A STUDY OF CERTAIN FACTORS WHICH

INFLUENCE THE RESISTANCE OF

STAPHYLOCOCCUS AUREUS WHEN GROWN

IN BEEF EXTRACT BROTH

by

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INTRODUCTION

Our knowledge of the resistance of bacteria to adverse conditions
has not kept pace with the rapid advances made in certain other branches
of bacteriology. Nevertheless bacterial resistance has considerable
significance in a number of fields of bacteriology and is of paramount
importance in the study of germicides and disinfection. So little syste-
matic study has been given to this particular field that today we possess
sufficient knowledge to specify the resistance of only two species of
microorganisms when these are to be employed as test organisms for deter-
mining the efficiency of germicides. In the U. S. Food and Drug Adminis-
tration, which regulates the claims made for germicides shipped in
interstate commerce more carefully than any other public agency, these
two microorganisms have been employed extensively for a number of years
as an aid in judging the efficiency of disinfectants and other germicides.*
They are <u>Eberthella typhosa</u> (Zopf) Weldin and <u>Staphylococcus aureus</u>
(Rosenbach).

In as much as the use of <u>Staph. aureus</u> supplies a better measure
of germicidal efficiency of certain types of products professing to be
germicides than does <u>E. typhosa</u>, and because it is the more fastidious
of the two in its growth requirements, the causes of the fluctuations
in resistance, which may be observed to be constantly occurring, assume
considerable importance. In addition, a large number of workers have
experienced difficulty in maintaining cultures of this organism at a
suitable level of resistance. It is fully appreciated that causes other
than those found in the culture media may be responsible for the small

* The current conception of the terms "germicide", "disinfect", "antiseptic
  etc. are defined by the lexicographer A. B. Patterson in the Amer. J.
  Pub. Health 22 (5) 465-72 (1932).

fluctuations in resistance of an organism which has maintained its general stability over a period of years. However, the possible effect of the ingredients of a prescribed culture medium on the resistance of Staph. aureus appeared to constitute a subject of investigation of practical as well as academic importance.

The present study is an investigation of the variation in resistance of a strain of Staph. aureus (209) resulting from minor changes in cultural methods and in the constituents of a medium prescribed to maintain the resistance of this culture. The organism used in these studies is designated as Staphylococcus aureus (209) and was isolated at the Army Medical School, Walter Reed Hospital, Washington, D. C. in 1922. Its resistance has not changed essentially since that time, and for a number of years it has been widely used for testing purposes.

HISTORICAL

The resistance of bacteria has been considered chiefly from an objective point of view and therefore many of the most enlightening references on the subject are intimately bound with the study of disinfection. Among those investigations which do deal primarily with resistance, the majority have been devoted to studies with bacterial spores.

Since long before the dawn of bacteriology as a science, an interest has been maintained in the control of disease-producing microorganisms. Although a number of early records of investigations with antiseptic substances are to be found and it was early appreciated that certain conditions such as the presence of large amounts of organic matter rendered the control of putrefaction more difficult, a definite interest in over-

coming the resistance of various bacteria did not come into evidence
in this country until 1874. At its meeting in St. Louis in 1874, the
American Public Health Association resolved to appoint a committee to
investigate disinfectants and, as a consequence, a report (1) was pub-
lished which shed considerable light on the proper use of disinfectants
although no specific method of testing germicides was recorded. It was
not until Koch and his coworkers (2) (3) (4) experimented with the killing
of pure cultures and described his methods with systematic precision, that
any studies on disinfection were capable of verification. Consequently
the history of disinfection may properly be said to have started with
Koch's experiments, and although he is generally known for his experiments
with anthrax spores dried on silk threads and transplanted on solid media
(gelatine), actually he worked with a number of organisms and used various
methods for testing and sub-culturing.

Apparently stimulated by the work of Koch (2), Geppert (5) and
Esmarch (6) published work in 1889 of lasting significance in which they
used anthrax spores as test organisms. The efforts of both not only
pointed out certain falacies in Koch's methods but contributed essential
considerations necessary in all germicidal work. Sternberg in 1892 (7)
claimed that 12 years previously he had inaugurated the drop method of
testing germicides and this method is essentially that most generally used
today. The prolonged popularity of the Rideal-Walker method (8) has been
responsible for the customary assignment of credit for the initiation of
the drop method to Rideal and Walker (9). Kronig and Paul's (10) classical
work on the killing of Staph. aureus and particularly anthrax spores, which
were dried on garnets, served as a basis and stimulus for later work deal-

ing with the dynamics of disinfection.

Methods of Testing Disinfectants

The first methods employed in testing germicidal action were
quite unsatisfactory for they consisted merely of determining the amount
of an antiseptic necessary to control fermentation or putrefaction in some
arbitrarily selected medium containing unknown numbers and mixtures of
organisms. While Koch's (2) method of dipping silk threads in cultures
of pure organisms and then laying these on gelatine media after definite
intervals of exposure to the lethal agent offered a significant advance
in disinfection testing technique, it nevertheless entailed the disad-
vantage of allowing inhibitory agents to remain on the organisms at the
site of implantation and thus preventing germination and growth. This
was pointed out by Geppert (5) who advocated implantation into fluid media
and animal inoculation to disperse the inhibitory substances. Sternberg's (7)
method of inoculating the germicidal substance with definite amounts of
a broth culture of the test-organisms was much like the methods most used
today but it lacked sufficient controls. Rideal and Walker (9) in their
proposed method utilized phenol as a control measure and compared the
resistance of the test-organism against appropriate dilutions of phenol
to its resistance against the disinfectant being tested. This came to be
known as the phenol coefficient method of testing disinfectants and is
extensively utilized at the present. Although Escherichia coli was first
used in this test the organism was soon changed to E. typhosa. A commission
appointed by the editors of Lancet recognized that the R. W. (Rideal-
Walker) (8) method ignored the difference in time required for the action
of various germicides and devised the Lancet Method (11) which utilized

the mean phenol coefficients obtained over a wider range of time periods. This method utilized E. coli for testing purposes. In the same year the Chick-Martin test (12) was offered as an improvement over the R. W. Test and organic matter (feces) was introduced into the mixture of test-culture and disinfectant as a means of rendering the phenol coefficient a more practical method of measuring the true worth of disinfectants. Anderson and McClintic (13) working at the U. S. Hygienic Laboratory combined what they considered the worthy features of the Lancet and R. W. phenol coefficient tests and, after utilizing some observations of their own, presented a new phenol coefficient test designated as the Hygienic Laboratory or H. L. method (14) of testing disinfectants. This test was adopted by the laboratory section of the American Public Health Association in 1912 as the official procedure to be used in disinfectant testing. In spite of its short-comings, the R. W. method exceeded the Lancet and the Chick-Martin tests in popularity and has continued ever since in England to be the popular method of testing disinfectants. In the last few years Garrod (15) has proposed a modified Chick-Martin test using yeast cells as organic matter but this test has not been well received. In this country the R. W. test has shared its popularity with the H. L. Method and until the middle of the last decade both were much used. Revisions of these tests were made in 1921. In 1918 the American Public Health Association (16) recognized objectionable features in both the H. L. and R. W. methods and published a method of its own based to a great extent on the findings of Wright (17) but this procedure failed to gain general acceptance. Perhaps, prompted by the efforts of the American Public Health Association, Shippen (18) proposed a method of testing disinfectants for the Insecticide

