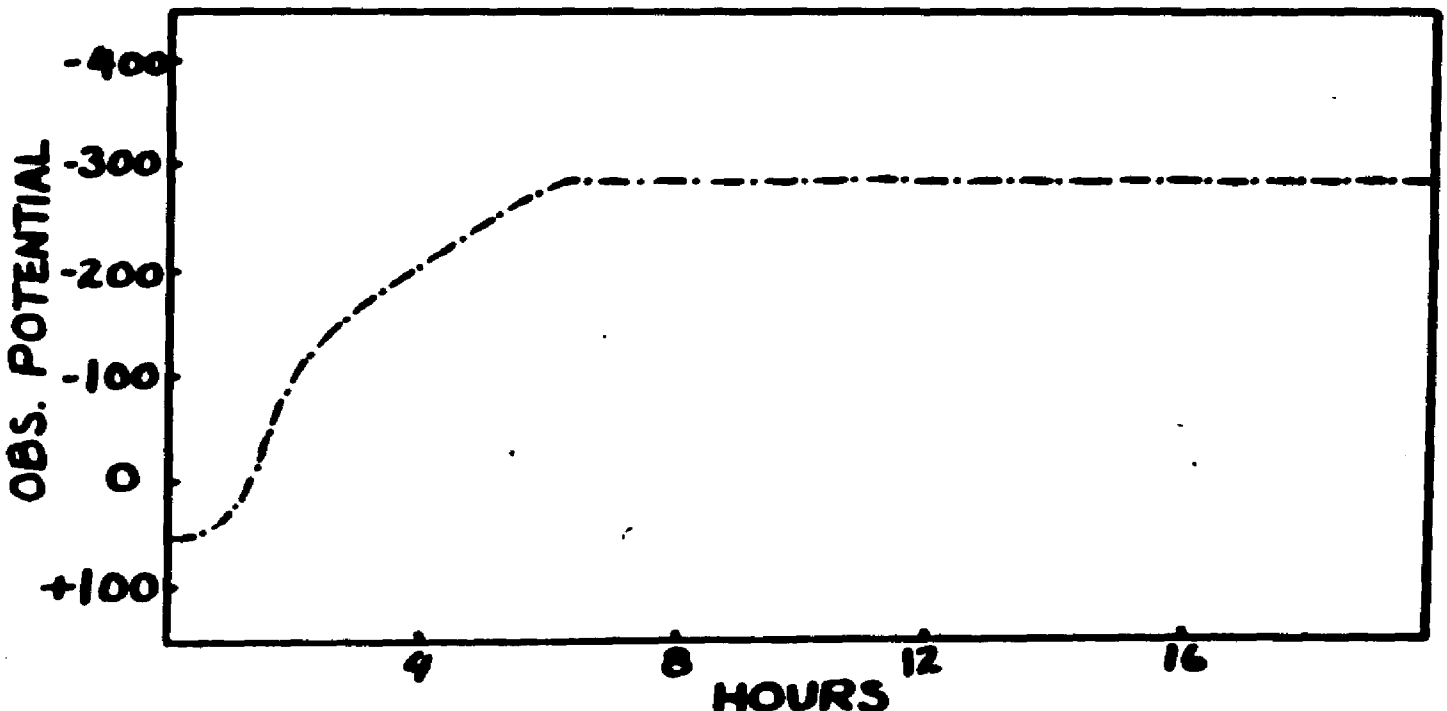


FIG. 6. GROWTH RESPONSE OF SAL. TYPHOSA.

appropriate to illustrate with scaled-down reproductions. For the most part, the normal time-potential curves are self-explanatory, but the variation in different test organism responses deserves some discussion. Fig. 5 is a curve obtained by growing M. pyogenes var. aureus. This organism has a lag phase of 3 hours in redox medium. During its most active proliferation, a slight hump is detected which is centered at about 5 hours after inoculation. Following this hump, the initial reduction rate of 100 mv. per hour is decreased to 50 mv. This reducing intensity prevails until 9 hours when it levels off at a constant potential of - 290 mv. Throughout the rest of the recording, this reducing intensity remains constant.

Fig. 6 is an illustration of a typical uninhibited response of Sal. typhosa. The time required to reach a minimum potential is approximately 6 hours as compared to 8 hours for M. pyogenes var. aureus, although the characteristic potential for both organisms is - 290 mv. The second response differs from Fig. 5 during the initial stages of reproduction by a 2 hour shorter lag phase and a slightly greater deviation in initial slope. One would expect greatest similarity between these test organisms because the medium was developed with the aim of equalizing their responses.

Fig. 7 presents the time-potential curve for Ps. aeruginosa. This organism has no appreciable lag in establishing reducing conditions, however, 16 hours were required to reach its

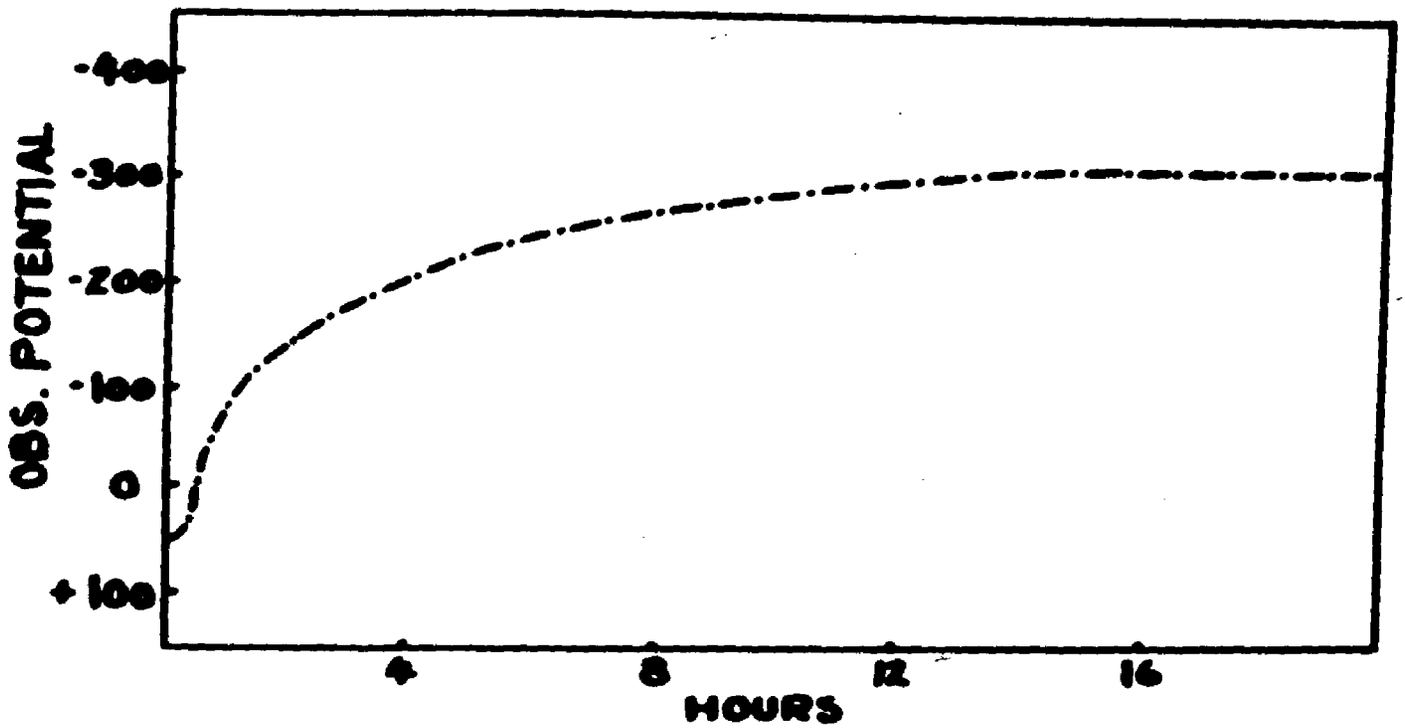


FIG. 7. GROWTH RESPONSE OF PS. AERUGINOSA.

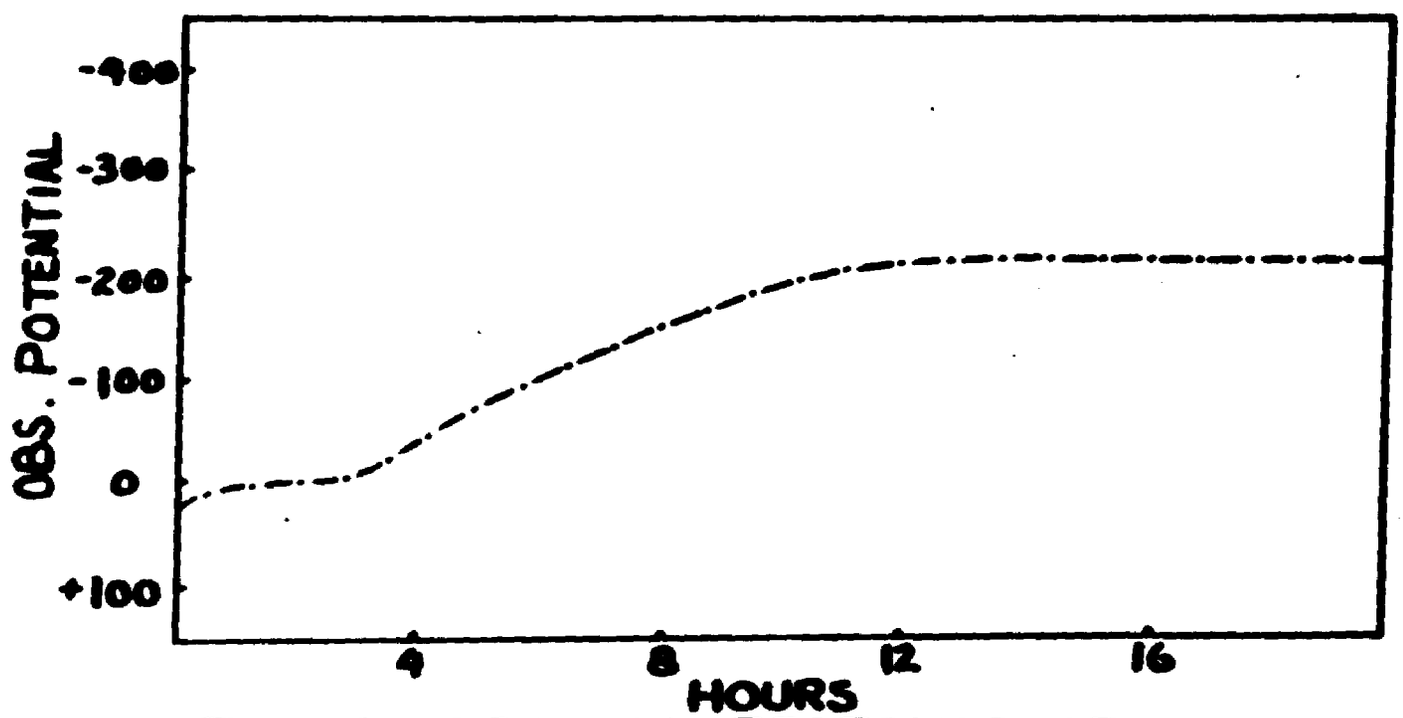


FIG. 8. GROWTH RESPONSE OF SAL. PULLORUM.

maximum reducing intensity of -310 mv. A changing rate of reduction during the initial growth phase is most pronounced with this organism.

The response of Sal. pullorum is illustrated in Fig. 8. It has a lag phase lasting for 3 hours in redox medium. Active proliferation takes place with a rise of 25 mv. per hour which continues for 12 hours. The maximum intensity is reached at -220 mv.

Fig. 9 graphically describes the response of E. coli in redox medium. This organism produces a very uniform response curve with a short lag phase, a constantly rising slope, and the greatest reducing intensity of the organisms studied, equilibrating at -370 mv. E. coli obviously adapts rapidly to the medium.

Having determined the general shape of the response curves, in terms of electrode potential, some relation must be found between this method and one obtained by standard plating procedure to establish the validity of the electrometric technique. A comparison of the response of M. pyogenes var. aureus by these two methods is presented in Fig. 10. The curves were obtained by examination of the growth, inhibition, neutralization, and partial recovery of the test organism by the observed electrode potential and standard plating techniques. For the moment, neglecting the general shapes of the curves, a duplication of each change in direction is

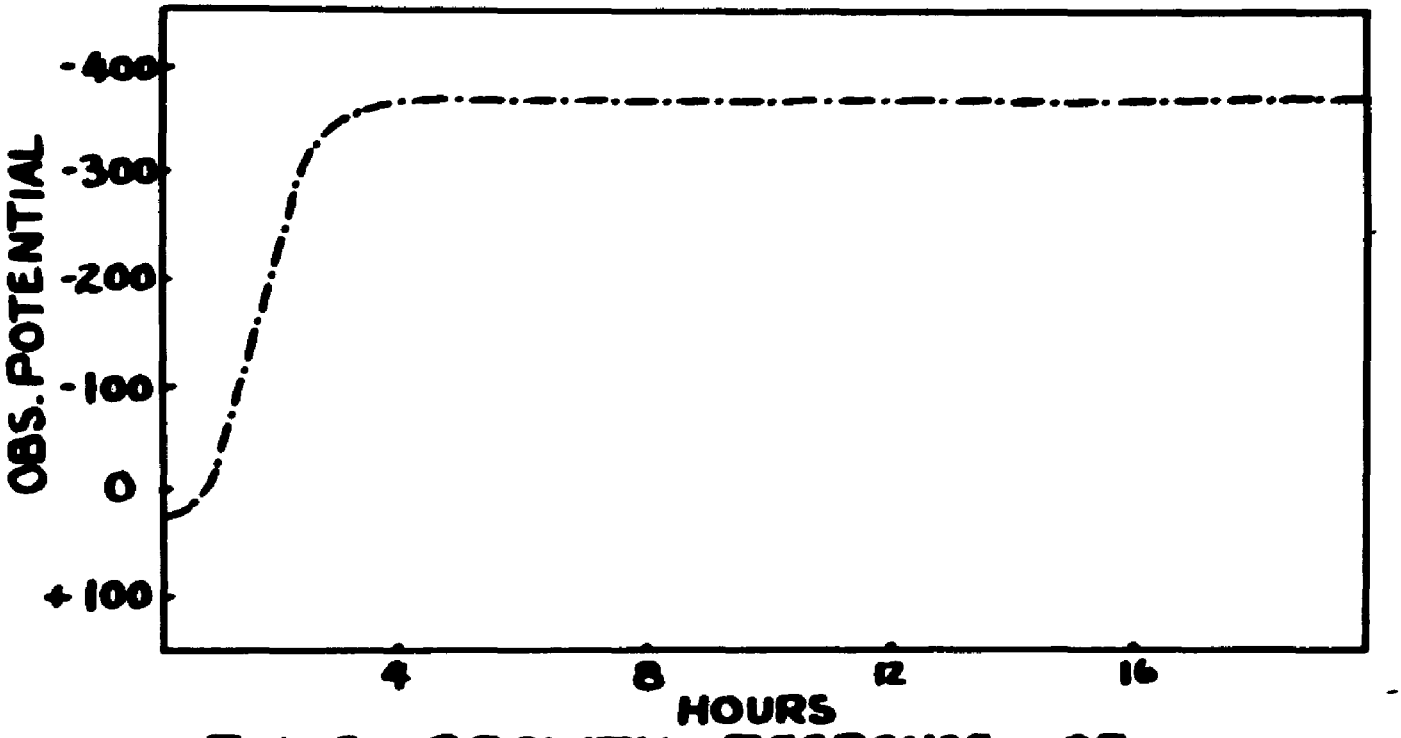


FIG. 9. GROWTH RESPONSE OF E. COLI.

discussed. These curves were obtained simultaneously so that the same organisms giving rise to the different reducing conditions are also removed at specified intervals and plated on tryptone glucose agar. The graphs were constructed with the observed potential on the left vertical axis, time on the horizontal axis, and the logarithm of the plate count on the right vertical axis. The log scale was made to coincide with the initial and maximum observed potential so that the curves are comparable in size. Under these conditions there is a lag in response in the electrode potential curve, if the plate count is considered the best method of measurement. This lag in potential change is most pronounced in abrupt changes of direction. The resistance to change may be due to (1) a poising capacity of the medium as evidenced in the initial growth response where the change is in a negative direction, and (2) the finite time required for the medium to be reoxidized by the air. The second cause is in effect when the potential is rapidly changing in a positive direction and is in total disagreement with the concept that reducing conditions are established by the cell elaborating reducing substances. This idea, advanced by Coulter and Isaacs (1929), would suggest that the electrode potential should go further negative upon the addition of a bacteriolytic substance. The author is of the opinion that incongruities shown in this correlation could be minimized by the use of a sufficiently barren medium, non-resistant