Board (which administered the U. S. Insecticide and Fungicide Act of 1910)
and designated it the R. W. modified technique. This method was published in
1927 by Reddish (19). The test then became popularly known as the Reddish
method and is essentially the same test now employed by the U. S. Food and
Drug Administration. It is now known as the F. D. A. test (20). Appended
to the phenol coefficient test as published and now used almost exclusively
in the United States is a series of special tests for antiseptics and germi-
cides which make use of Staph. aureus as a test organism. Many of these
tests are of unknown origin and were described in the Department of Agri-
culture Circular No. 198 (20) for the purpose of offering a uniform method
of test to augment chemical and pharmacological tests as well a s medical
opinion in judging the value of certain types of products according to
the claims made for them on their labels. Unfortunately it has become the
general conception, that these are official methods serving as criteria
for complete antiseptic or germicidal value. This, they are incapable of
doing. More recently, and even more unfortunately, certain of the methods
with modifications are being incorporated in the National Formulary as
official methods of bacteriological assay.

The methods mentioned above have all laid down more or less care-
fully prescribed limits regulating temperature, time, ratio of test material
to culture, etc., with a consequence that much uniform material has been
gathered on disinfecting agents. Since, however, the test organism has
usually been designated, very little light has been shed on the comparative
study of resistance by these methods.

Variation in Resistances of species and strains of organisms and factors
influencing resistance

In investigating bacterial resistance experimentation has proceeded

along individual and arbitrary lines. The use of an assortment of methods has been necessary because of the absence of standardized organisms, the wide variety of physical and chemical agents investigated, and the diversity of purposes and objectives prompting the investigations. Although our knowledge has been greatly enriched by the varied quality of the work accomplished much contradictory evidence has been presented on the resistance of bacteria mainly because of lack of uniform methods of control.

The greatest number of observations leading to pertinent facts on resistance have resulted from attempts to study the germicidal effieiency of various agents and these observations have contributed largely to our knowledge concering the difference in resistance among certain species of organisms. A more limited number of workers have shed light on the subject through studies of the dynamic action of germicides and to these investigations we owe our knowledge of the general reactions of bacteria to germicidal agents. More particularly they have directed attention to the important consideration of the variation which may exist among bacterial cells in the same culture. A number of bacteriologists interested in the physiology of bacteria have, more or less incidentally, brought out the fact that young cultures are more susceptible than old cultures to outside influences. A few investigators have attempted to select an organism representing the standard or resistance for a particular species and by so doing have provided us with a knowledge of the extreme variability among different strains of the same organism.

A. Variation in resistance among species

When Kronig and Paul (10) utilized Staph. aureus along with the anthrax bacillus as test organisms for drying on beads it became known that certain vegetative cells were much more resistant to drying than were others. Since

then it has been gathered from the great bulk of evidence that resistance among bacterial species varies tremendously. It is well known that the lability to heat of the gonococcus and syphilis organisms prevents their withstanding, even briefly, temperatures reaching 47°C. (21). In marked contrast certain "earth spores" have been found to resist flowing steam for 30 hours (Rodenbeck) (22) or survive 110°C. in the autoclave for 2 hrs., 120°C. for 8 minutes (Konrich) (23); 115°C. for 15 minutes (Williams) (24) and 120°C. for 8 minutes (Curran) (25). A comparable resistance to heat has been found with spores of Clostridium botulinum by Estey and Meyer (26). In contrast to the susceptibility of vegetative cells to alcohol, bacterial spores have been found to be viable after 46 years in pathological specimens preserved in alcohol (27). In general it has become recognized that the more parasitic bacteria are the more susceptible they are to adverse conditions. This common observation, however, is not without exception, since the tubercle bacillus, although moderately susceptible to some chemical agents is so unusually resistant to others that it can be separated from other vegetative bacteria by chemical means.

It can be shown in general from the history of disinfection that those organisms most selective in their growth requirements are the most susceptible to unfavorable influences. This general rule, however, also has its exceptions since it is well known that although Brucella abortus is extremely selective in its growth requirements this organism grows well in the presence of dyes which inhibit the growth of less fastidious organisms.

B. Variation of resistance among strains

While the difference in resistance among species is well recognized, on the other hand, the difference in resistance among strains of the same species

is frequently overlooked. Von Esmarch (6) in 1889 was the first to note the great variation in resistance among strains of the same organism, which he demonstrated with anthrax spores. Since that time little attention has been paid to the selection of strains of organisms for experiments dealing with resistance and, as a result the literature contains many contradictions concerning the resistance of bacteria of the same species to various agents. It is not uncommon to find statements in the literature to the effect "that a 24 hour broth culture of B. coli was used" or "washings from 48 hour plates of anthrax cultures which were shown to contain a high percentage of spores were used in the following tests". Obviously this type of experimental work has been in part at least responsible for the confusion that exists.

Anderson and McClintic (13) recognized the variation in resistance among strains of the same species and were probably the first workers to give consideration to the importance of selecting a strain of vegetative organisms for use as test-cultures in disinfection and resistance studies. They found less variation among strains of typhoid organisms than among strains of E. coli, and consequently chose the Hopkins' strain of E. typhosa for use in the Hygienic Laboratory phenol coefficient test as representing the resistance of the species. This strain is now used almost exclusively in this country for the determination of phenol coefficients. That such a selected strain can not represent the resistance of its species, however, was clearly shown by Schaffer and Tilley (28). They obtained wide variations in phenol coefficients of the same chemical compound by using different strains of E. typhosa.

Reddish (29) in 1924 determined the resistance of 22 strains of Staph. aureus and found that a majority gave resistance closely represented by Strain 209 which was being used as a test-organism. Since then, of approximately 18 strains of Staph. aureus tested at the Food and Drug Administration 13 have met the prescribed resistance when grown on the prescribed media. However most of these strains have failed to maintain this level of resistance.

C. Factors affecting resistance

Factors such as nutrition, pH, temperature, etc., which have a profound affect on the growth of bacteria so obviously affect their resistance also that no discussion of their influence is necessary here. Once having obtained a culture showing excellent growth under optimal conditions it has been quite generally assumed that such a culture at the age of maximum population possesses good or near optimal resistance. While this is not true in the case of Staph. aureus, as will be shown later, it is necessary to turn to work that has been done on bacterial spores to find most of the contradictory evidence to this general assumption.

Esmarch (6) showed that not only did different strains of anthrax produce spores of different resistance but that by the cultivation of the same strain on different media, a difference in the resistance of the spores was obtained. Williams (24) working with the spores of B. subtilis found that different brands of peptone used in the medium affected resistance of spores. He also found that the addition of vegetable extracts improved the resistance of these spores. Dickson, et al (30) with the spores of Cl. botulinum, observed the same affect and found that different strains of this organism required different media for the production of resistant

spores. The writer has improved the resistance of anthrax and cereus spores by the addition of soil extract to the culture media. Brunstetter and Magoon (31) state that the cultural background of a strain is in part responsible for its sporogenic qualities. These writers have improved the heat resistance of the spores of Bacillus mycoides by aeration. Leifson (32) influenced the production and resistance of spores by the addition of dilute solutions of certain salts. Reiter (33) and Mundel and Schmidt (34) increased the heat resistance of spores by raising the temperature of cultivation to 46°C. Reiter (33) improved the resistance of anthrax spores with the addition of wheat extract to the medium. Curran (25) has modified the resistance of spores by removing them from the original culture medium and storing them under different conditions. Magoon (35) also obtained similar results by storing spores.

Reddish and Burlingame (36) have shown that the use of different brands of peptone affect the resistance of Staph. aureus and Philbrick (37) reported that different batches of the same brand of peptone give differences in resistance of this organism. Wright (17) found that by increasing the peptone (Witte's) in his culture media from 1.0% to 2% the resistance of E. typhosa was slightly increased but was slightly decreased by the addition of 3% peptone. He obtained contrary results when increasing the amount of beef extract from 0.3% to 0.5% and although he obtained heavy growth he showed a slight decrease in the resistance of the organism to phenol.

It is evident from the foregoing that the production of a constant resistance in spores, at least, is a difficult problem and that without controlled testing their resistance cannot be estimated. Evidence has shown that the occurrence of spores of unusually high resistance are quite rare.

Oesterle (38) claims that "earth spores" of comparative high resistance occur in certain types of soil, especially heavily manured ground. Konrich (23) found resistant spores in a few samples of soil gathered from various locations in Germany. Rodenbeck (22) found native spores in garden soil resisting flowing steam for 30 hours while 48 hour cultures of the same organism on agar plates succumbed in from 5 to 25 minutes. Estey and Meyer (26) found that the majority of spores of Cl. botulinum lacked exceptional heat resistance. Townsend et al (39) state "The maximum heat resistance of spores of this organism in standard phosphate solution has been fairly well established *** unfortunately it is difficult to produce spore crops of strains of Cl. botulinum having such high resistance, even a medium resistance being difficult of attainment".

The considerations which have been touched upon in the preceding paragraphs serve to call attention to the care that must be exercised in the selection and culturing of a microorganism to be used in studies on resistance if dependable and reproducible results are to be obtained.

METHODS

Organism used

As stated above the organism used in this investigation was obtained from the Walter Reed Hospital in 1922 as a freshly isolated strain of Staph. aureus (209) from a pathological process. It was put into immediate use as a test organism by Shippen (18). Its resistance against phenol was found to be representative of a number of strains of Staph. aureus cultures when tested by Reddish (29). Since 1922 this strain has, in general, maintained a constant resistance to phenol. According to Slocum (40) the culture still retains hemolytic properties to a fair degree. Although a number of Staph.

aureus cultures have been shown to exhibit approximately the same resistance as strain 209, quite a large portion of these have failed to remain constant in their resistance over a period of time, and have been discarded. Whether other strains would have settled into a state of constant resistance on prolonged cultivation on artificial media is not known. Strain 209 freely dissociates producing cells which give rise to white colonies on plating on agar. After several weeks of daily transfer in nutrient broth the white colonies (rough variants) increase and the resistance of the culture correspondingly decreases. Pinner (41) reported that this change of Staph. aureus to the "albus" form occurs frequently in daily transfers in beef infusion broth. Smythe (42) reports a sharp change in the character of the culture of strain 209 at about the 20th daily transfer in broth with macroscopic changes in pellicle formation and an increase in proportion of organisms capable of producing white colonies. Such a sharp differentiation has not been observed in this laboratory; indeed noticeable changes in broth cultures have as a rule been observed only at widely varying intervals. Smythe (42) further observed that the daily culture closest to removal from the agar slant generally shows the highest death rate when tested with phenol though no transplants from single yellow or white colonies were tested for resistance. According to Boland, O'meara and Bigger (43) variants produced in broth culture tend to breed true. This has been found to be the case of white variants carried in this laboratory. The white variants are invariably less resistant than the original culture.

Culture media used

Wright (17) found that Armour's peptone (with Difco a poor second) was an adequate substitute for Witte peptone as a base for the culture media

when using E. typhosa as a test organism in the study of disinfectants. As a consequence Armour's peptone has been prescribed, in both the H. L. and the F. D. A. methods, as the peptone to be used for culturing the test organism. Armour's peptone which is specially prepared for disinfectant testing has been the subject of several investigations, dealing with its superiority in maintaining the resistance of Staph. aureus reported by Philbrick (37).

The most variable ingredient of the media prescribed for the F. D. A. method is Liebigs beef extract as was pointed out by Wright (17). Difco extract is not only much more soluble but is also much more uniform and should be designated in place of Liebigs. As will be shown later, the amount of beef extract prescribed is so far in excess of the amount needed for growth requirements of the organism that any advantage to be gained from the use of either brand is distinctly in favor of the more soluble and uniform product.

In the following work the F. D. A. method has been followed in so far as circumstances allowed. The subculture medium has been the prescribed F. D. A. broth, the 4 mm. platinum loop has been used for transfer, and the 20°C. temperature of medication and the 1-60 dilutions of carefully prepared phenol have been adhered to. The variable factors have consisted of varying amounts of beef extract or substitutes, varying amounts of peptone, varying time intervals, varying sizes and shapes of containers for culturing the organism and varying conditions of culture for the test organism. All references to peptone in the text refer to Armour's (special for disinfecting testing).

EXPERIMENTAL

The standard prescribed in the F. D. A. method, for the resistance of

Staph. aureus is such that when 0.5 cc. of a 24 hour culture is placed in 5 cc. of a 1-60 dilution of phenol, enough cells shall survive a 5 minute exposure at 20°C. to produce growth in a subculture medium of F. D. A. broth when one 4 mm. loopful is transferred from the mixture of culture and phenol into the subculture medium, or that under the same circumstances growth shall occur in the subculture after a 5 minute exposure to a 1-80 dilution of phenol at 37°C.

The affect of numbers of cells as influenced by enrichment substances on the resistance.

Since numerous reports indicated that cultures of the test organism frequently fall below the prescribed standard, it was thought desirable to attempt to raise the resistance of the culture sufficiently to meet the standard with more regularity. The literature indicates that in accord with the Law of Mass Action, other conditions being equal, a greater number of organisms will survive longer than a smaller number. It was thought therefore that by enriching the medium a sufficient increase in the number of cells could be produced to meet the demands of a satisfactory survival time more consistently. Consequently, media containing 1% peptone, 0.5% NaCl and various substitutes for the regular 0.5% beef extract, were inoculated from a broth culture of Staph. aureus (209) showing the proper resistance to phenol at 37°C. After daily transferring into the respective media, the resistance of 24 hour organisms was tested at 20°C. against a 1-60 dilution of phenol. Time intervals were reduced to one minute from the regular 5 minute time interval so as to demonstrate more accurately differences in resistance. The tests were made after the 4th, 5th and the 9th daily transfers. Viable counts were made on the 4th and 9th transfers.

As has already been pointed out, daily transfers vary in resistance and in using one minute intervals it is seldom that the same resistance is consistently obtained in a series of tests. Table I shows the resistance of Staph. aureus (209) grown in the standard medium containing various substitutes for the prescribed 0.5% beef extract. Three determinations were made for each test condition. (See Table I).