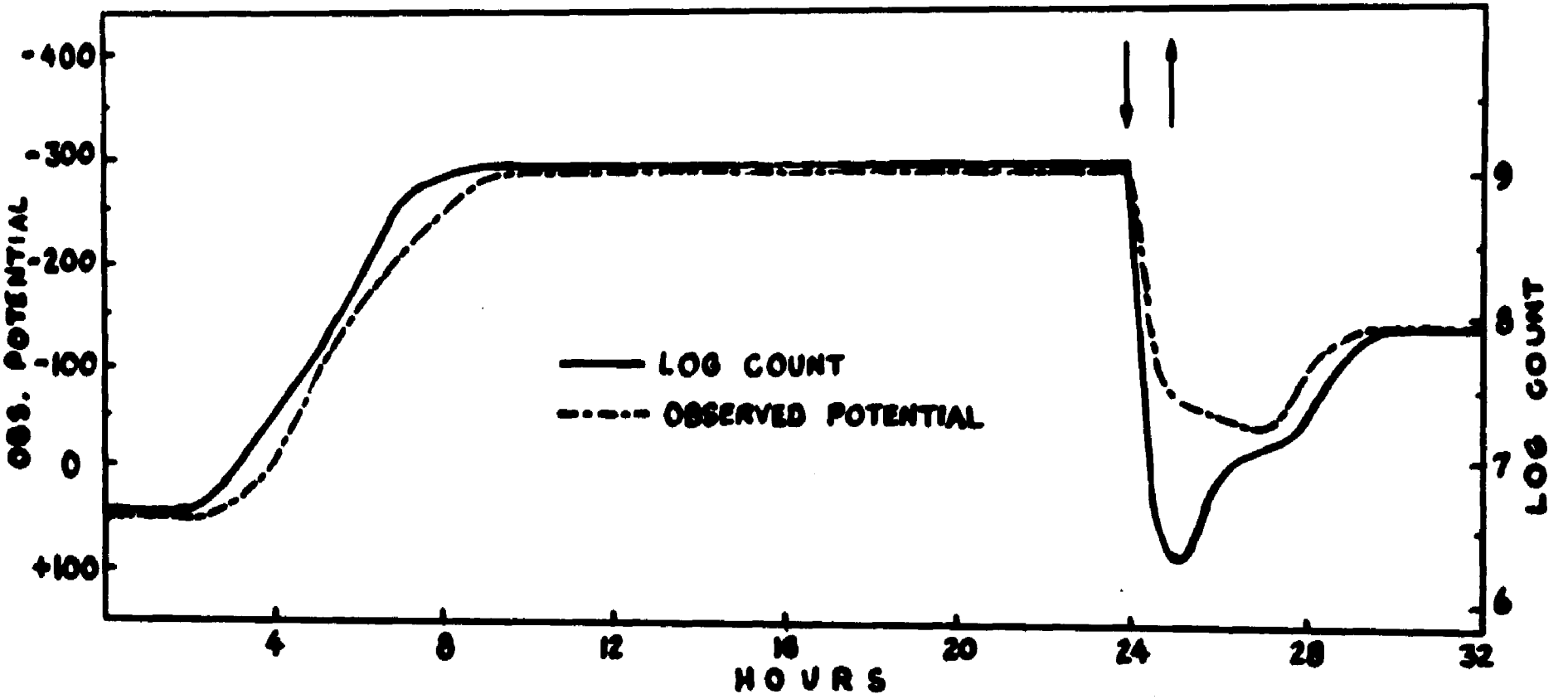


FIG. 10. COMPARISON OF POTENTIAL AND PLATE COUNT CURVES OF M. PYOGENES var. AUREUS.

to change in potential, and requiring a minimum reoxidation time. Such a medium having these qualities and still retaining its prime function as a provider of growth factors is hardly possible. The redox medium developed by the author is an approach to minimal poising but has its limitations. These statements are based on results obtained by plating methods. These too have their limitations but are beyond the scope of this paper.

Fig. 11 and 12 illustrate similar incongruities when using Sal. typhosa and Ps. aeruginosa respectively as the test organisms. These correlations would seem to indicate that any deviation of electrode potentials from reflecting the logarithm of the number of organisms is a limitation of the medium in which the organisms are grown. These differences are more pronounced in older cultures and during periods of abrupt changes in cultural conditions.

To give some indication of the oxygen absorbing intensities of freshly prepared medium and one which has been "spent" by a bacterial suspension, the following experiment was conducted. A quantity of redox medium was prepared in the usual manner. One-half of this quantity was placed in duplicate electrode vessels; the other half was placed in two other electrode vessels. All four of these cells were autoclaved and cooled to 37°C. Two cells were inoculated with 1 ml. of a 24-hour suspension of M. pyogenes var. aureus. The other two cells were left uninoculated. All cells were incubated and mixed for 24

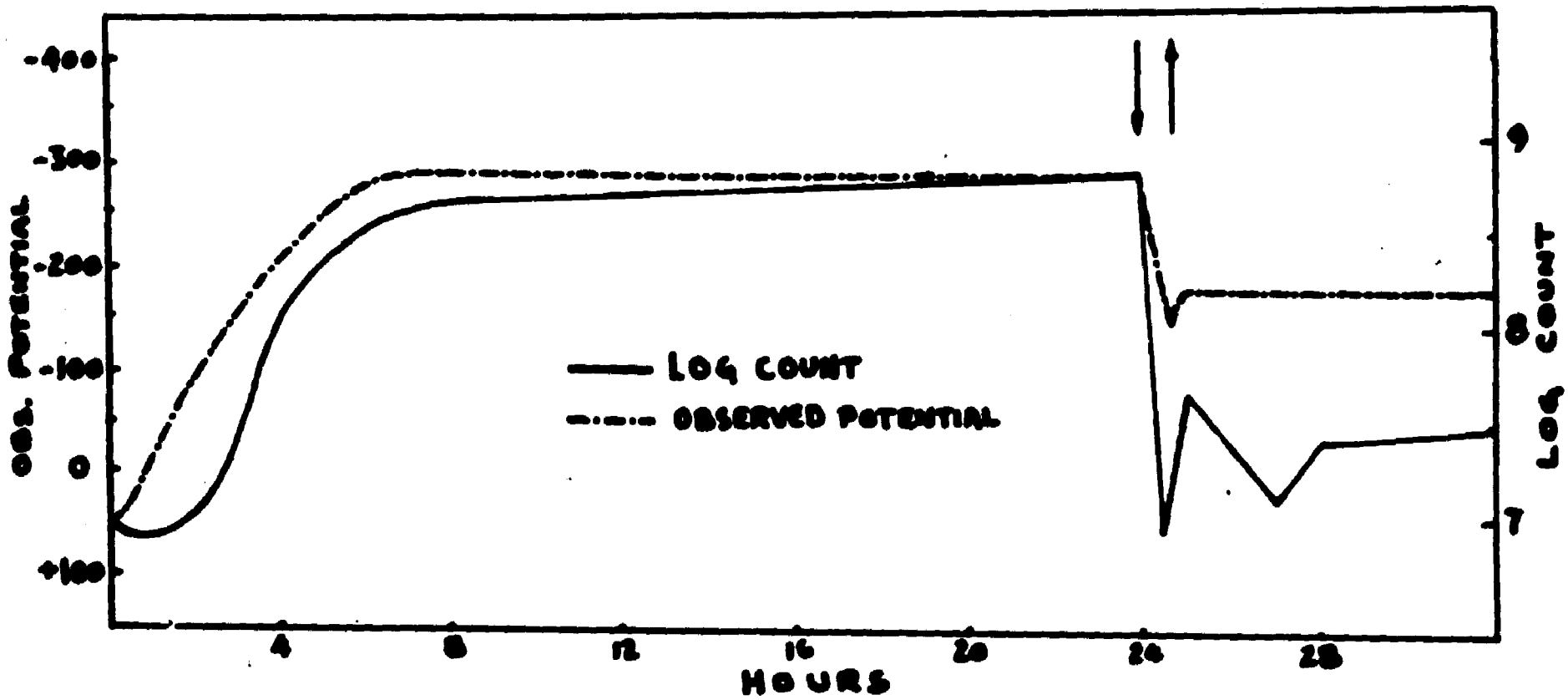


FIG. 11. COMPARISON OF POTENTIAL AND PLATE COUNT CURVES OF SAL. TYPHOSA

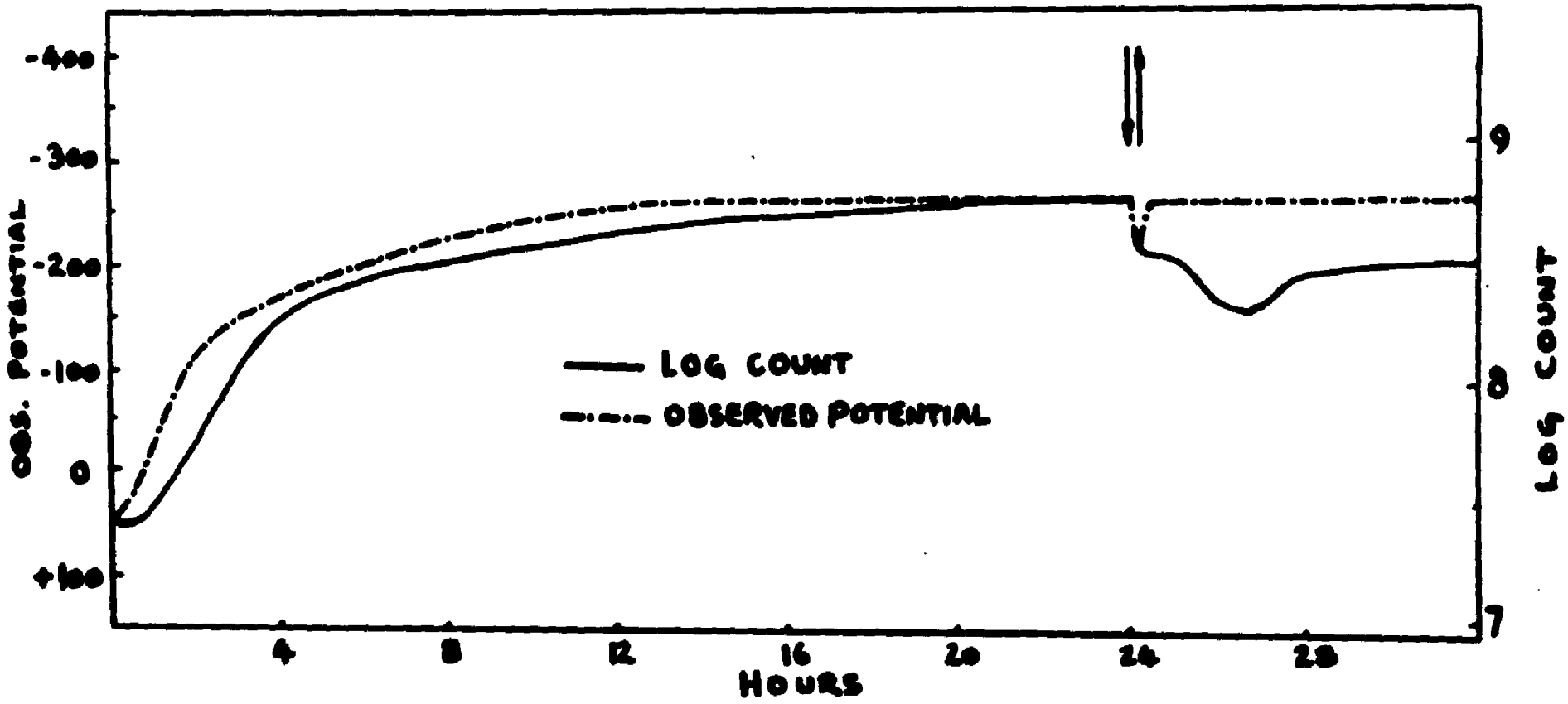


FIG. 12. COMPARISON OF POTENTIAL AND PLATE COUNT CURVES OF Ps. AEROGENES.

hours. The two cells containing the organisms were disassembled and the bacteria removed by centrifugation. The supernatant fluid, or "spent" medium, was returned to the two electrode vessles and all four were reautoclaved at 10 pounds pressure for 10 minutes. This operation resterilizes the "spent" medium and drives out the dissolved oxygen from both these and the uninoculated medium. The electrode vessels were immediately returned to a 37°C water bath, electrodes connected to the recorder and time-potential curves made to determine the rate of oxygen consumption, as evidenced by the observed potential. Fig. 13 is a reproduction of the recording obtained in this manner. The initial potential of the fresh medium was -200 mv., while that of the medium which has supported bacterial growth was -60 mv. The rate of oxygen absorption in fresh medium was 130 mv. per hour as compared to 40 mv. per hour for the "spent" medium. The potential difference in the two media became constant in about 4 hours and stabilized at a potential of -20 mv. for the fresh medium and -50 mv. for the "spent" medium in about 9 hours. These differences in the two media must be attributed to changes in composition due to bacterial metabolism, since all other conditions which might cause such a change have been eliminated by identical treatment throughout. It will be noted that the rate of reoxidation of the fresh medium was 3.25 times greater than that of the

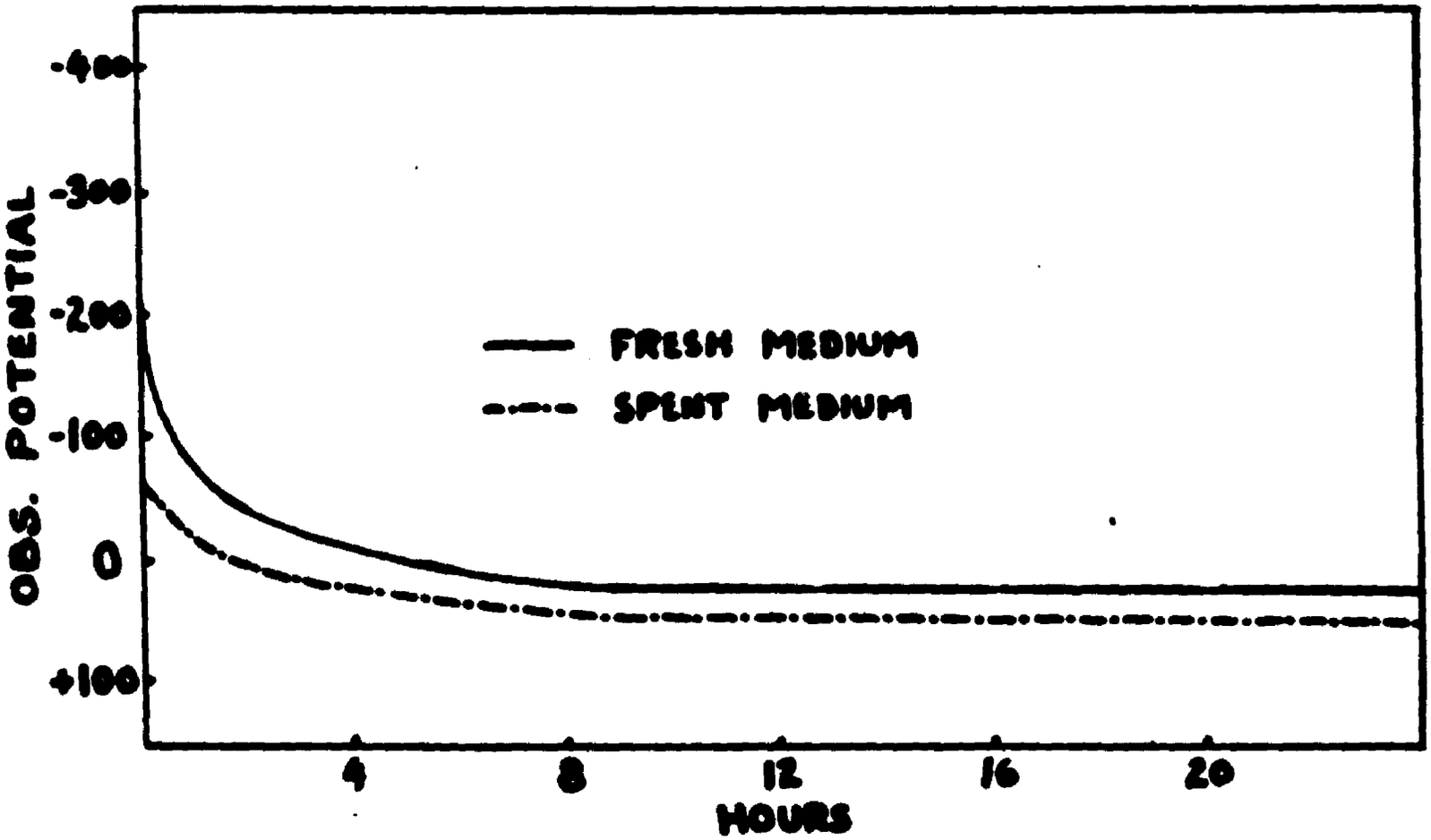


FIG. 13. COMPARISON IN RATES OF REOXIDATION OF FRESH AND SPENT MEDIUM.

"spent" medium. It, therefore, seems plausible to attribute sluggish electrode potential responses to metabolic products which poise the medium. The interference from these substances is greatest in older cultures and during the first hour of rapid change in cultural conditions. The effect becomes constant after about 5 hours. The amount of this material is less in younger cultures. It, therefore, would seem that it can be minimized by testing for germicidal activity as soon as the maximum reducing intensity is reached. In recalling the normal growth curves of the test organisms used in this study, this time varies with the organism. From these data it can be concluded that increased sensitivity to electrode potential measurements can be achieved by minimizing initial lag phases and increasing the slope of the logarithmic growth phase.

Sensitivity of test organisms

When testing a disinfectant in the laboratory for its germicidal activity, the relative sensitivity of several species must be known. The importance of providing a sufficient safety factor in a testing procedure, to account for the action of a given disinfectant against several test organisms is pointed out by Reddish (1950). An example of outstanding resistance to most germicides is the organism, Ps. aeruginosa Reddish states that "... it is quite apparent that this resistant species

(Ps. aeruginosa) should be used in all tests for antiseptic activity".

The test organisms used in this study to demonstrate the relative sensitivities to the standard germicide, hyamine 1622, are M. pyogenes var. aureus, Sal. typhosa, and Ps. aeruginosa. These organisms were inoculated into three separate electrode vessels and incubated at 37°C. until a constant minimum electrode potential was noted for each. At this time, the germicide was added in a 1 - 10,000 dilution and the rise in potential recorded. Fig. 14 is a reproduction of this recording. The resistance of Ps. aeruginosa was clearly demonstrated by this method of testing. Hyamine 1622 was added at the 4-hour mark. The reducing intensity of this test organism was unaffected by this disinfectant in a 1 - 10,000 dilution. Sal. typhosa showed intermediate resistance, with a rise in potential of 70 mv. per hour for the first hour and held in bacteriostasis at -200 mv. The greatest sensitivity was demonstrated by M. pyogenes var. aureus, which had a rise in potential of 230 mv. in the first hour and was completely inactivated at a constant potential of -10 mv. This order or resistance to quaternaries has been demonstrated by other methods.