TABLE I

The Effect of Enrichment Substances on Resistance

Culture Medium		Resistance to 1-60 phenol in minutes						Average Count
Enrichment	Peptone	2	3	4	5	6	7	Millions cc
None	2%	*3	3	3	2	2	2	***590
2% Beef extract	-	1	0	0	0	0	0	1200
1% Beef extract	1%	3	0	0	0	0	0	1500
2% Yeast Extract	-	0	0	0	0	0	0	1800
1% Yeast extract	1%	3	1	0	0	0	0	1200
Beef infusion	1%	3	2	1	0	0	0	1500
1% Dried blood**	1%	3	3	2	1	1	0	44
1% Bile	1%	0	0	0	0	0	0	47
1% Fresh horse serum	1%	3	2	1	0	0	0	200
1% Fresh rabbit blood	1%	3	3	2	1	0	0	250
0.5% Beef extract (regular media) (control)	1%	3	3	2	1	1	0	1500

* Each condition was tested three times. The figures under the minute columns represent the number of times the test culture (grown in the medium listed on the left) was found to survive for the number of minutes listed.

** Dried blood hydrolized in alkali then neutralized.

***The count in millions/cc. represents the average number of organisms in the test culture.

The first thing to be noticed from the results in Table I is that apparently there is no correlation between numbers of organisms and their survival time - the premise (based on the Law of Mass Action) which dictated the test is completely overshadowed by other more important factors which are responsible for resistance. With the exception of the culture media containing bile it will be noticed that the cultures of beef and yeast extract with no peptone produced organisms with the least resistance while the culture consisting of peptone alone with no enrichment produced organisms having the highest resistance, though the bacterial count was less than half of that of the beef and yeast extract cultures. Again the media containing hydrolyzed blood produced but 44 million cells per cc. as compared with the 1500 million in the regular F. D. A. broth yet both registered the same resistance.

Effect of varying concentration of beef extract and peptone on growth
and resistance

Inasmuch as beef and yeast extract as enrichment materials produce luxuriant growth but cells of poor resistance, experiments were carried out to determine the effect that various percentages of beef or yeast extracts in the nutrient media might have on the resistance of the cells. The results obtained are shown in Table II.

TABLE II

Survival time of a 24 hour culture in a 1-60 solution of
phenol at 20 degrees C. of Staph. aureus grown in 1% peptone
containing various amounts of Beef extract and Yeast extract

Per cent Beef Extract		Average Count		Survival time in minutes						
					2	3	4	5	6	7	8
1.0	*(5)	2150	*(10)	**10	8	4	1	0	0	0
0.5	(5)	1290	(10)	10	10	7	7	3	0	0
0.3			(10)	10	10	8	8	4	4	1
0.1	(5)	980	(10)	10	10	8	7	7	5	2
0.05	(3)	650	(4)	4	4	3	2	1	1	1
0.01	(3)	770	(3)	3	3	3	2	1	1	0

Per cent Yeast Extract											
1.0	(3)	1970	(5)	4	1	0	0	0	0	0
0.5	(3)	2010	(5)	5	2	0	0	0	0	0
0.1	(3)	850	(5)	5	5	4	2	2	0	0
0.05	(3)	550	(5)	5	5	4	4	2	1	1
0.01	(3)	670	(5)	5	4	4	3	2	2	0

* Figures in ( ) indicate number of tests made

** Figures under "Survival time in minutes" indicate number of cultures
surviving for each time interval.

It is quite obvious from these results that the resistance of the organisms is in inverse ratio to the amount of beef extract present, down to 0.1%. Apparently yeast extract has the same relative action except the proportional effect on the resistance is not as apparent and the optimal amount of yeast extract appears to be less than in the case of the beef extract. Wright (17) has shown a very slight and gradual loss of resistance in E. typhosa with increasing amounts of beef extract up to 3% but his results are in contrast to the sharp gradiant of those in the above table. The results can not be explained on the basis of a toxic principle in the beef and yeast extract since a rise in actual count accompanied the increase in concentration of extract. Both yeast and beef extract therefore increase growth but decrease resistance. It should be pointed out that the character of the growth occurring in beef and yeast extract broth is quite distinct from that obtained in peptone broth. Growth in the former medium is character- ized by a pellicle formation, which is rather fragile, and by a heavy flocculant precipitate, often with a rather clear intermediate zone in the middle of the column of medium. In peptone a uniform distribution of growth is obtained with a heavy deposit appearing only after several days incubation. Although it would appear that the flocculant and granular masses of culture should offer more resistance this was not found to be the case. Apparently some stronger factor abrogates this effect.

Not only does beef extract increase the population of 24 hour cultures as compared with 24 hour cultures grown in peptone broth; but the early phases of the growth curve begin sooner and rise faster when Staph. aureus is inoculated into media containing beef extract. The effect on the bacterial cound when Staph. aureus is grown in beef extract media and in peptone broth is shown in Table III.

TABLE III

Bacterial Counts in the early stages of growth
in beef extract and in peptone media.

Age of culture in hours	Number of viable cells per cc.		
	1% Beef Extract + 1% Beptone	2% Peptone No beef extract	0.5% Beef
	Millions	Millions	
0	12	9	
2 1/2	17.5	12	
5	116	50	
Exp. 1 7	520	240	
10	800	370	
12	1200	820	
15	2260	840	
0	2.6	2.3	
2 1/2	3.9	2.7	
Exp. 2 5	25.4	14.8	
7 1/2	227	128	
0	1.4	1.4	1.4
3	2.1	1.5	1.3
Exp. 3 4	4.3	2.8	2.2
6 1/2	45.4	23.8	41.6

It will be noted that there is a much greater increase in numbers of organisms as the culture increases in age in the beef extract media than in the peptone media.

According to Topley and Wilson (21), Bail suggests in his "M Concentration" theory that in any one culture, depending on its physiological requirements, once a certain concentration of cells is reached this limiting concentration cannot be exceeded. The thought occurred that some such factor might be responsible for the condition attending the crowded growth in culture media containing increased amounts of beef extract. Since beef extract is a concentrated food it was thought possibly that the cell population might reach a degree disadvantageous for complete development and maturity of individual cells because of an excess of nutriment. To increase the food supply and test the above theory additional peptone was used. The results obtained are recorded in Table IV.

TABLE IV

Survival time of a 24 hour culture in a 1-60 solution
of phenol at 20 degrees C. of Staph. aureus grown in
varying amounts of peptone.

Per cent Peptone	Count in millions		Survival time in minutes						
			2	3	4	5	6	7	8
3.0	(7) 1100	*(7)	**7	7	7	5	5	3	2
2.0	(10) 750	(10)	10	9	8	7	7	5	3
1.0	(6) 480	(10)	10	8	7	7	4	4	1

* Figures in ( ) indicate number of tests made.

** Figures under "Survival time in minutes" indicate number of cultures surviving for each time interval.

As can be readily noted (Table IV) the increased amounts of peptone increased the number of organisms developing as did the increase in the amounts of beef extract (Table II), though to a somewhat lesser degree. On the other hand the heavier concentrations of peptone had just the opposite action on the reistance of the culture - increasing rather than diminishing it. It follows therefore that the resistance of Staph. aureus does not depend alone on food concentration.

Although it appears that cultures of Staph. aureus can attain a greater resistance in peptone, beef extract on the other hand possesses some factor which has an accelerating effect on the growth of the organism which is not exhibited by peptone. Thus beef extract offers a better "recovery" medium. Experience has shown that when a culture is subjected to a germicide and sub-cultured in beef extract media the survival time appears to become longer than when sub-cultured in peptone broth. The apparent differences in resistance of the same culture of Staph. aureus when tested against a 1-60 solution of phenol by the F. D. A. technique and then sub-cultured in different media is shown in Table V.

TABLE V

Apparent differences in resistance of the same 24 hour culture tested against a 1-60 solution of phenol by the F. D. A. technique and sub-cultured in different media.

Test No.	Sub-culture media					Survival time in minutes			
						5	6	7	8
No. 1	1% beef	extract	broth	1%	peptone	+	+	+	+
	0.5% "	"	"	1%	"	+	+	+	-
	0.0% "	"	"	2%	"	+	+	-	-
No. 2	1% "	"	"	1%	"	+	-	-	-
	0.5% "	"	"	1%	"	+	-	-	-
	0.0% "	"	"	2%	"	-	-	-	-
No. 3	1% "	"	"	1%	"	+	+	+	-
	0.0% "	"	"	2%	"	+	+	-	-
No. 4	1% "	"	"	1%	"	+	+	-	-
	0.0% "	"	"	2%	"	+	-	-	-

+ Growth
- No growth

It is obvious from the results tabulated that beef extract broth constitutes a more favorable medium for the growth of Staph. aureus after it has been subjected to the action of a germicide than does peptone broth.

Relation of Oxygen Tension to Resistance

Since Staph. aureus is a faculative anaerobe, growth of this organism is curtailed to some extent under anaerobic conditions. Richardson (44) has shown that the addition of uracil to synthetic media is needed for anaerobic but not for aerobic growth.

Preliminary tests indicated that aerobic growth increased resistance, so it appeared possible that a supply of oxygen more abundant than that accessible to cultures grown under the usual conditions might increase the

resistance of <u>Staph.</u> <u>aureus</u> cultures. To study this problem, 10 cc. amounts of F. D. A. media were dispersed in tubes of different diameters and inoculated with a single 4 mm. loopful of a 24 hour broth culture of <u>Staph.</u> <u>aureus.</u> After a growth period of 24 hours each culture was tested for resistance to the standard 1-60 phenol. The results obtained are given in Table VI where, in tubes of different diameters, each containing a volume of 10 cc., the relationship of the calculated surface-depth ratio to the resistance of the cultures has been shown.

TABLE VI

Relationship Between Surface-Depth-Ratio and Resistance in 1% Peptone Broth With added Beef Extract

Surface Depth ratio	% Beef Extract	24 hour culture Survival time in minutes								48 hour culture Survival time in min.						72 hour culture Survival time in min				
		2	3	4	5	6	7	8	10	5	6	7	8	9	10	8	9	10	11	12
0.18 (15 mm. tube)	1.0	+	-	-	-	-	-			+	-	-				-	-	-	-	-
	0.5	+	+	-	-	-	-			+	+	-	-			-	-	-	-	-
	0.1	+	+	-	-	-	-			+	+	-	-			-	-	-	-	-
0.62** (20 mm. tube)	1.0	+	-	-	-	-	-			+	-	-	-	-	-	+	+	+	-	-
	0.5	+	+	+	-	-	-			+	+	+	+	-	-	+	+	+	+	+
	0.1	+	+	+	-	-	-			+	+	+	+	-	-	+	+	+	+	+
1.4 (25 mm. tube)	1.0	+	+	-	-	-	-			+	+	+	-	-	-	+	+	-	-	-
	0.5	+	+	+	-	-	-			+	+	+	+	-	-	+	+	+	+	+
	0.1	+	+	+	+	-	-			+	+	+	+	+	-	+	+	+	+	+

Surface Depth ratio	% Beef Extract	24 hour culture Survival time in minutes								48 hour culture Survival time in min.				
		2	3	4	5	6	7	8	10	5	7	10	12	15
0.18	1.0	+	-	-	-	-	-			+	-	-	-	-
	0.5	+	+	-	-	-	-			+	-	-	-	-
	0.1	+	+	+	-	-	-			+	-	-	-	-
0.62	1.0	+	+	+	-	-	-			+	-	-	-	
	0.5	+	+	+	+	-	-			+	+	+	-	-
	0.1	+	+	+	+	+	-			+	+	+	+	-
1.4	1.0	+	+	+	-	-	-			+	+	+	-	-
	0.5	+	+	+	-	-	-			+	+	+	+	-
	0.1	+	+	+	+	+	-			+	+	+	+	+
2.7	1.0	+	+	+	-	-	-							
	0.5	+	+	+	+	+	-							
	0.1	+	+	+	+	+	+							
13.8	1.0				+	+	-	-	-					
	0.5				+	+	+	+	-					
	0.1				+	+	+	+	+					

+ Growth in sub-culture

- No growth in sub-culture

* Note - The surface depth ratio is obtained by dividing the area of the surface of the medium in each tube by the depth of the medium.

** Represents surface depth ratio of standard 20 mm. tube.

The relationship of surface-depth ratio to resistance can be readily recognized. It is evident that an abundance of available atmospheric oxygen enhances resistance. It can be seen from the results of the experiments recorded in Table VI that an increase in area of the media-air interface per unit volume of media not only increases resistance in the 24 hour culture but that the available oxygen supply continues to effect the resistance in older cultures. Table VI also reaffirms the conclusion drawn from Table II, i. e. that a decrease in the amount of beef extract present in a 1% peptone media results in an increase in the resistance of the cultures. These results indicate that there are at least three important factors affecting the resistance of a culture of Staph. aureus; (1) the amount of beef extract, (2) the age and (3) the available oxygen. It would appear that cultures of Staph. aureus grown in media containing a comparatively small amount of beef extract (0.1%), confined in vessels affording a large surface and shallow depth, and incubated for several days will show increased resistance over those treated by the standard technique.

Brunstetter and Magoon (31) have shown that the oxygen-food relationship is important in the growth and spore forming characteristics of a number of aerobic spore formers. Clifton (46) and Hershey and Bronfenbrenner (45) have shown that the food-oxygen ratio is of paramount importance as limiting growth factors in Es. coli cultures. Braun (47) using the colon, paratyphoid and proteus organisms, has shown quite distinctive growth characteristics depending entirely upon the available oxygen in ratio to the available carbon. The oxygen-food relationship to resistance has been left unstudied heretofore.

The incubation of cultures in 10 cc. of media placed in comparatively large Erlenmeyer flasks (200 cc. capacity) doubtless offers optimum access of

atmospheric oxygen, nevertheless, Hershey and Bronfenbrenner (45) have shown that a depth of media amounting to only a few millimeters can interfere with the diffusion of oxygen. Since Brunstetter and Magoon (31), Hewitt (48) and others have utilized aeration, it was decided to determine the affect of aeration on the resistance of Staph. aureus by continuously bubbling air through the media. It seemed probable that the ordinary inoculum transferred with a 4 mm. loop (in the neighborhood of 20 million cells) would exert sufficient reducing activity to lower the reduction potential necessary for the initiation of metabolic activity even in the presence of a constant stream of oxygen. That such an assumption was justified is shown by the results given in Table VII.