The relative rates of germicidal activity to a single test organism, M. pyogenes var. aureus, is illustrated in Fig. 15. The disinfectant, in specified concentrations, was introduced

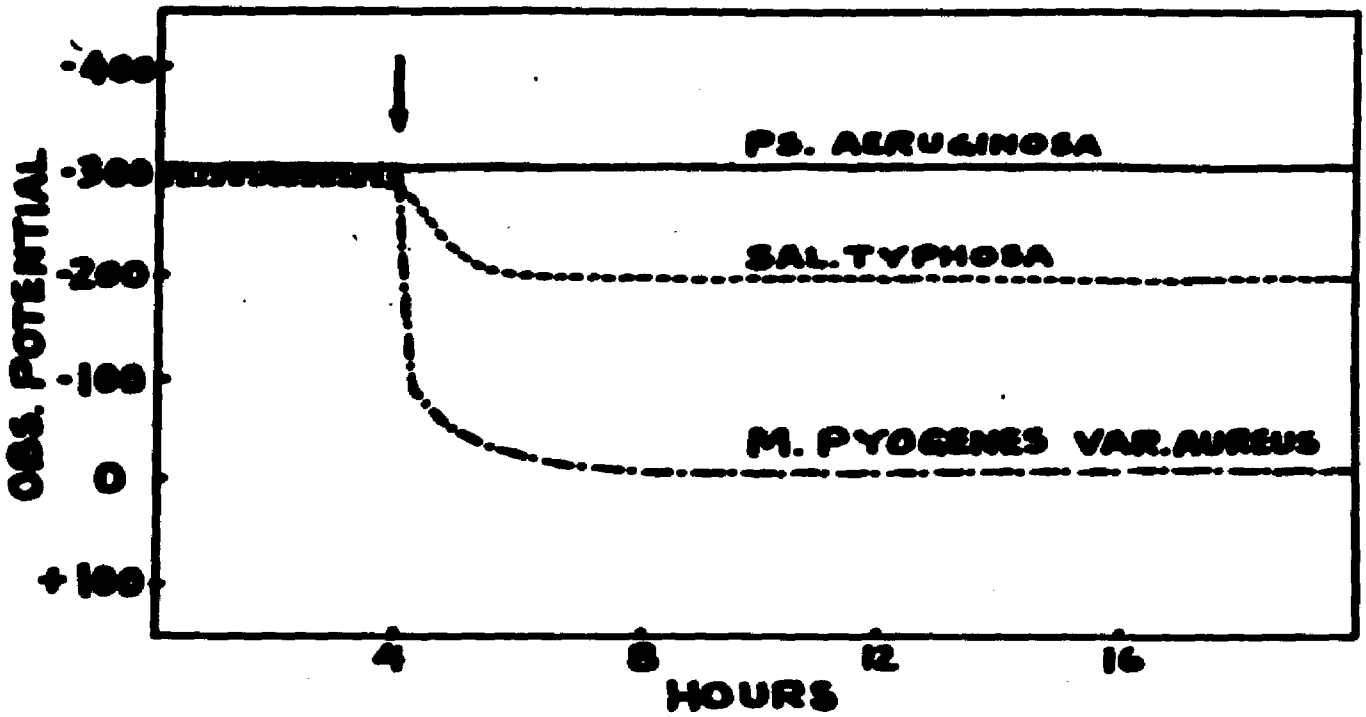


FIG. 14. ORGANISM SENSITIVITY TO QUATERNARY.

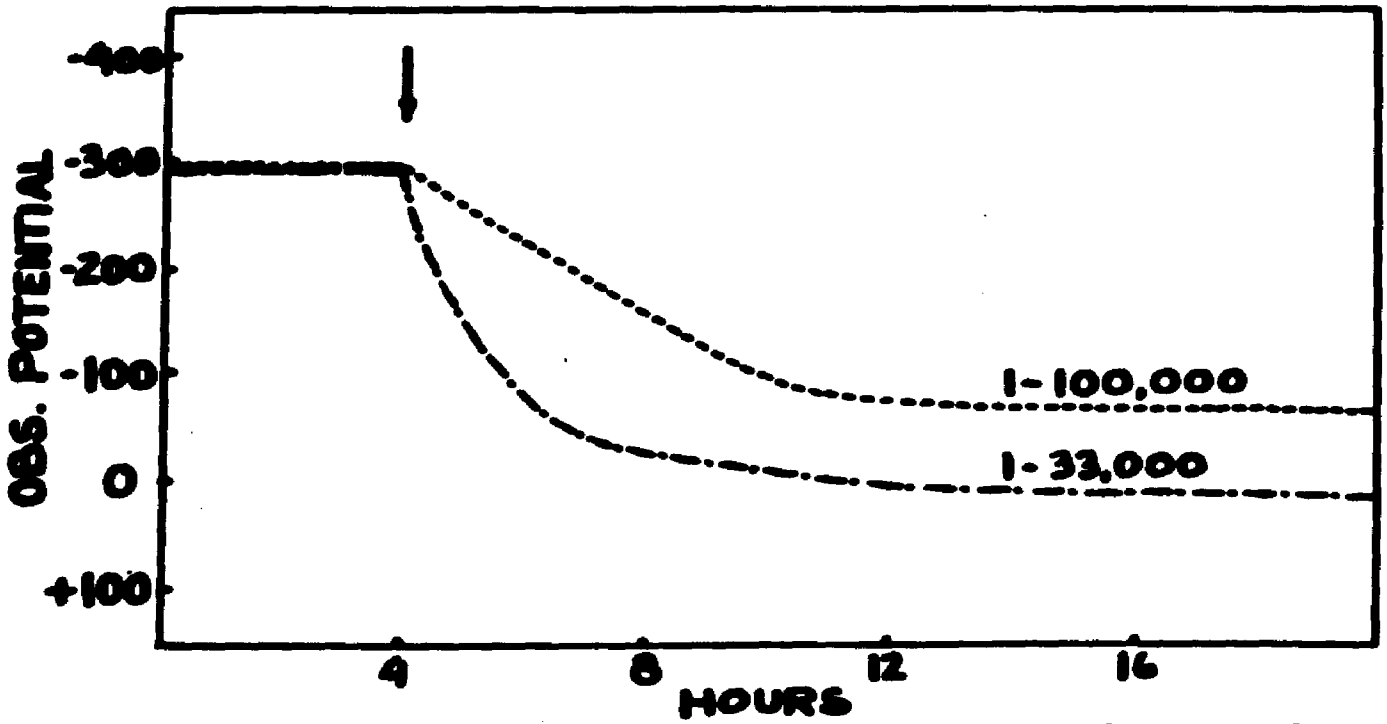


FIG. 15. EFFECT OF THE DISINFECTANT CONCENTRATION ON M. PYOGENES VAR. AUREUS.

at the 4-hour mark. When the quaternary concentration was set at 1 - 100,000, the response showed a gradual and rather constant increase in potential at a rate of 30 mv. per hour, which lasted for about 7 hours. After this interval, the potential equilibrated at -70 mv. A 1 ml. portion of this treated suspension initrated growth when sub-transferred to fresh medium, showing that the organism was held in a state of bacteriostasis at this potential. When the quaternary concentration was increased to 1 - 33,000, a regularly increasing potential occurred, rising a total of 300 mv., with an initial rise of 150 mv. after the first hour of exposure. No organisms could be recovered by sub-transfer after 2 hours. A disinfectant concentration increased to a dilution of 1 - 10,000 effected a rapid rise in potential of 280 mv. total, with the initial rate of 220 mv. taking place in the first one-half hour. No organisms could be recovered after this time. It is interesting to note that the rate of potential rise subsides abruptly when it has risen to approximately that of the "spent" medium in Fig. 13. Obviously, the poising metabolic products are influencing this rate during rapid rises which occur within a 1-hour exposure period.

Neutralization of germicidal activity

It was not long after recognizing the antiseptic qualities of quaternary ammonium compounds that observations were noted

which seemed to indicate limitations of this class of compounds. Lawrence (1950), in his book on surface active quaternary ammonium germicides, lists some 29 chemical compounds which form a complex precipitate or otherwise inactivate them. Valko and DuBois (1944) have described quaternary inactivation and found that bacteria, apparently killed by the action of quaternaries, could be revived by the addition of a neutralizing agent, provided the neutralization takes place within 30 minutes. One of the main objections to the use of the standard phenol coefficient of Ruehle and Brewer (1931) is the inability of the test to distinguish between bacteriostatic and bacteriocidal action. In an attempt to give a more truthful representation of the antibacterial activity of these compounds, it has been suggested by Weber and Black (1948) that neutralizing be added to the treated cell suspensions to nullify the bacteriostatic action after carefully controlled exposure times. Many such inactivators have been used, but those most commonly employed have been soap by Domagk (1935), synthetic anionics and phospholipids by Baker, Harrison, and Miller (1941), oxgall by Klarman and Wright (1948), azolectin and tween 80 by Quisno, Gibby, and Foter (1946), sodium suramine by Lawrence (1947), activated charcoal by Mueller, Seeley, and Larkin (1947), a sodium salt of a condensed aryl sulfonic acid, tamol N by Goetchius (1948), and many others. Such an imposing list of

quaternary inactivators attests to the present lack of a single, universal inactivator which has the property of reviving cells which have been inactivated by quaternary germicides.

Inasmuch as some rather startling claims have been made for several of the quaternary antidotes, a study of some of the more common ones has been made. Electrode potential measurements were used as the indicator of reestablished metabolic activity. By this procedure, if the antidote is of doubtful value, the observed potential should continue to use in a positive direction at the same rate as it would in a treated cell suspension without neutralization. If the neutralizer is effective in arresting bactericidal activity, the potential should stabilize at a level commensurate with the instantaneous potential at the time of addition. If the antidote provides for a true reversal of bacteriological activity, the potential, previously raised by the addition of quaternary, should rapidly return to its minimum value, characteristic of the test organism prior to metabolic attenuation.

The effect of neutralizing the bactericidal activity of hyamine 1622 with tamol N is shown in Fig. 16. This curve was prepared by inoculating redox medium in an electrode vessel with 1 ml. of a 24-hour suspension of M. pyogenes var. aureus. The observed potential was recorded against time. When the suspension was 24-hours old, hyamine 1622 was added

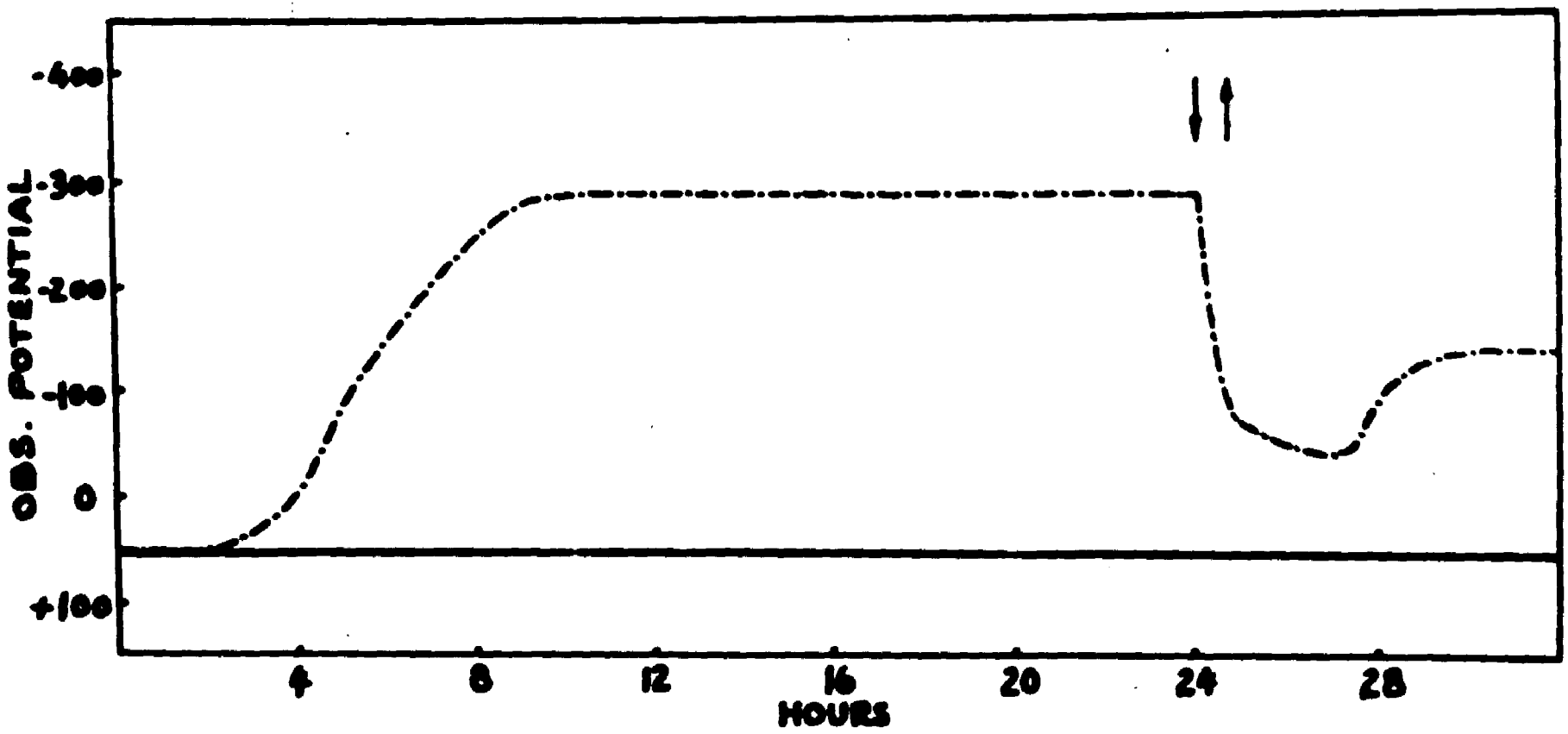


FIG. 16. NEUTRALIZATION OF HYAMINE 1622 WITH TAMOL N, USING M. PYOGENES VAR. AUREUS.

to give a final dilution of 1 - 30,000. The exposure was for 1 hour and then ten times this amount of the neutralizer, tamol N was added. The recorder was operated continually during these additions and the curves to be described were produced. A rapid rate of germicidal activity was again noted for M. pyogenes var. aureus which is apparently very susceptible to this quaternary. A sharp decrease in the rate of reoxidation was shown immediately after the addition of the antidote. For the next two and one-half hours this reduced rate prevailed, although still in a positive direction. After this time, the potential returned to a somewhat lower reducing intensity at about 150 mv. higher than the minimum stabilized level. Since the culture maintained a new stabilized level which was higher than the characteristic potential, this condition is interpreted as being due to those organism which escaped the lethal action of the germicide and are now living in an attenuated state. Similar results were obtained with Sal. typhosa in Fig. 17 and with E. coli in Fig. 18.

A sample recording, reproduced in full scale, showing the relative sensitivities of E. coli, Sal. pullorium, and M. pyogenes var. aureus to hyamine 1622 and the efficiency of tamol N to neutralize its action, is presented in Fig. 19. It will be noted that neither the addition of hyamine 1622 nor tamol N caused any alteration in the potential of the uninoculated medium.

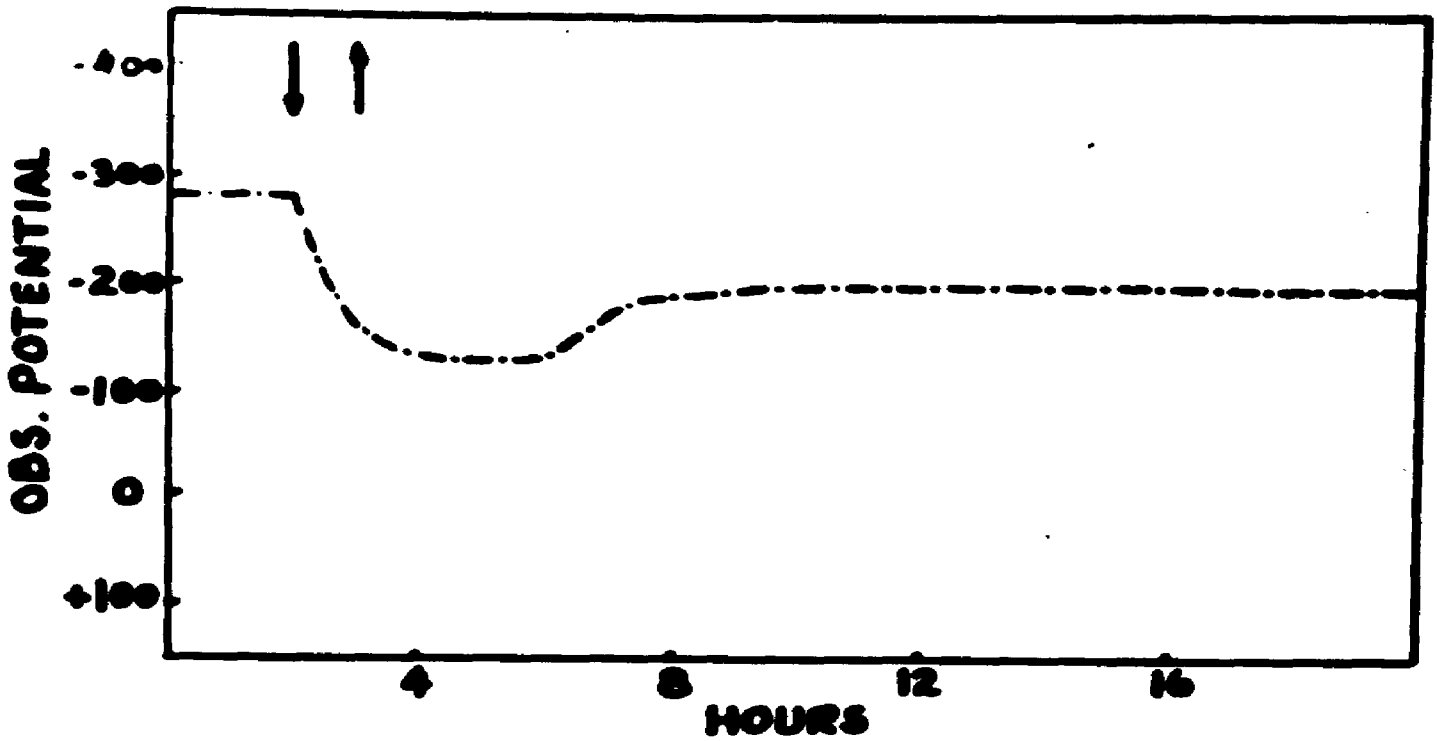


FIG. 17. NEUTRALIZATION OF HYAMINE 1622 WITH TAMOL N USING SAL. TYPHOSA.