TABLE VII

Effect of **Abnormal** aerobic conditions and
of aeration on bacterial count and resistance

Amount of beef extract added to 1% Peptone	Method of Cultivation	Count in millions/cc. of 24 hr. culture	Survival in 1-60 phenol at 20°C. culture 24 hrs. old	Survival in 1-60 phenol at 20°C. culture 48 hrs. old
			Minutes	Minutes
1%	Tube*	2100	3	4
	Flask**	4600	5	6
0.5%	Tube	1550	5	7
	Flask	2500	7	7
0.0 (2% peptone)	Tube	840	6	10
	Flask	1100	8	20
	Control+	1700	5	
1%	Tube	2500	4	5
	" aerated	10000+	8	10
0.5%	Tube	1350	5	6
	" aerated	10000	9	9
0.0 (2% peptone)	Tube	700	8	12
	" aerated	6300	20	30+
	Control+	1900	5	

* All tubes were standard 20 x 150 mm. bacteriological tubes.
**All flasks were 200 cc. capacity Erlenmeyer flasks.

+ Control represents the normal daily culture of 10 cc. of standard 0.5 beef extract broth in a 20 x 150 mm. tube.

+ After the figure represents a figure greater than the one shown, 10 cc. amounts of media were inoculated with one 4 mm. loopful of a standard 24 hr. broth culture of the organism and incubated at 37°C.

Table VII represents two individual tests. One compares the resistance of cultures grown under normal conditions with that of those grown in wide bottomed flasks to give a shallow depth and a large surface. The other shows the effect of bubbling a continuous stream of air through the medium. The tests shown in the table were chosen to represent a number of similar tests made under similar conditions. In the section showing the results of cultivation in flasks, the example is quite representative. In this test the medium in which the organism was grown was freshly prepared medium warmed to approximately 37°C. before inoculation. The medium used in the aeration experiment was treated similarly; but the example cited is not so indicative of the results obtained in other similar tests. The variation of results obtained was due primarily to the deficient technique and equipment used for aerating. Air under pressure was forced through capillaries and the flow was controlled by pinch-cocks on the rubber tubing connections. Due to the fact that the capillaries were not held in a fixed position and slight variations in diameter caused different rates of air flow, it was found difficult to adjest and more difficult to maintain, an equal and even flow of air through all cultures. It was found that too intense a rate of aeration resulted in a poor development and low resistance of the cultures, especially in those containing no beef extract. This was due no doubt to the long delay in the initiation of growth under highly aerobic conditions. The above figures therefore represent only the tests in which nearly optimum control was maintained.

To show the contrast in resistance which might be obtained in cultures when no attention is paid to the condition of the medium inoculated, the following examples are given. The tests shown were chosen from experiments

run at different times, but the control in each case showed the prescribed resistance. These results are shown in Table VII (a).

TABLE VII (a)

Effect of condition of culture medium at the time of
inoculation on the resistance of the organism when
grown under highly aerobic conditions -10 cc. medium
in 200 cc. Erlenmeyer Flasks.

Culture Medium	Condition of Culture Medium	Survival time in 1-60 phenol at 20°C.		
		24 hrs. old Minutes	48 hrs. old Minutes	72 hrs.old Minutes
0.5% beef extract in 1% peptone broth	1. 10 days old	6	8	10
	2. Boiled, cooled to 37°C. inoculated,incubated for 5 hrs. then transfer to flask	5	6	6
0.0% beef extract in 2% peptone broth	1. 12 days old	4	5	12
	2. Boiled, cooled to 37°C. inoculated 5 hrs. then transferred to flask	11	25	35

In Table VII it is clearly demonstrated that an abundance of atmospheric oxygen raises the resistance of all cultures. It reiterates that the presence of beef extract (in normal amounts) prevents the attainment of the high resistance reached by cultures in peptone broth, this difference became more apparent in older cultures. In spite of the fact that aeration and an excess of available air increases the numbers of organisms per cc and also increases resistance, the results again indicate that resistance is not dependent alone on an increase in the number of bacteria.

While the results shown in Table VII (a) are not comparable, having been selected from tests made at different times, they are typical of a number of tests. The use of recently de-aerated beef extract medium may or may not improve the resistance of the cultures when grown under highly aerobic conditions; but improved resistance of cultures is a typical reaction to the de-aerating of peptone broth before use as a culture medium under higher aerobic conditions. It would appear from this that initial metabolism and proliferation may be retarded in cultures with no beef extract under highly aerobic or aerated conditions so that the culture is still comparatively young at the 24 hr. period. Winslow, et. al. (49) have shown that aeration retards the logarithmic phase in Es. coli, grown in peptone, but produces a steeper curve after multiplication has started. Apparently the cells of the beef extract cultures reach maturity much earlier than those in peptone cultures, if resistance is an indication of maturity. In the former, the 24 hour period is almost optimum for resistance, while the peptone cultures continue to increase in resistance on further incubation. While this observation would indicate that cultures in beef extract broth exhibit a shorter lag phase and reach the stationary stage earlier than cultures in peptone, especially under completely aerobic conditions, it still fails to explain the generally higher resistance obtainable in peptone, cultures, and will be further commented upon later.

Consumption of Atmospheric Oxygen

Since Staph. aureus when grown in beef extract broth forms a pellicle, it is possible that this would act as either a mechanical or physiological barrier against the diffusion of oxygen into the sub-surface layers, and

the organisms in such cultures will have less atmospheric oxygen for

consumption than cultures which do not show pellicle formation. Since

no such surface layer of growth is produced in peptone cultures, this

might afford an explanation of their increased resistance. A few simple

determinations in Warburg-Barcroft manometers sufficed to show that

on the contrary beef extract cultures in spite of the pellicle formation

consume more oxygen more rapidly than those cultures grown in peptone

broth. The determinations were carried out at 37°C. and the flasks

were not shaken since such manipulation is not customary in the regular

production of test cultures. Several such determinations are given

in Table VIII.

TABLE VIII

Consumption of Oxygen by Cultures in
Beef-Extract and in Peptone Media.†
(Readings in centimeters of mercury)

Media	3 hrs.	18 hrs.	38 hrs.			
No. 1.						
2% Peptone	0.6	3.2	5.4			
1% Beef extract	1.7	6.7	14.0			

	18 hrs.	30 hrs.	42 hrs.	62 hrs.	80 hrs.	96 hrs.
No. 2						
2% Peptone	3.2	5.2	8.5	16.5	20.3	22.3
1% Beef extract	7.5	9.5	16.5	25.0	27.5	29.1

	16 hrs.	24 hrs.	36 hrs.			
No. 3						
1.5% Peptone	2.3	4.2	9.1			
.5% Beef extract	5.3	8.2	16.4			

	18 hrs.	24 hrs.	40 hrs.	68 hrs.	80 hrs.	96 hrs.
No. 4						
2% Peptone	2.5	3.5	7.6	9.9	12.0	13.6
1% Beef extract	2.7	5.5	8.5	11.6	14.3	16.2
2% Beef extract	4.5	7.5	13.4	15.3	18.6	20.6

	2 hrs.	20 hrs.	45 hrs.	85 hrs.	100 hrs.	150 hrs.	195 hrs.
No. 5							
2% Peptone	.07	2.4	5.0	7.7	8.7	9.9	11.7
1% Beef extract	.15	7.1	11.1	13.6	15.2	18.8	21.5

Footnote —*Flasks inoculated with 1 loopful of a 24 hour culture of Staph.aureus,
stopcocks left open for 1 hour to allow adjustment of temperature.
Tests No. 1 and No. 3 were made with 5 cc. of media, while in No. 2 and
No. 4, 4 cc. of media were used. A renewed supply of air was drawn
into all the flasks when the mercury column connected with any of them
approached a height of 10 cm.