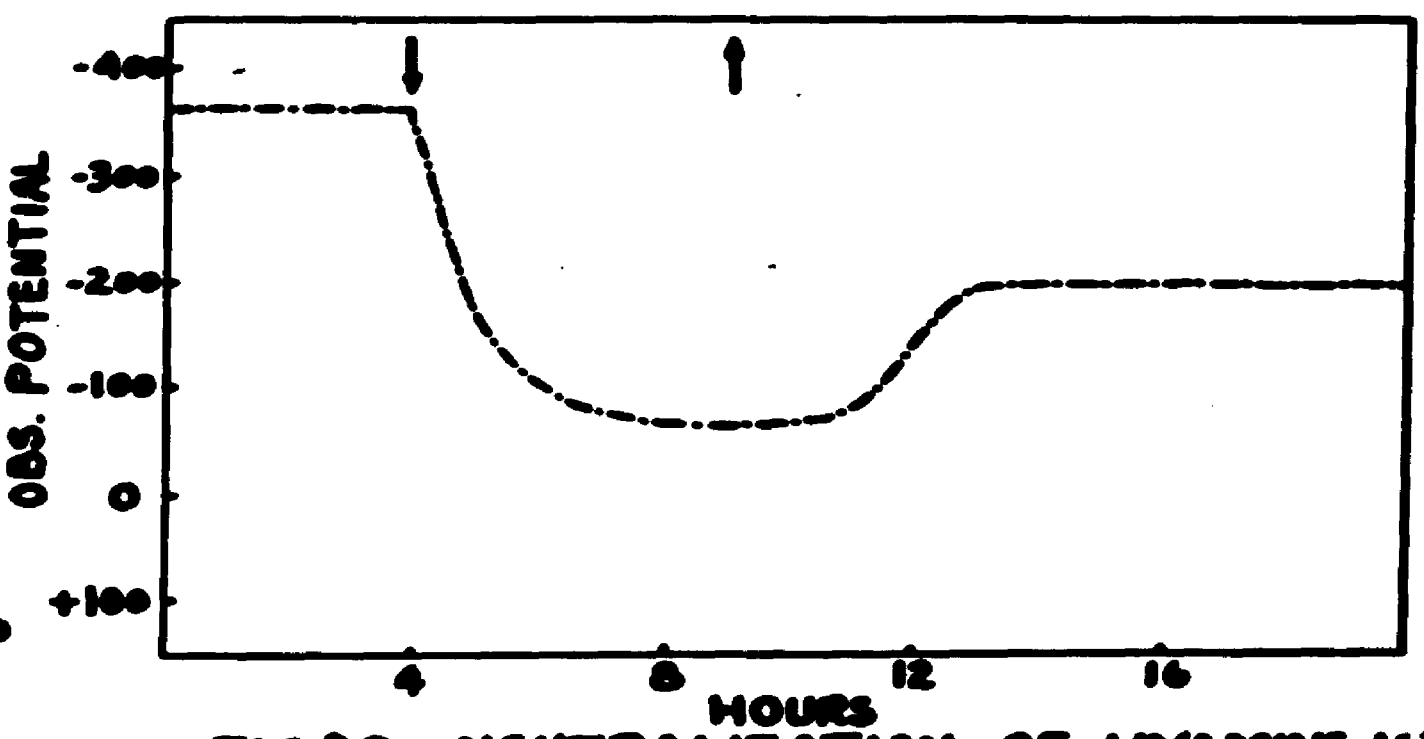
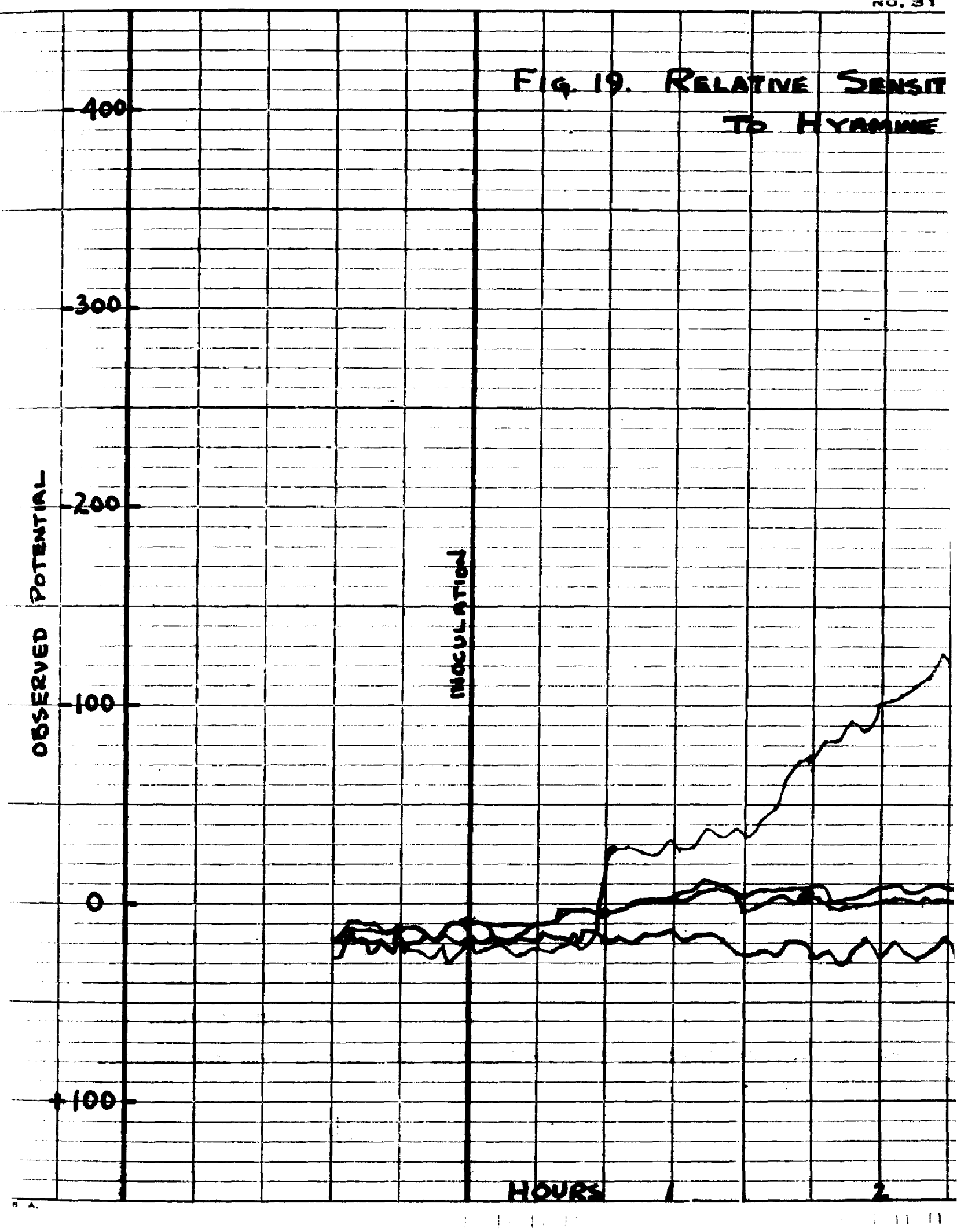


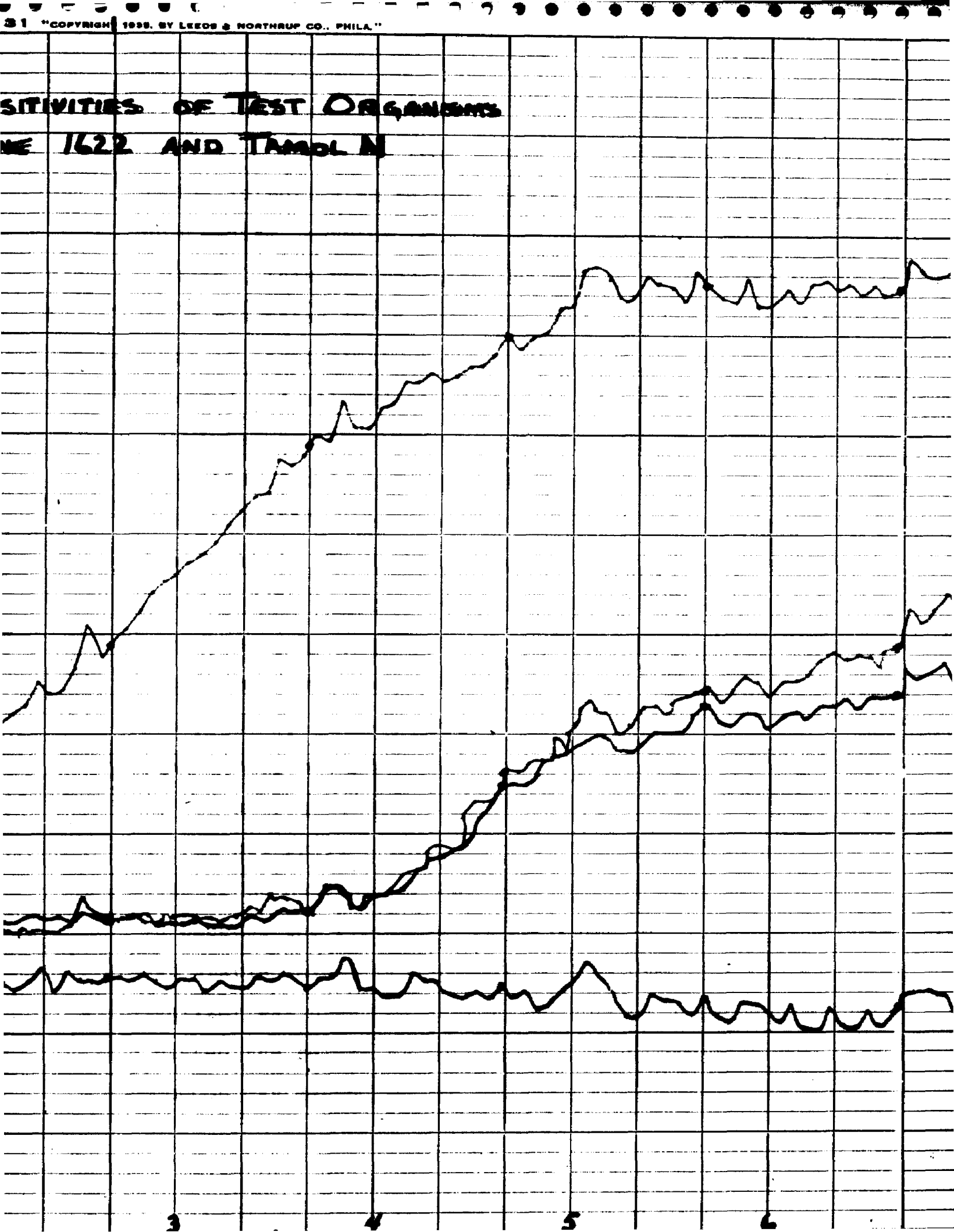
FIG. 18. NEUTRALIZATION OF HYAMINE 1622 WITH TAMOL N USING E. COLI.