A study of the results given in Table VIII indicates that in general there is nearly twice as much consumption of oxygen in the beef extract cultures as in those cultures grown in peptone broth. This difference is more exaggerated in the early stages of growth, as might be expected from the more rapid growth rate and the greater number of cells in cultures grown in beef extract media. However the increased rate of oxygen consumption which is continued in the beef extract cultures does not correspond to their almost stationary resistance, while the reverse condition is to be noted in the peptone cultures which havebeen shown to continue to increase in resistance.

Age as a Factor in Resistance

As has been noted in the historical section of this study it has been well established that old bacteria are more resistant than young. In many of the observations made in connection with determining this fact, it has been considered that 24 hour cultures are old. The term "old" as applied to cultures of Staph. aureus might properly be applied to 24 hour culture if reference is made to the so called stationary phase of the growth cycle or even loosely to the period of maximum population. On the other hand, a 24 hour culture is not necessarily an old culture with reference to its maximum resistance. The relationship of age to resistance of this organism has been shown more or less incidentally in one or two of the foregoing tables. It can best be demonstrated perhaps, uncomplicated by other considerations, as is done in Table IX.

TABLE IX

Relation of Age to Resistance and Population

% of Beef Extract added to 1% peptone broth	Age of culture in hours									
	24		48		72		96		144	
	Survival time*	Count **	Survival time*	Count **	Survival time*	Count **	Survival time*	Count **	Survival time*	Count **
1.0	3	2150	5	1900	5	588				
0.5	4	1900	7	2100	8	1760				
0.1	5	1000	8	1000	10	770				
0.0	6	550	10	650	15	450				
1.0	4	1900	-5	2000	-5	1350				
0.5	4	1700	7	1500	7	1200				
0.1	7	400	12	700	15	1000				
0.0	6	500	10	750	20	750				
0.5	3 1/2	1350	5	1400			5	380	7	350
0.1	5	760	6 1/2	750			15	760	20	500
0.0	6 1/2	580	8	930			+15	900	+20	750
0.5 f	6 1/2	1770	10	1100			10	1500	7 1/2	1430
0.1 f	10	1370	15	850			20	820	30	1120
0.0 f	10	1000	20	850			+30	1100	-50	1100

* survival time in 1 to 60 phenol at 20°C. in minutes.

**number of viable cells per cc. in millions.

f indicates that the culture was grown in 200 ml. flasks - depth of 10 cc. of media = approximately 3 m.m.

- Before a figure indicates that the survival time was less than the figure indicated.

+ Before a figure indicates that the survival time was greater than the figure indicated.

It is apparent from Table IX that cultures of Staph. aureus in general become more resistant with increasing age, though the number of organisms tends to decrease after 48 hours. Of particular interest, however, is the fact that cultures grown in 1% beef extract fail to gain in resistance commensurate with those grown on media containing small amounts or no beef extract. It appears therefore that while age has a direct influence on resistance, it is not a constant relationship but varies with the medium used for growing the cultures. Most of the work correlating resistance and age has been done with cultures not older than 24 hours, in which case a fair degree of uniformity with respect to the individual cells still prevails. The lack of correlation between age and resistance of cultures grown in different media suggests that the age of the individual cells might be the vital factor connected with resistance and not the age of the culture as a whole.

Experiments with growth promoting substances

Hughes (50) obtained an activating substance from beef extract which promoted growth of Staph. aureus when 1 micro-milligram was added to 5 cc. of casein hydrolysate. That peptone is deficient in certain growth promoting substances which are present in beef extract is a possible assumption from the effect gained when a small amount of beef extract is included in peptone medium, namely - an increase in population of the 24 hour culture as can be demonstrated by comparing Tables II and IV. It has also been indicated that the early phases of the growth curve begin sooner and show greater increases in the presence of beef extract than in peptone broth alone. The increase in the bacterial count in the initial stages of

growth when Staph. aureus is cultured in beef extract media as compared
with peptone media was shown in Table III. The heavy sediment and more
dense appearance of the beef extract cultures as well as their greater
ability to promote the growth of cells previously exposed to a germicide
(Table V) offers additional indication that beef extract may contain a
growth stimulating substance which is either absent or insufficiently
available in peptone to promote rapid growth. It has already been shown
that additional supplies of food substances (Table IV) and increased
oxygen supply (Tables VI and VII) will increase the number of cells per cc.
but fail to produce the growth and resistance characteristics of beef
extract cultures. The higher rate of oxygen consumption which continues
well past the stage of maximum population and into the phase of declining
numbers (Table IX) is an indication that metabolism and growth continues
to take place in beef extract cultures, in spite of the reduction in the
number of viable cells.

A consideration of the above indications of the difference in
action between beef extract and peptone together with the reported
extraction of a necessary growth factor from beef extract, by Hughes (50),
presented the possibility that such a growth factor might be correlated with
the characteristic inability of cultures grown in beef extract to gain in
resistance with age. Consequently it became of interest to add known growth
promoting substances, other than beef or yeast extracts, to peptone broth
inoculated with Staph. aureus to observe the influence on resistance.
Knight (51) has disclosed that thiamin and nicotinic acid are both necessary
adjuncts for the growth of Staph. aureus in synthetic media and Hills (52)
has shown thiamin to be necessary for the metabolism of Staph. aureus.

Nicotinic acid was identified spectroscopically by Holiday (53) as being contained in the growth promoting fractions essential for the development of Staph. aureus. Therefore these two substances were added to peptone broth in 1 gamma per cc. amounts which according to Knight (54) is more than ample to activate growth in synthetic media. After the addition of these substances, the cultures were tested for their resistance against a 1-60 solution of phenol. The results obtained are tabulated in Table X.

TABLE X

Effect of addition of Enzymes on Resistance

Substances added to 1% Peptone Broth	Age of Culture																												
	24 hrs.			48 hrs.			96 hrs.					144 hrs.						240 hrs.											
	Survival time in minutes in 1-60 phenol at 20°C.																												
	4	5	6	5	8	10	5	8	10	15	20	5	8	10	15	20	25	5	8	10	15	20	40	50					
None	+	+	–	+	+	–	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+	+	–					
1 gamma B1**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+					
2 " B1+N*	+	–	–	+	–	–	+	+	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–					
0.5% Beef Extr.	+	–	–	+	–	–	+	–	–	–	–	+	+	–	–	–	–	+	–	–	–	–	–	–					
2% Peptone																													
None	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+					
1 gamma B1	+	+	–	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	–					
2 " B1+N	+	+	–	+	–	–	+	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–					

Number of cells in millions per cc.