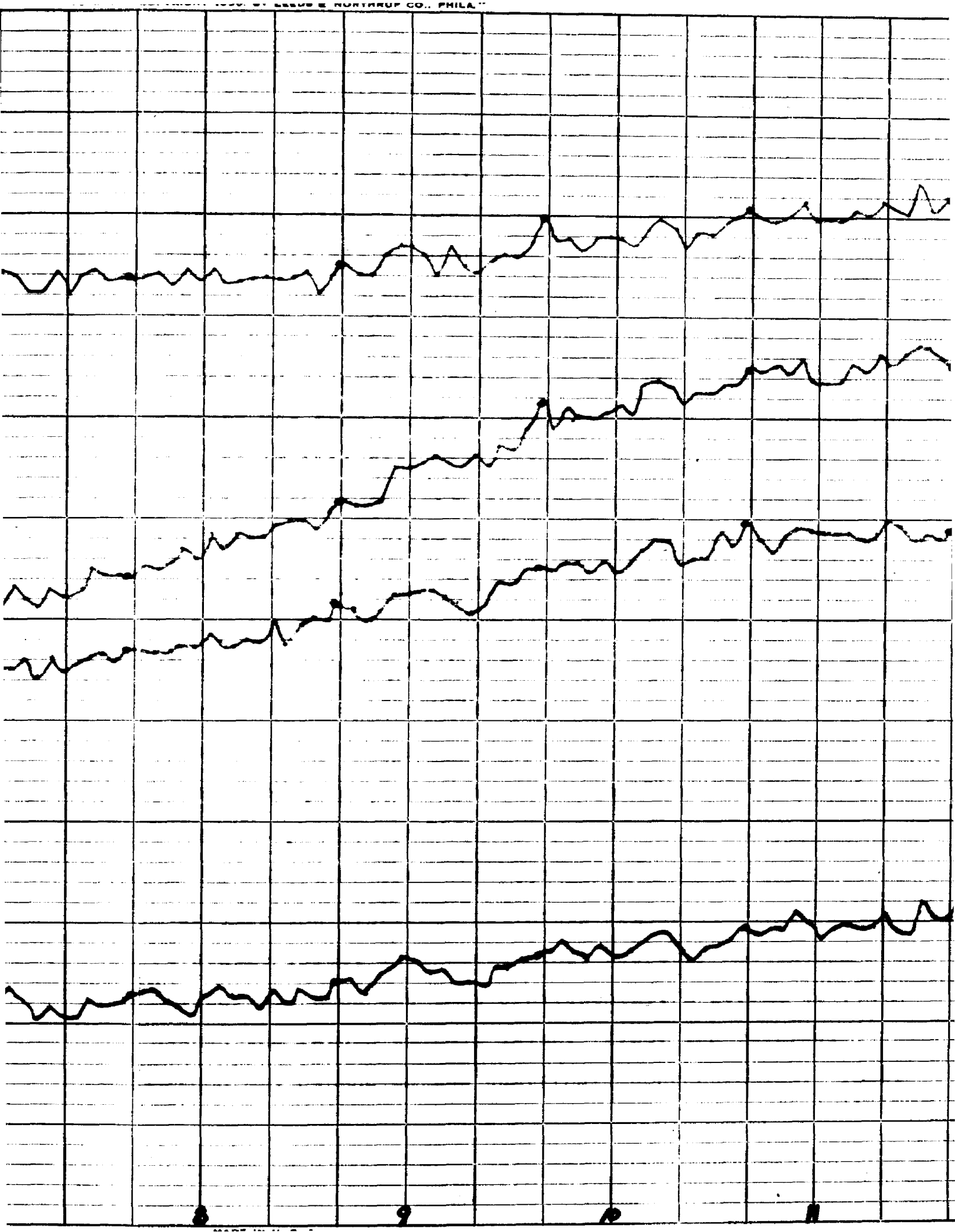
Fig. 19. Relative Sensit
To Hyamine

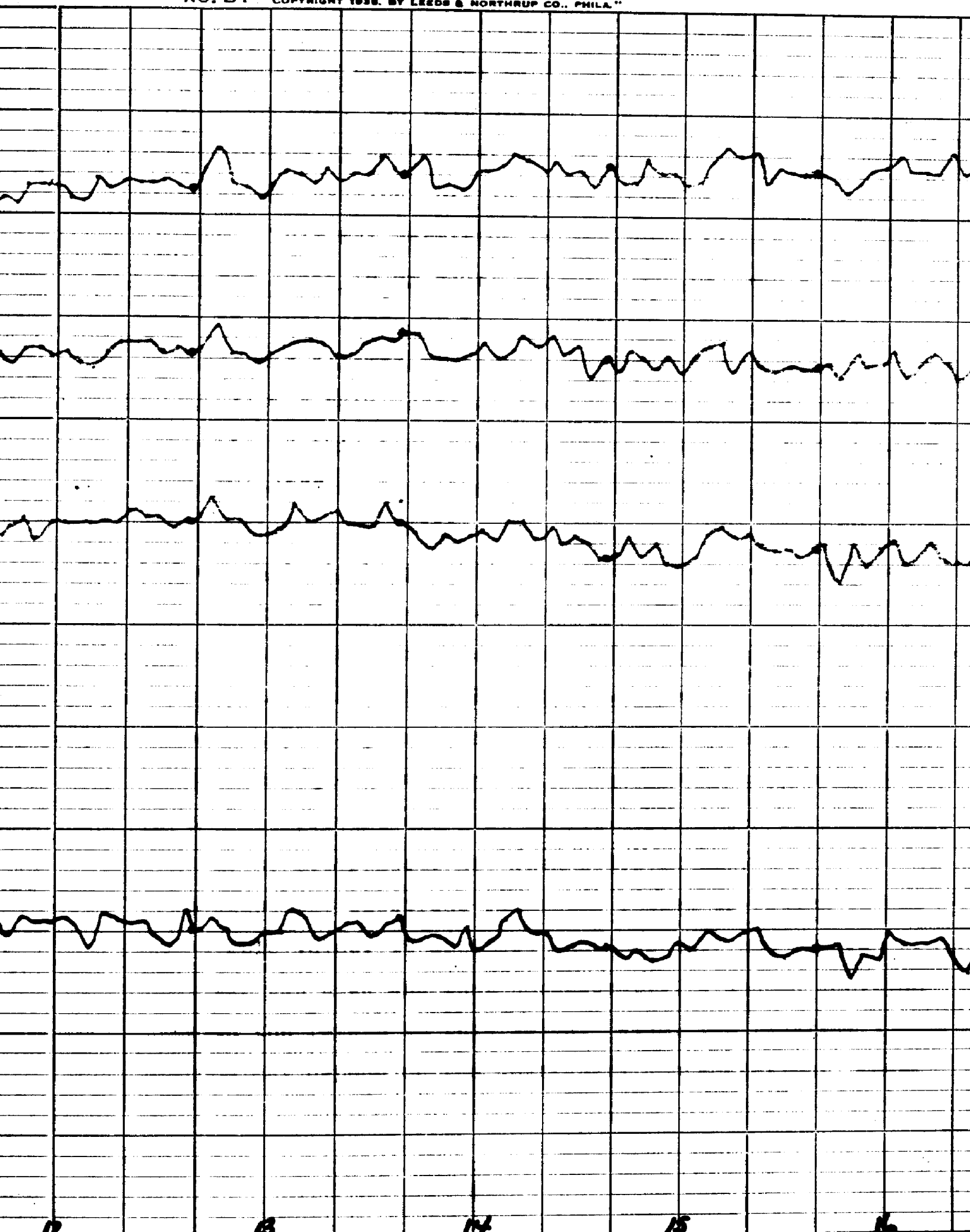


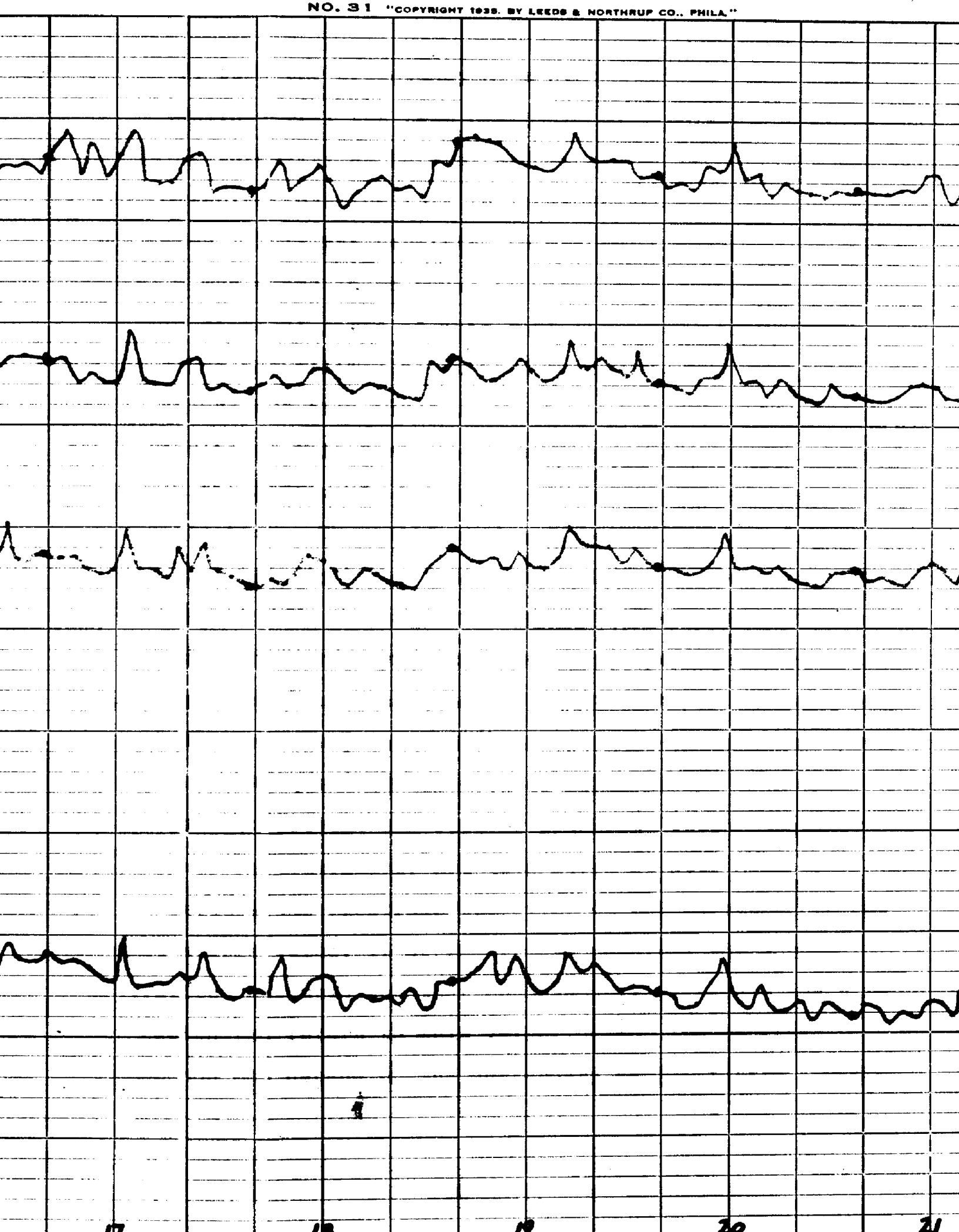
SITIVITIES OF TEST ORGANISMS

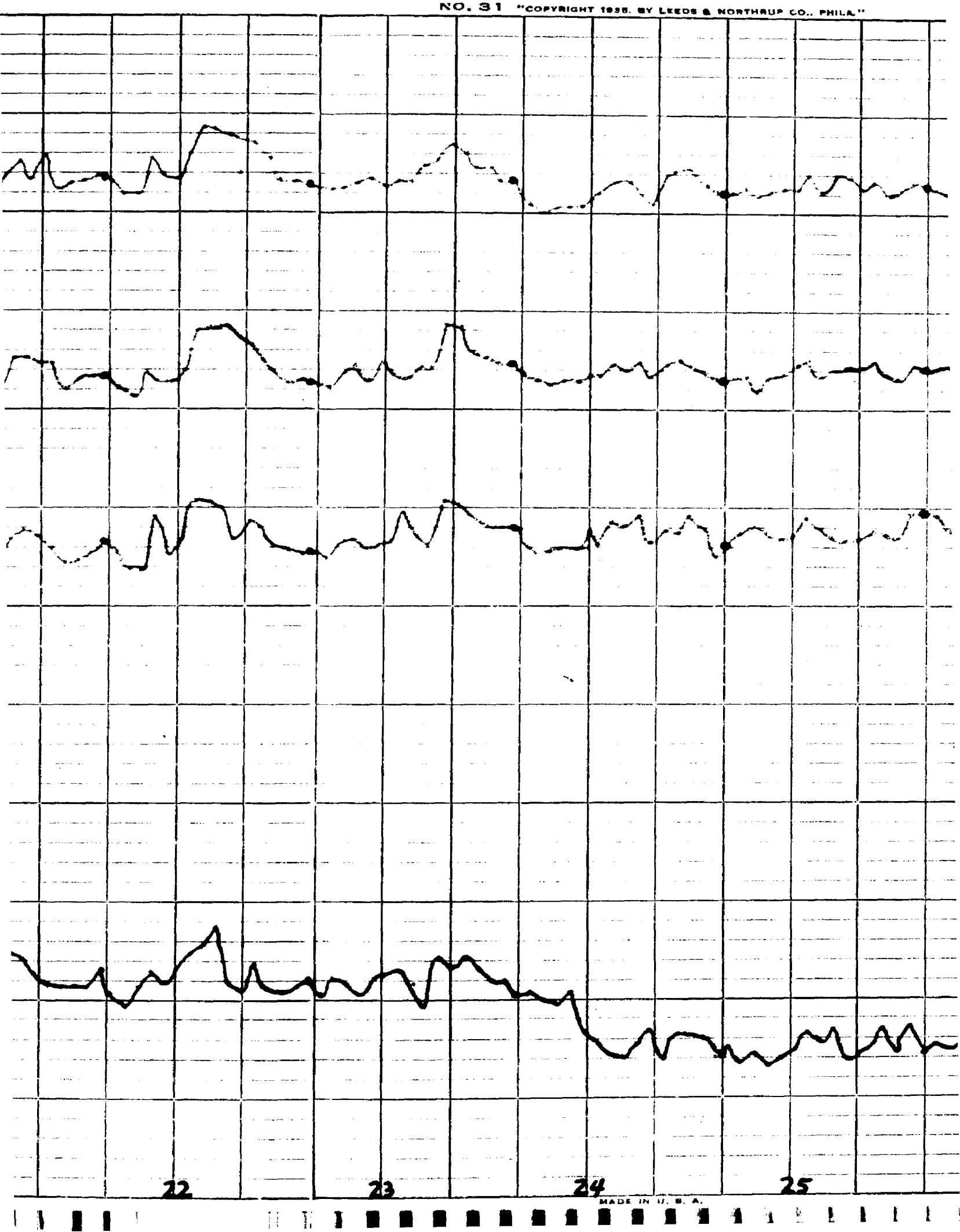
NE 1622 AND TAMOL N

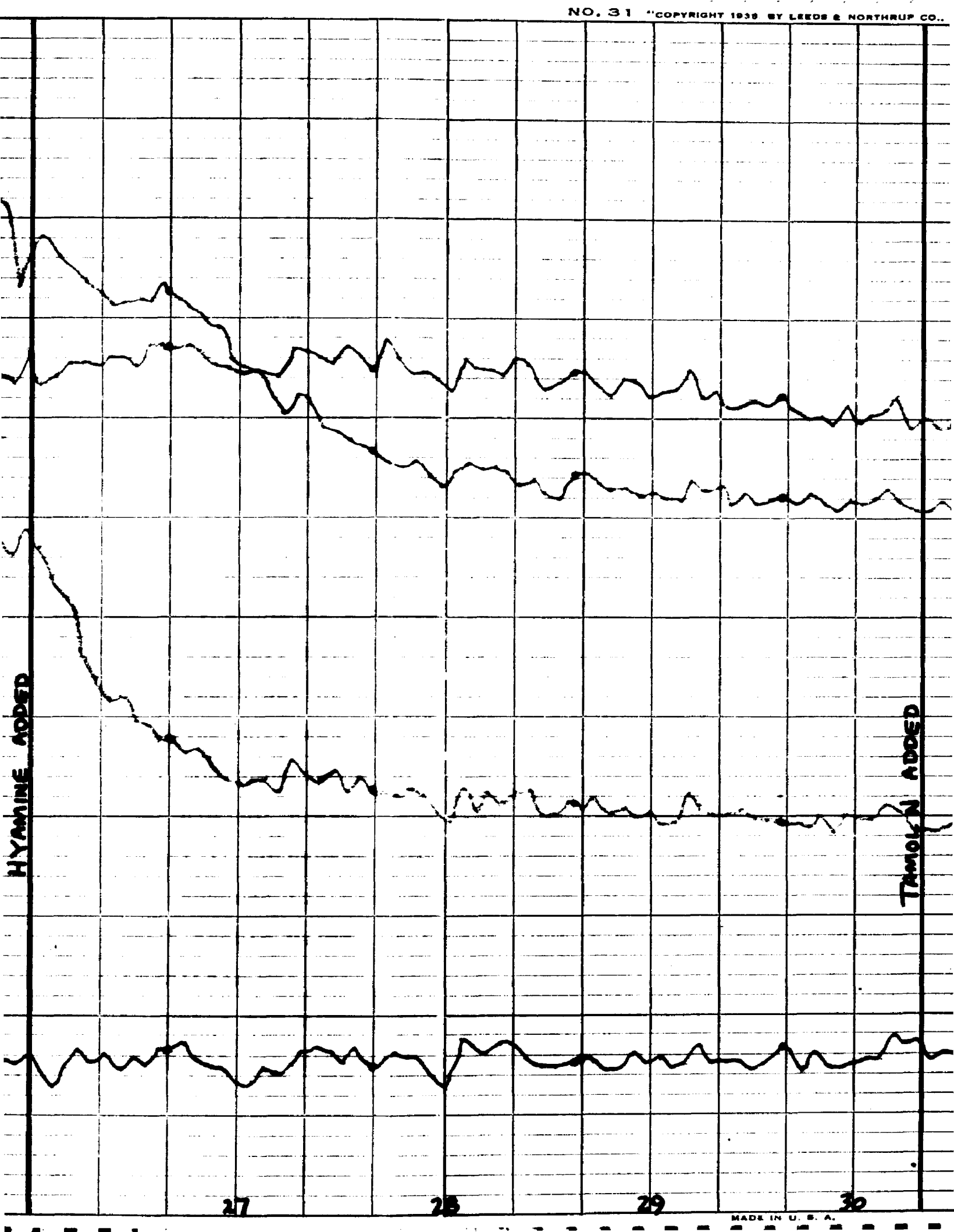


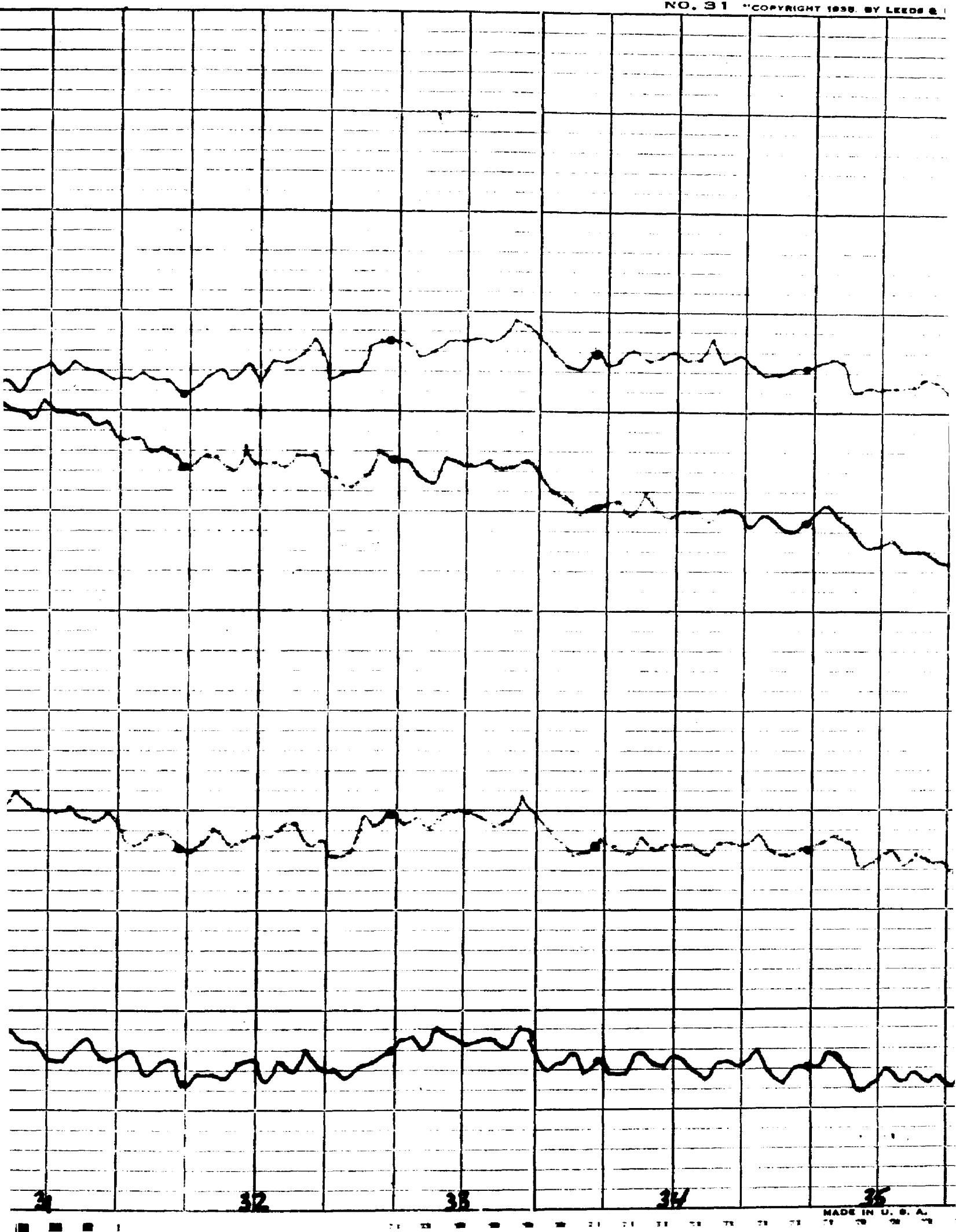








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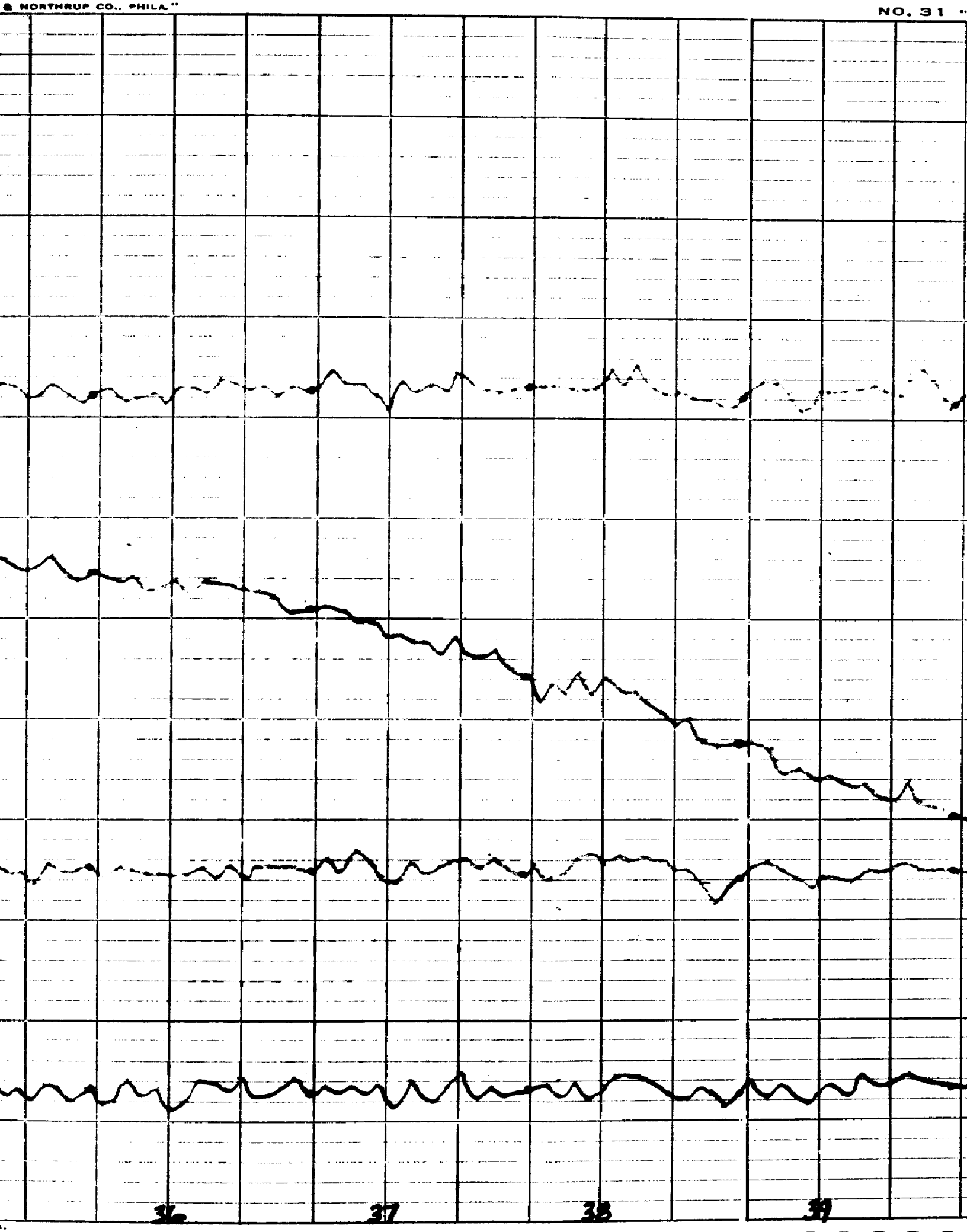
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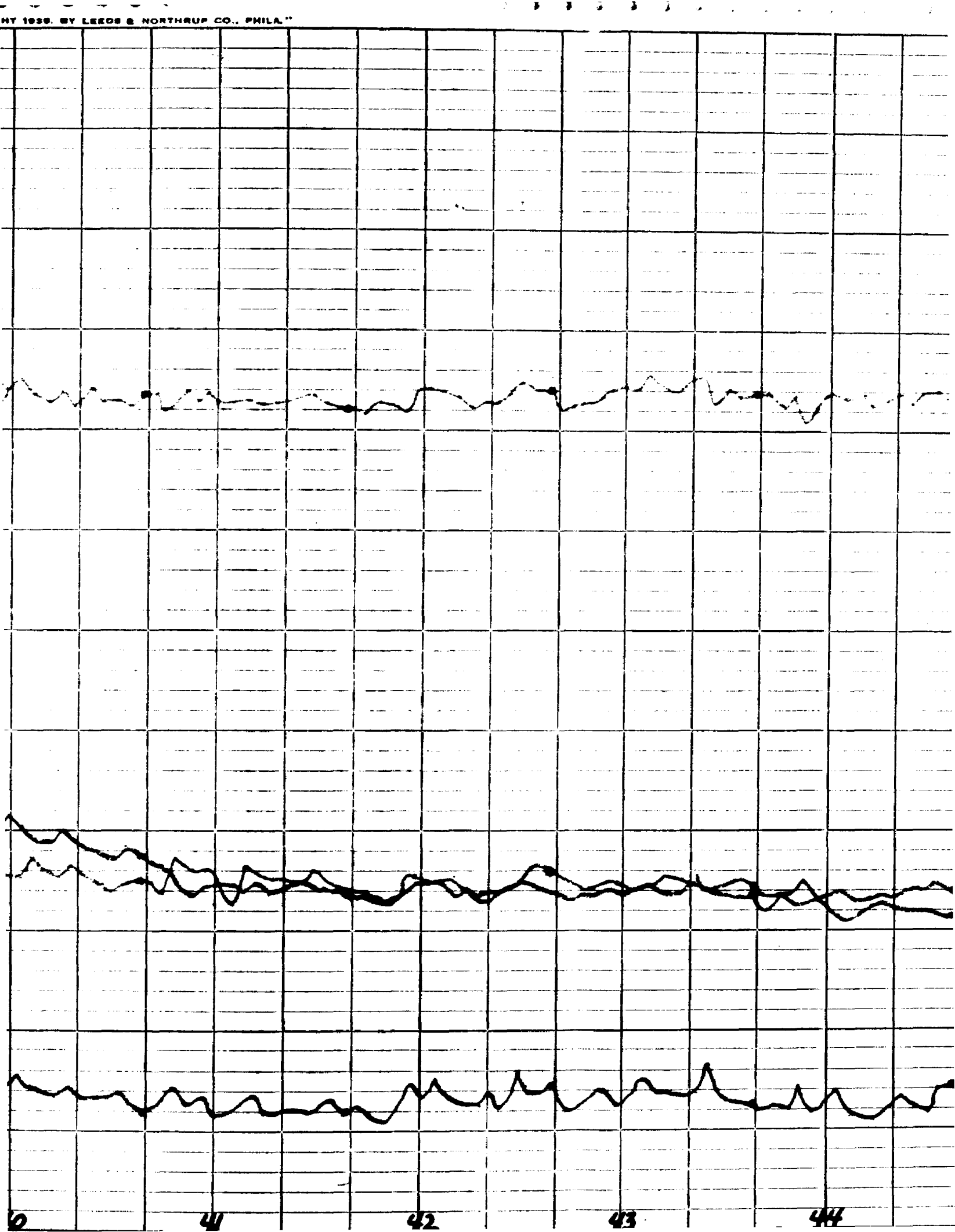

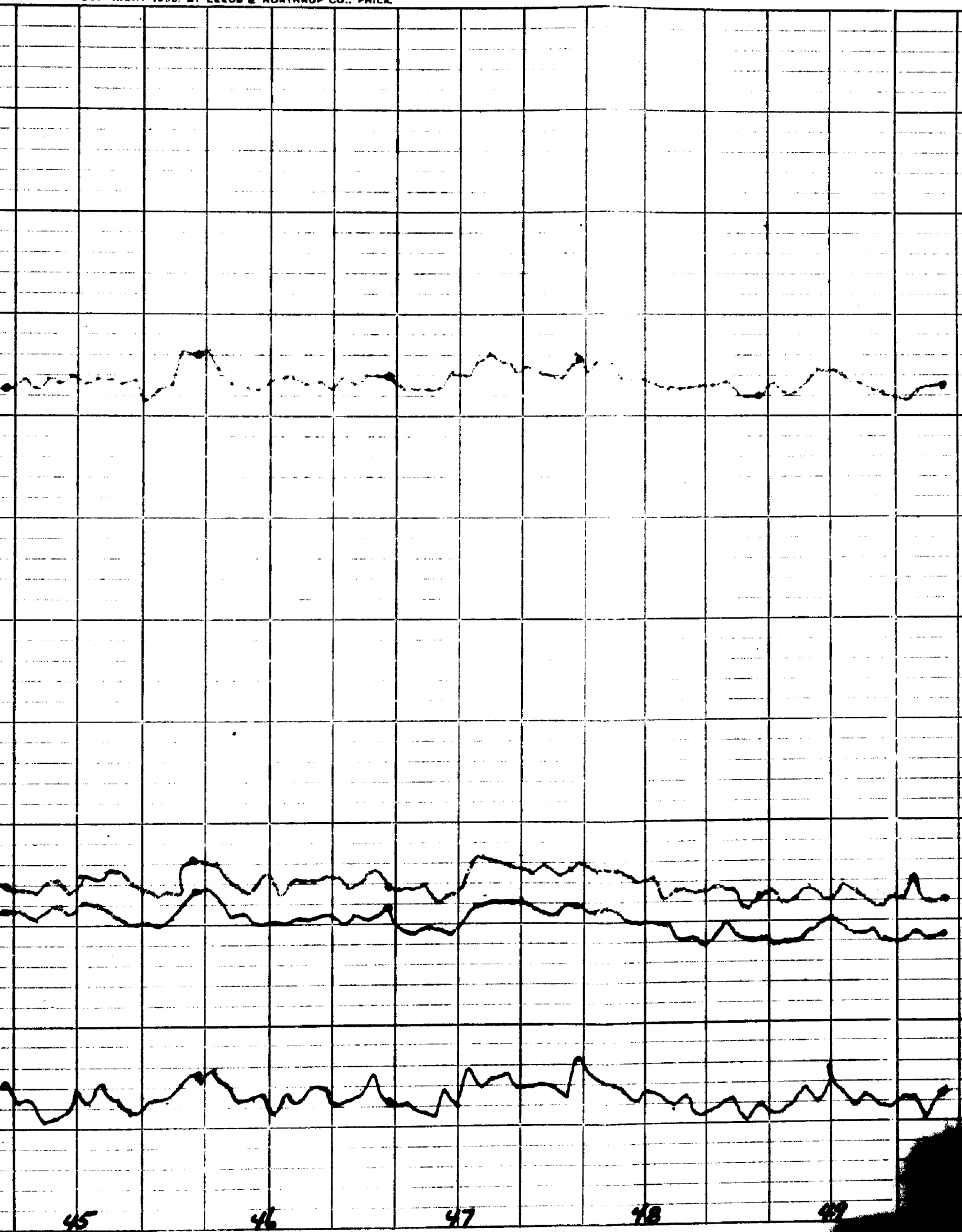

To test the efficiency of a one to one ratio of tamol N to hyamine 1622, the test organisms were grown to maximum reducing intensity as before, and to one suspension of each test organism, hyamine 1622 in final dilution of 1 - 10,000 was added. This was permitted to act for 2 minutes and then tamol N was added in an equal amount. At the same time, a mixture of equal parts of this quaternary and neutralizer were mixed and allowed to stand together for 2 minutes. The mixture was then added to the test organism suspensions. The results of this test are presented in Fig. 20 and is meant to show the difference between a chemical and a biological neutralization. The solid curves represent this difference when using Sal. typhosa, while the dash-dot curves represent the test using M. pyogenes var. aureus. When the neutralizer and quaternary were mixed together before adding to the organism suspension, no noticeable effect was noted. However, when the quaternary was permitted to act on the bacteria for 2 minutes, the subsequent addition of an equal amount of neutralizer did not prevent the rise in potential which responded just as if no neutralizer had been added at all.

Because the electrode potential failed to return to the lowest stable value, it appeared that possibly not enough neutralizer was used and that consequently, a second addition may hasten the return to this value. Also, the reversal phenomenon may depend on the quaternary concentration. These

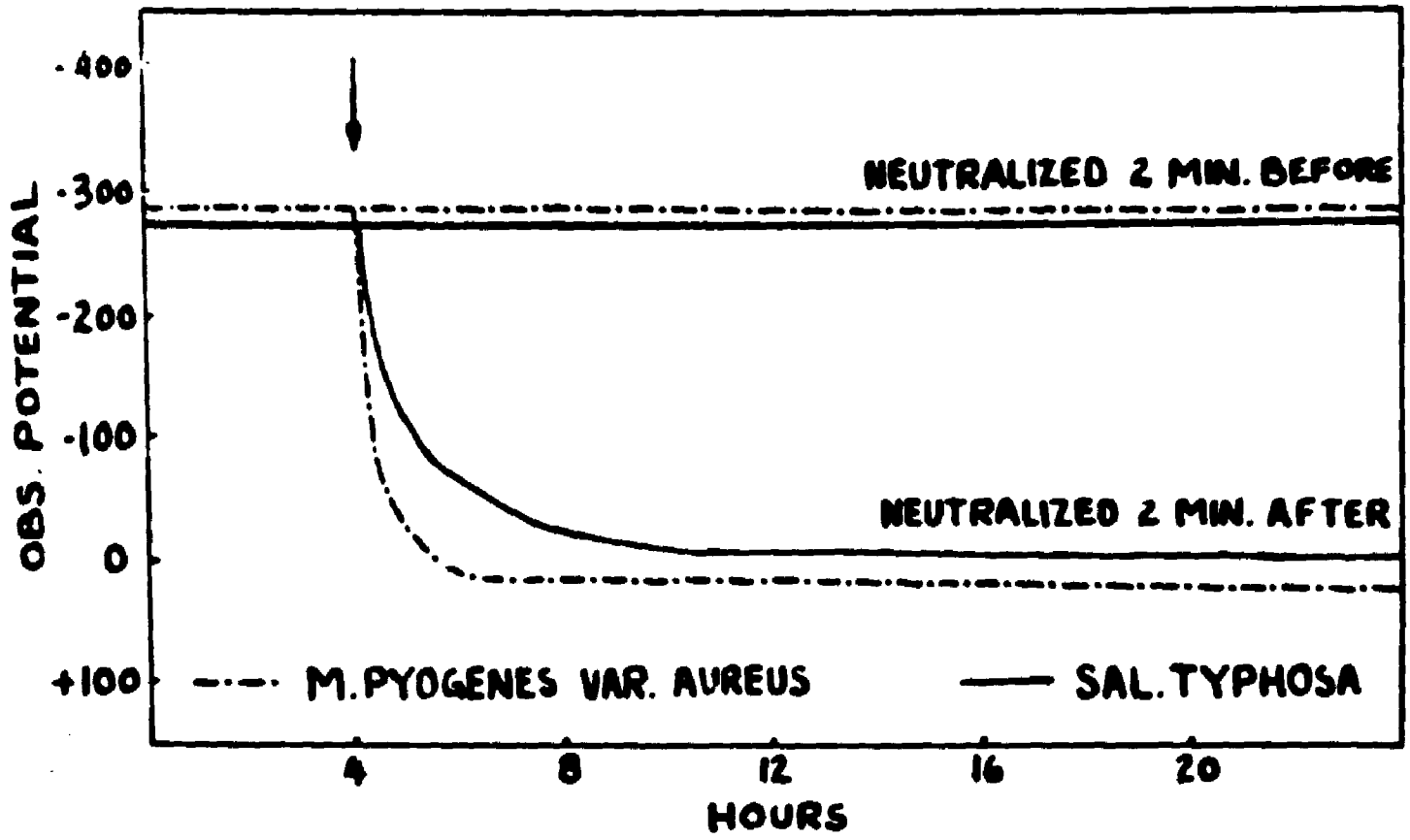

FIG. 20. NEUTRALIZATION OF QUATERNARY BEFORE AND AFTER ADDITION TO A BACTERIAL SUSPENSION.

points were tested in three separate electrode vessels inoculated with M. pyogenes var. aureus. One of these suspensions was treated with hyamine 1622, 1 - 50,000, another with 1 - 25,000, and the third with 1 - 15,000. After a 1-hour exposure, tamol N in final dilution of 1 - 15,000 was added and 6 hours later, a second addition of tamol N was added, bringing the final concentration to 1 - 3,500. The observed electro potentials were recorded in the three vessels simultaneously. The effects of double neutralization on three concentrations of quaternary are presented in Fig. 21. During the first hour of exposure, the observed potential rose at a rate which is proportional to the concentration of quaternary. In the case of 1 - 50,000 dilution the initial rise was immediately stopped by the first addition of neutralizer and the curve flattened out at a potential which was 50 mv. positive with respect to the uninhibited cell suspension. The second addition of neutralizer caused a gradual restoration of metabolic activity for the next 4 hours. After this time, reoxidation took place again, which would indicate that free quaternary was still present. When the quaternary was increased to a 1 - 25,000 dilution, this effect was more pronounced. The initial potential rose to 210 mv. when hyamine 1622 was added. The first addition of tamol N caused a change in direction which, for the next hour looked as if some reversal of germicidal activity had actually taken place. However, the potential stabilized

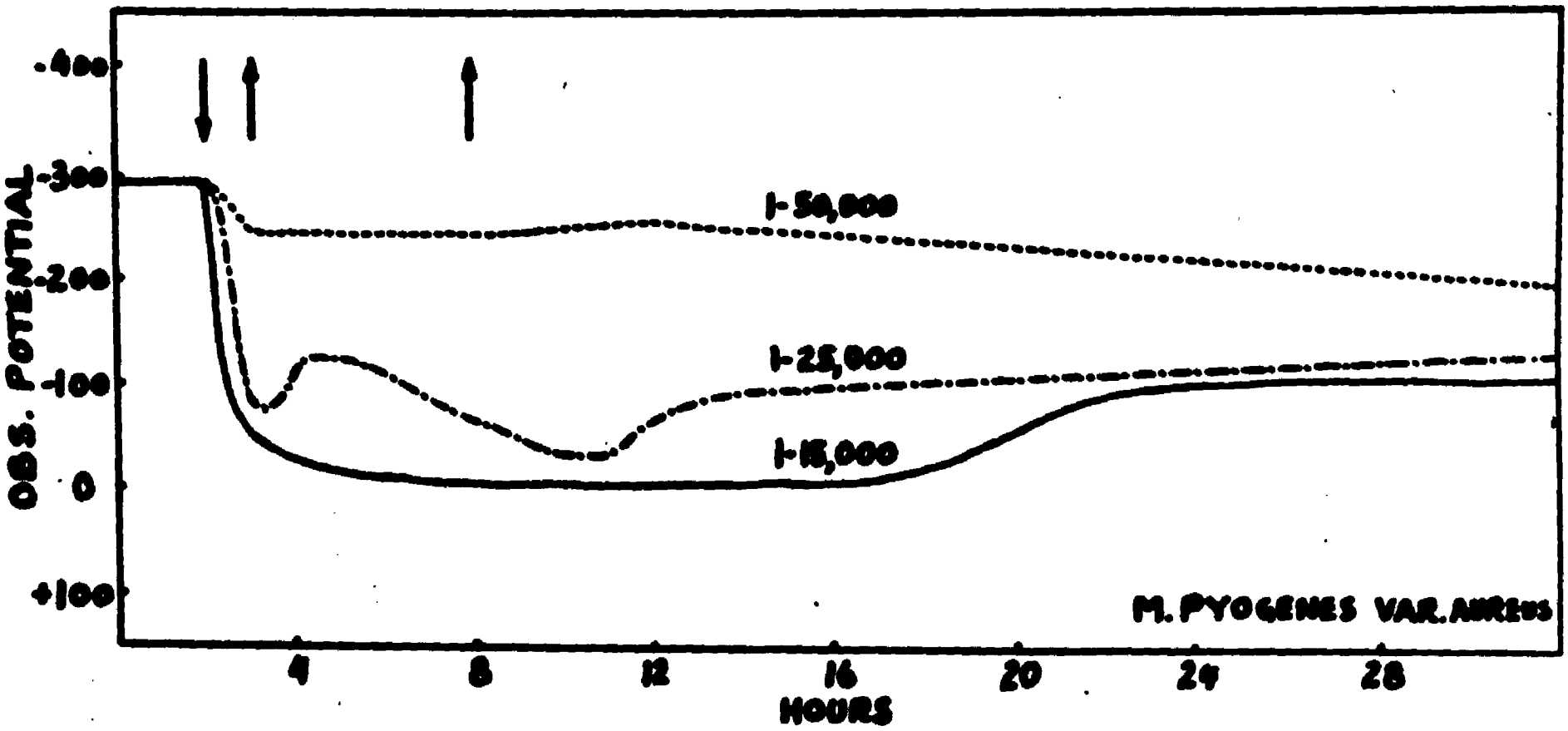

FIG. 21. DOUBLE NEUTRALIZATION OF VARIUS CONCENTRATIONS OF QUATERNARY.

at -120 mv. and remained there for 1-1/2 hours. After this time, it once more began to rise. Upon the second addition of neutralizer, no immediate effect was noted but 3 hours after the addition, the reducing conditions were intensifying. The potential leveled off at -120 mv. which is 170 mv. short of complete restoration of metabolic activity. The suspension treated with a 1 - 15,000 dilution of hyamine 1622 produced a very abrupt change in potential. Neither the first nor second additions of neutralizer had any effect until 11 hours after the first addition. At this time, a slow, feeble growth was initiated which is suggestive of an attenuated organism. The minimum potential reached at this time was -100 mv. which is 190 mv. less than the reducing intensity reached by the untreated suspension.

Sodium suramin is a quaternary inhibitor which has been used in much the same way as tamol N. This compound is a naphthalene sulfonic acid derivative and is also known as Bayer 205, Germanin, or sodium naphuride. A comparison of sodium suramin and tamol N by electrometric technique is given in Fig. 22. These curves were prepared by treating a suspension of E. coli with a 1 - 15,000 dilution of hyamine 1622 and neutralizing this action with the antidotes after 5 minutes exposure to the germicide. One suspension was not neutralized to demonstrate the germicidal activity of hyamine 1622 in this concentration. Neutralization with tamol N

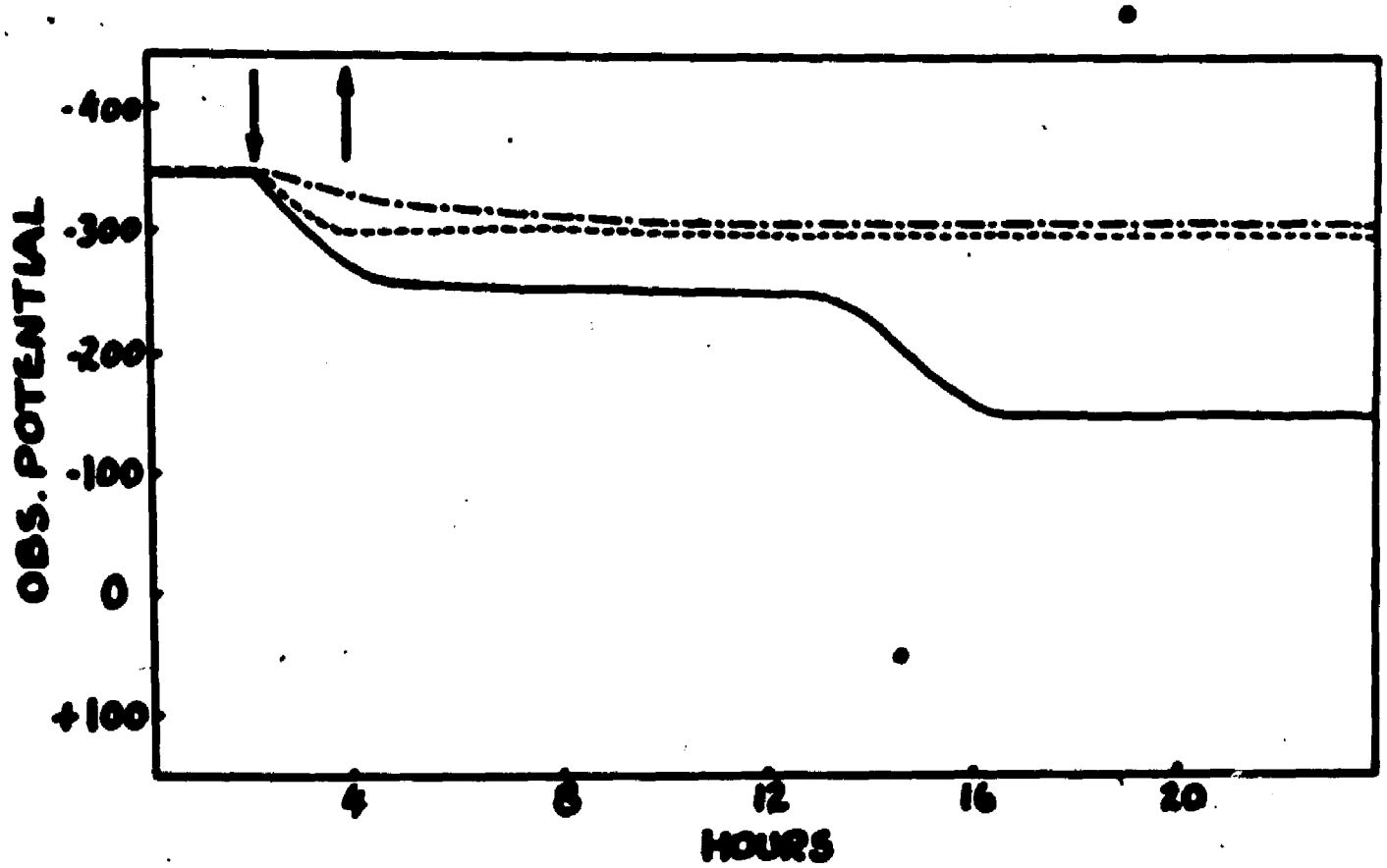

FIG. 22. COMPARISON OF TAMOL N AND SODIUM SURAMIN USING E. COLI.

resulted in a delayed reaction with the potential rising 40 mv. in 7 hours. Sodium suramin in the same concentration showed little neutralization until the potential had increased 50 mv. This rise required approximately 1 hour and 20 minutes. From this point on, the potential remained constant at -300 mv. for 24 hours. The unneutralized quaternary in 1 - 15,000 dilution caused a continuation in initial rate of kill up to a -260 mv. level. The potential increased 10 mv. in the next 8 hours. After this time, another rise of 200 mv. occurred. It remained constant for the rest of the experiment.

The next experiment was designed to determine whether sodium suramin could neutralize after the quaternary has been acting on a cell suspension for a long period of time. After a 1 - 10,000 dilution of hyamine 1622 had acted for 22 hours, a 1 - 5,000 dilution of sodium suramin was added to the system. The results of this experiment are recorded in Fig. 23. In the case of Ps. aeruginosa, the curve was not altered from its previous potential by either the addition of hyamine 1622 or sodium suramin. Sal. pullorum is intermediate in its response to the germicide, although its metabolic activity was decreased to a minimum in 18 hours. At 22 hours the neutralizing agent was added. The decrease in electrode potential indicates a re-establishment of growth of a few resistant organisms rather than true reversal. If this was a case of

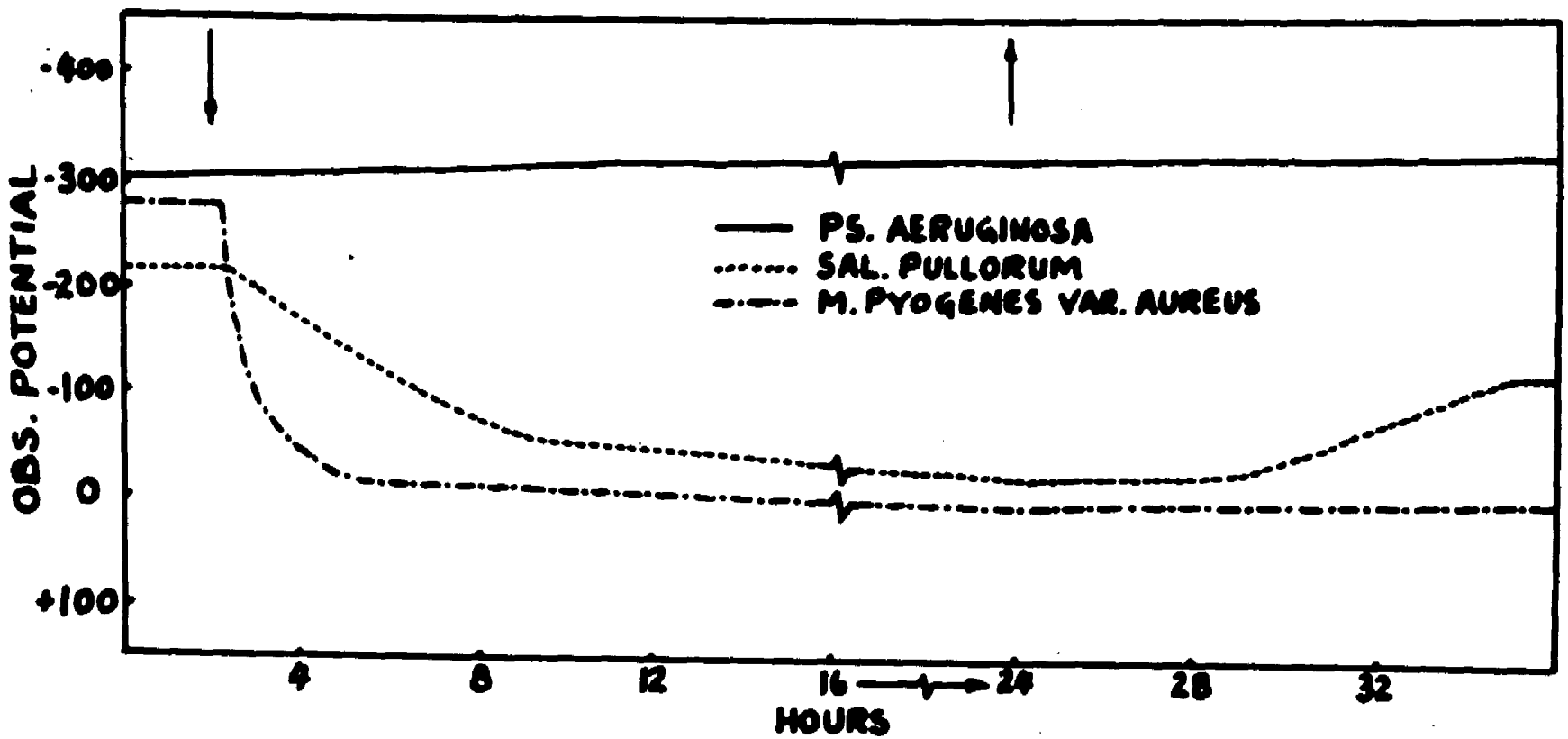

FIG. 23. DELAYED NEUTRALIZATION OF HYAMINE 1622 WITH SODIUM SURAMIN.

reversal, a more rapid return to the reduced state would be expected. The returning potential stabilized at -110 mv., representing about 50% of the original activity. M. pyogenes var. aureus, the most susceptible of the test organisms, was oxidized rapidly and its reducing capacity could not be reinstated by this method.

During the course of study on sodium suramin, it was noted that this quaternary inhibitor formed a finely divided precipitate when mixed with hyamine 1622. Undoubtedly, one of the mechanisms responsible for the germicidal activity of quaternaries is adsorption. Quaternary adsorption has previously been mentioned in connection with glass surfaces. A test of quaternary inactivation by selective adsorption was attempted by introducing activated charcoal and kieselguhr into a system of bacteria and quaternary. The organisms were treated with a 1 - 10,000 dilution of hyamine 1622 and 5 minutes later, charcoal in aqueous suspension and kieselguhr were added. A control tube was not interrupted so that a comparison could be made. Neither activated charcoal nor kieselguhr altered the shapes of the electrode potential curves in any manner different from the germicide control. One must conclude that, on the basis of these tests, the quaternary is preferentially adsorbed on the bacterial surface and held with a force greater than the attraction offered by either of these adsorbents.

Figs. 24 and 25 show the relative neutralizing properties of tamol N and duponol, a synthetic anionic, lauryl sulfate. Neutralization by duponol was attempted on Sal. typhosa and Ps. aeruginosa. Anionics inhibit the metabolism of Gm (+) organisms so it would be difficult to ascertain whether inhibition was due to quaternary or excessive anionic if M. pyogenes var. aureus were used. Referring to Fig. 24, it will be noted that a 1 - 3,000 dilution of tamol N is about 30% more effective in stopping a rise in observed potential caused by the addition of a 1 - 15,000 dilution of hyamine 1622 to a suspension of Sal. typhosa. In this experiment, the suspension was exposed to quaternary for a period of 30 minutes before neutralization was attempted. The observed potential had increased from -280 m to -160 mv. in this time. After the addition of tamol N, the potential stabilized at -170 mv. But when duponol was used, the potential continued to rise to a level of -75 mv. It remained at this point for the next 5 hours. When the quaternary was increased to 1 - 7,500 and added to Ps. aeruginosa, the characteristic minimum was raised 60 mv. This is shown in Fig. 25. After the addition of either neutralizing agent, the electrode potentials returned to the minimum point. In this case, no significant difference in neutralization is noted between the two compounds.

Weber and Black (1948) in a study of quaternary inhibitors gave preference to a combination of lecithin and tween 80 for

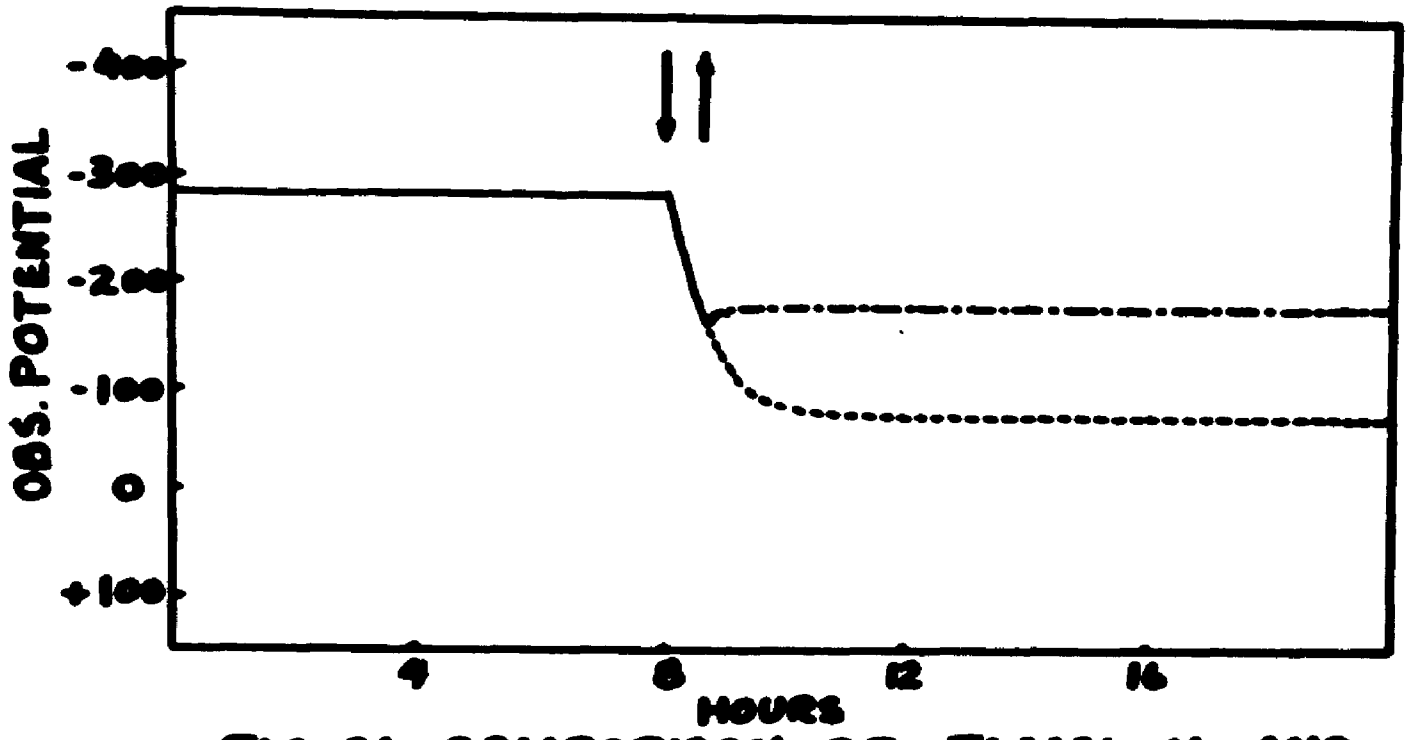

FIG. 24. COMPARISON OF TAMOL N AND DUPONOL NEUTRALIZATIONS USING SAL. TYPHOSA.

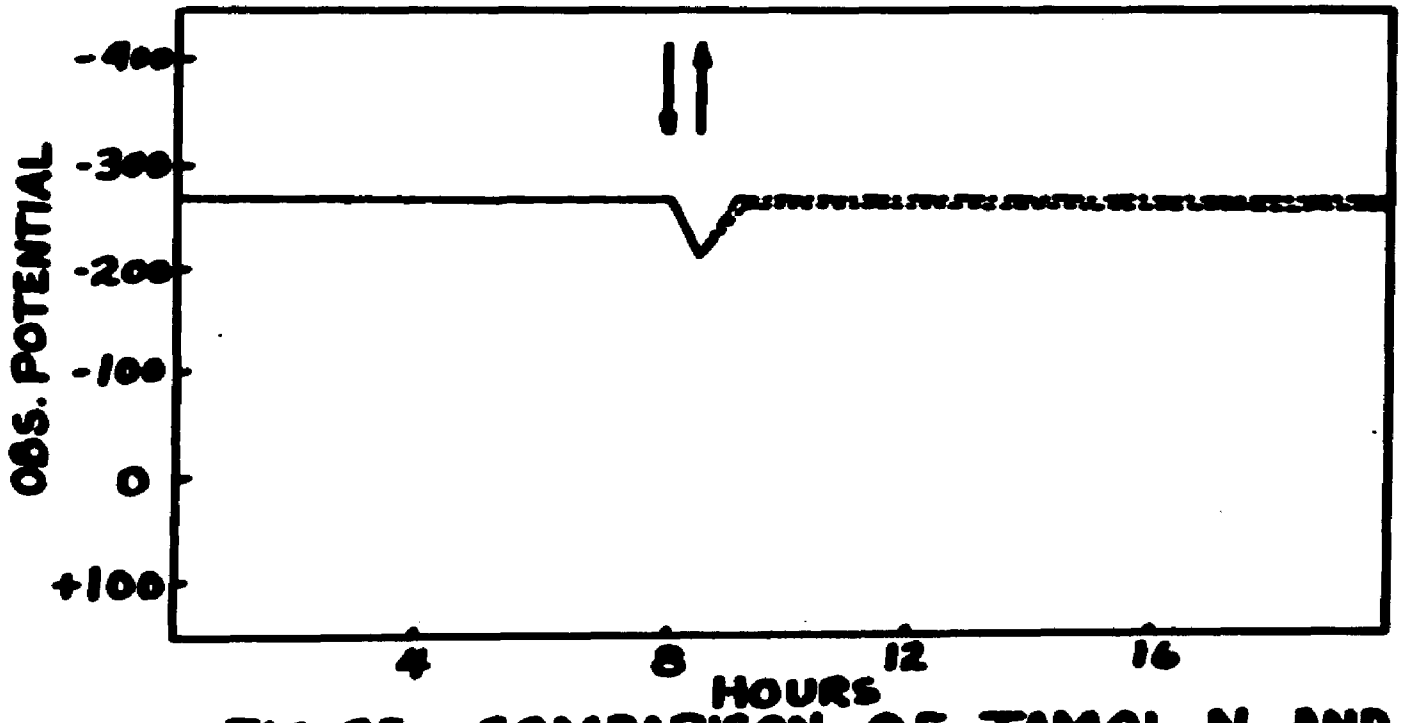

FIG. 25. COMPARISON OF TAMOL N AND DUPONOL NEUTRALIZATIONS USING PS. AERUGINOSA.

effective neutralization of quaternary ammonium compounds. They suggested that the sample be transferred to a diluent prepared by dissolving 2.2 g. azolectin and 15.6 ml. of tween 80 per liter, buffered to pH 7.2. It was reported that 9 ml. of this diluent was sufficient to neutralize 1 ml. of a 1 - 5,000 solution of quaternary.

In a test of the combination of azolectin and tween 80 described above, the same ratio of neutralizer was added to M. pyogenes var. aureus and to Sal. typhosa suspensions, previously treated with a 1 - 10,000 dilution of alkyl dimethyl benzyl ammonium chloride for 20 minutes. The results of this experiment are shown in Fig. 26. When the quaternary was added, the electrode potential came up 30 mv. in the case of Sal. typhosa and 190 mv. for M. pyogenes var. aureus. A second suspension of Sal. typhosa was not neutralized, and shows the inhibition of this concentration of quaternary. A comparison of these curves indicates that azolectin plus tween 80 is about 80% effective in prolonging the germicidal action if the test organism used is Sal. typhosa. After a 2-1/2 hour inhibition, the potential rapidly rises to the value of the quaternary treated control. Under the conditions of this experiment, no apparent neutralization was observed in the case of M. pyogenes var. aureus.

Germicides which are electrolytes in nature and enter into stoichiometric reactions with their antagonists, produce

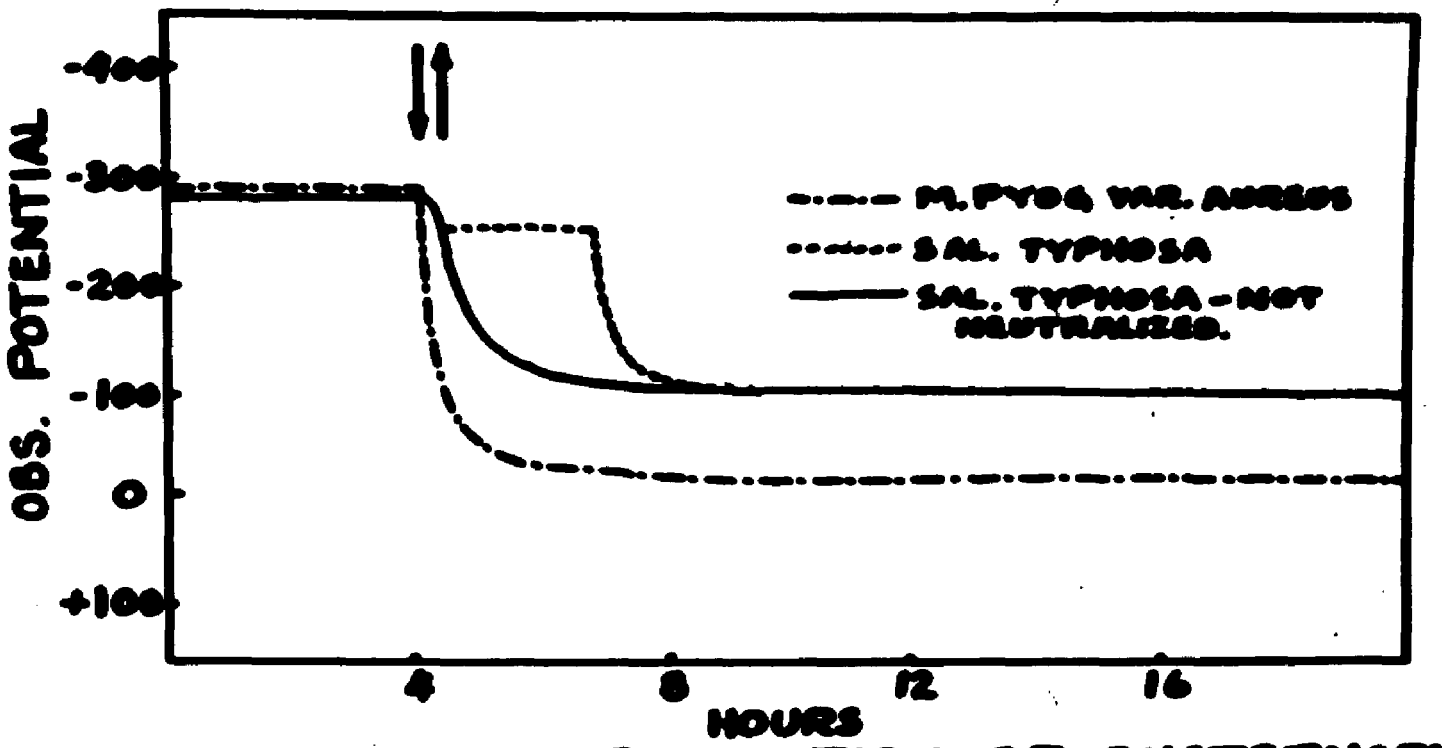

FIG. 26. NEUTRALIZATION OF QUATERNARY WITH ASOLECTIN PLUS TWEEN 80.

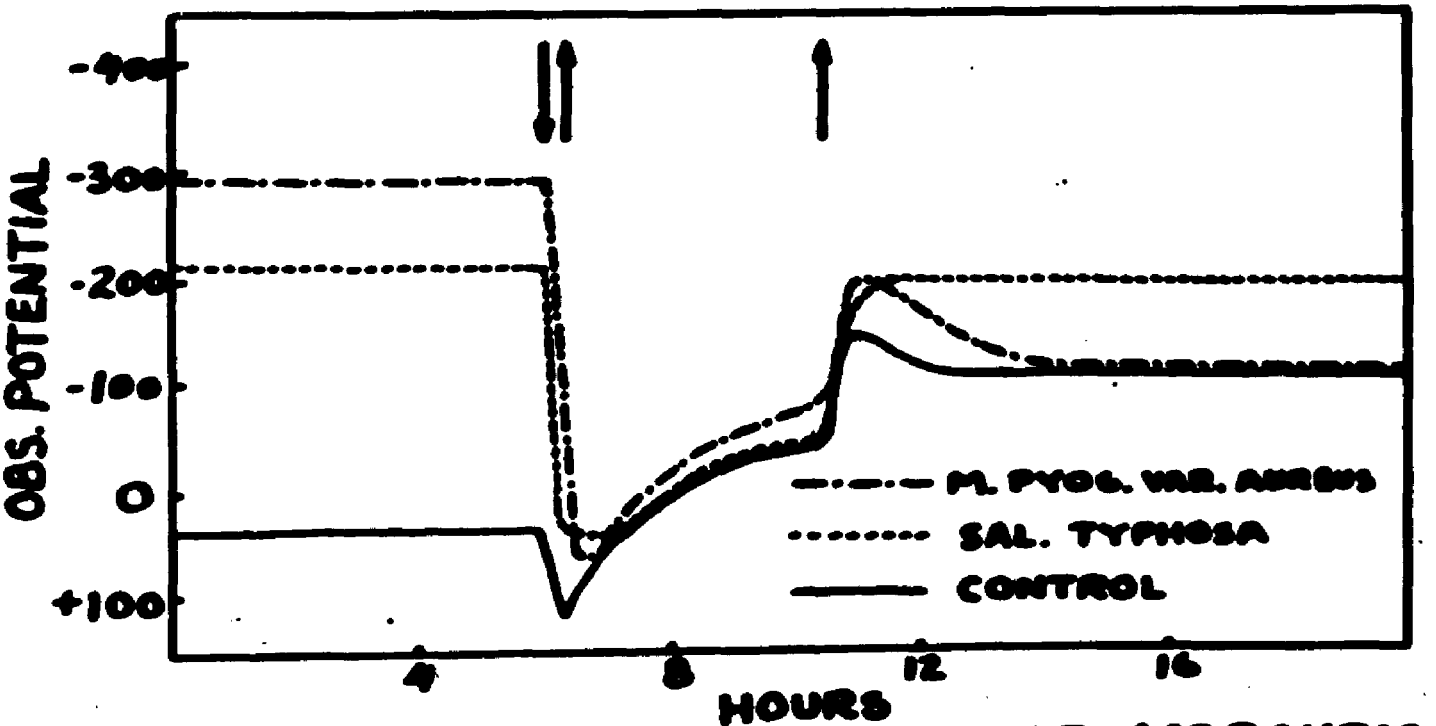

FIG. 27. NEUTRALIZATION OF MERCURIC CHLORIDE WITH AMMONIUM SULFIDE.

a different type of reoxidation curve. A limitation of the electrode potential method for following reaction rates of germicides of this type is given in Fig. 27. These curves were prepared by treating suspensions of M. pyogenes var. aureus and Sal. typhosa with a mercuric chloride solution and neutralizing with a solution of ammonium sulfide. The same treatment was also applied to an equal volume of uninoculated medium. At first observation, the curves would seem to indicate that about 50% reversal for M. pyogenes var. aureus and about 95% reversal was possible in these systems. However, it will be seen that the solid line, representing the uninoculated control, goes 80 mv. positive when mercuric chloride is added. The curve then takes a sharp drop stabilizing at -110 mv. upon the addition of potassium sulfide. These curves are meaningless as indicators of biological metabolism because of the similar changes induced in the uninoculated control by the additions of these types of germicides. It is necessary that the added substances cause no change in the uninoculated control. Only in this manner can it be assumed that the recorded changes in observed electrode potential are a result of biological metabolism.

SUMMARY AND CONCLUSIONS

Bacteria which are capable of actively metabolizing the constituents of a suitable medium are also capable of establishing

reducing conditions within this medium. If the organisms are inactivated under aerobic conditions, the constituents of the medium are reoxidized by the air. Preliminary observations determined that this is true regardless of how the metabolic activity is stopped. It occurred to the author that a method of recording electrode potential changes of a bacterial suspension inactivated with a disinfectant would provide a quantitative approach to a study of germicidal activity rates and the substances or factors which control these rates. An apparatus and a medium was developed and built which can be applied to the study of four separate bacterial suspensions, recording the data in terms of electrode potentials, and providing instantaneous indications of the environmental conditions to which the organisms are subjected.

A brief review of the fundamental concepts of oxidation reduction phenomena is presented together with various factors involved in its electrometric measurement.

The choice of test organisms is justified and the development of a suitable medium for oxidation-reduction potential studies is described.

An apparatus suited to the task of providing continuous electrode potential information in the form of a recording is discussed and a step-wise procedure for setting it in operation is given.

Normal time-potential curves of uninhibited test organisms grown in redox medium are shown. These curves demonstrate the various responses of 5 test organisms and establish the minimum reducing intensity of each.

Correlation of electrode potential data with standard plate counts are compared. Discrepancies are attributed to limitations of the medium and are apparent only during rapid changes in potential.

Differences in the sensitivity of the method during extremes of oxidation and reduction are pointed out.

The concentration of quaternary ammonium compound was found to be a function of the slope of the reoxidation curve.

Quaternary inhibitors were found to alter the shape of the reoxidation curve in a manner that provides a convenient method for assaying these compounds. Of the six quaternary inhibitors studied, one was able to partially reverse the action of quaternaries, two were able to arrest the action at the time they were added, and three were found to be of doubtful value. None of the quaternary inhibitors could revive vegetative cells once they have been exposed to the action of the germicide. When the neutralizer and quaternary are mixed prior to adding to the bacterial suspension, effective neutralization was noted.

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Abstract of Thesis

Oxidation-Reduction Potential Studies on the Rate of Germicidal Activity of Quaternary Ammonium Compounds

by

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This paper explores the possibilities of oxidation-reduction potential measurements used as quantitative indications of bacterial metabolism. A review of the literature leading to its use in the fields of chemistry and bacteriology is presented together with a discussion of methods of measurement and their limitations. Some common difficulties encountered in testing disinfectants in general and specifically those encountered in the testing of quaternary ammonium germicides are pointed out. The thesis is primarily concerned with the development of a medium and a suitable apparatus for the continuous recording of electrode potential data in the form of time-potential curves. The technique is based on the principle that bacteria, when inoculated into a suitable culture medium, give rise to reducing conditions within the medium. The maximum reducing intensity is established at a time when the reducing power of the organisms is in equilibrium with the oxidizing power of the air. As the death rate of these organisms exceeds the rate of cellular multiplication, in accordance with a growth curve obtained by viable counting procedures, the oxygen absorbing capacity of the medium exceeds the diminishing reducing capacity of the cells and the electrode potential begins to return to some point at or near the original potential of the uninoculated medium. If this normal metabolic course is interrupted in such a way that these cells are prevented from reproducing, then, the rate of return to the normal, or initial equilibrium should change accordingly.

This principle was employed to study the germicidal activity of benzene hexachloride, known chemically as gamma isomer of 1,2,3,4,5,6 hexachlorocyclohexane, a potentially potent therapeutic agent, and cetyl dimethyl benzyl ammonium chloride. The method was also used to study the effectiveness of several of the so-called quaternary inactivators.

The results obtained seem to indicate that under the conditions of this test the electrode potential changes with time in a manner similar to the logarithm of the number of surviving organisms. The slope of the potentiation curve was found to be a function of the quaternary concentration. Of the six quaternary inactivators studied, one was able to partially reverse germicidal action, two were able to prevent this action at the time the inhibitor was added, and three were found to be lacking in neutralizing properties.