None	370	510	620	500
1 gamma B1	540	680	640	600
2 " B1+N	810	790	390	220
0.5% Beef Ext.	1140	1350	380	350
2% Peptone				
None	640	930	960	750
1 gamma B1	---	---	---	---
2 " B1+N	500	830	1000	310

** B1 = thiamin   * N= Nicotinic acid   += growth   – = no growth

Table X presents very striking evidence that there is a close corre-lation between the resistance of cultures growing in media containing known growth-stimulating substances and those grown in media containing beef extract. In addition the macroscopic appearance of peptone cultures to which thiamin and nicotinic acid have been added is distinctly changed from the uniform turbidity characteristic of peptone cultures to that peculiar to beef extract cultures which, as noted before, show a pellicle formation with a heavy flocculant deposit. Since, as shown in Table X, the addition of certain known growth activating substances produce cultures of low resistance, it can be readily assumed that the growth activating constituents of beef extract might produce the same effect; and that it is these factors which are responsible for the low resistance of beef extract cultures as compared with peptone cultures. One of the known growth-stimulating factors (nicotinic acid) used in the experiments in Table X has been shown by Holiday (53) to be present in extracts which stimulate the growth of Staph. aureus. The other (Thiamin) is known to be contained in yeast extract which has the same effect on resistance as beef extract. This was shown in Table II.

It should be further noted (Table X) that the cultures grown in peptone broth alone, as would be expected from previous results, increased in resistance with the age of these cultures. Furthermore, the addition of thiamin alone to peptone broth tended to increase the resistance of the cultures grown in such broth over that obtained even in peptone broth without thiamin, at least in 1% peptone broth. Additional data shows that the addition of thiamin may frequently increase the resistance when added to 2% peptone as well, though this is not shown in the table. While again

it was not shown in the table, it should be stated that the addition of nicotinic acid alone failed to produce any apparent change in the characteristic growth or the resistance of peptone cultures. However, the combined thiamin and nicotinic acid, though having no effect when added to beef extract cultures, when added to peptone broth prevented the expected increase in resistance which usually results in peptone cultures with age. Similar results were obtained, as will be noted, when 2% peptone broth was used. Additional data showed that the same relative effect was obtained when the same series of cultures were grown under more aerobic conditions (in flasks); the count was approximately doubled as was the resistance, except in the cultures of beef extract and thiamin and nicotinic acid combined which showed an approximate 50% gain in resistance over the tube cultures. The 50 min. time limit of the test was reached in the case of peptone and peptone plus thiamin cultures in flasks sometime between the 96 and 144 hr. tests.

When cultures of Staph. aureus (209) were grown in peptone to which thiamin alone had been added, a definite change in the character of the growth was noted. A pellicle was formed but this was lighter and more fragile than that found in standard beef extract broth. On the other hand, the pellicle formed in media containing both thiamin and nicotinic acid was of a similar character to that observed in beef extract broth. Although cultures grown in both the thiamin media and the thiamin plus nicotinic acid media exhibited deposits, in the former the deposit was light, granular and chromogenic in both young and old cultures, whereas in the media containing both enzymes the deposit was heavy and flocculent simulating in this respect the type of deposit obtained with cultures grown in beef

extract broth, as noted before.

Tests indicated that addition of these substances in the small amounts used had no measurable effect on the reduction-oxidation potentials of either sterile or inoculated media. This is demonstrated in Table XI.

TABLE XI

Reduction–oxidation potential measurements
of media with and without the addition of
certain nutritional and activating substances

Substance added to 1% sterile peptone media	Before adding substances	5 min.	30 min.	60 min.	120 min.
		Potentiometer readings in volts			
		After adding substances			
.3 cc of 3% peptone	+ .025	+.025	+.025	+.026	+.030
20 gamma B_1* + N**	+ .029	+.022	+.021	+.022	+.024
.1 cc. of 10% beef extract	+.023	+.020	+.020	+.023	+.028
To 24 hr. cultures in 1% peptone					
.3 cc. of 3% peptone	- .155	-.143	-.141	-.145	-.147
2.0 gamma B_1 + N	- .176	-.166	-.171	-.177	-.180
.1 cc. of 10% Beef extract	- .175	-.168	-.171	-.180	-.181

* B_1 - thiamin
**N - nicotinic acid.

Measurements were made on a Leeds and Northrop potentiometer calibrated
for a saturated KCl calomel electrol at 25°C. The galvonometer used was
a Leeds & Northrop reflecting type. The readings recorded are uncorrected,
since the only purpose of the experiment was to note any change effected
by the addition of certain substances.

It is apparent from Table XI that no appreciable effect on the
reduction-oxidation potential of media was obtained by the addition
of the small quantities of substances (when measured under aerobic con-
ditions) which nevertheless changed the resistance of the cultures.

It has already been brought out that old cultures are more
resistant to the action of disinfectants than young ones and it has been
demonstrated in this investigation that the old cultures in media
apparently lacking certain growth promoting substances are very much
more resistant than those in beef extract. On the other hand cultures
grown in media containing complete growth promoting substance, thiamin
plus nicotinic acid, does not increase materially in resistance with
age (Table X) simulating in this respect cultures grown in heavy con-
centrations of beef extract.

The small amounts of thiamin and nicotinic acid that are required
to change the resistance of peptone cultures, do not change the redox
potential of peptone broth and it is difficult to conceive of such
amounts of an enzyme having any gross effect on the physical conditions
of the culture as a whole. There is the possibility, however, that such
an amount of enzymes might have a physiological effect on the cells which
constitute the culture. If it can be assumed that the greater resistance
of older cultures is due to the greater age of the cells constituting such
a culture, as the work of Sherman and Albus (55) and others has strongly
indicated, then it can be further postulated that a difference in age
of the cells might be responsible for the difference in resistance of
cultures which contain growth stimulating substances and those which do
not. Since the consumption of oxygen continues at a comparatively high

rate in cultures containing beef extract as shown in Table VIII[I] it would appear quite possible that there is continuous production of new cells in cultures containing beef extract. Such a continuous production of new cells would produce a culture constituted largely of young cells and could readily account for the low resistance of such cultures. If the growth stimulating factor in beef extract does bring about a continuous renewal of population in cultures of Staph. aureus, by stimulating the production of young cells even in physically old cultures, then the addition of known growth stimulating substances should do the same thing when added to cultures which are already mature but contain only small amounts or no growth stimulating factors. This effec should then be reflected in the degree of resistance which such cultures normally attain on further aging. It therefore seemed advisable to add to 24, 48 and 72 hour cultures adequate concentrations of growth promoting substances to determine their effect on the resistance to phenol after continued incubation. In Tables XII, XII-a and XII-b the effect of adding various suxiliary substances,(beef extract, thiamin and nicotinic acid combined and peptone) to such cultures is shown.

TABLE XII

Resistance measurements on the effect of adding
auxiliary substances to 24 hour cultures of
Staph. aureus.

Substances added to 10 cc. 24 hour culture in 1% Peptone.	Survival time in minutes in 1-60 phenol at 20°C.			
	24 hrs.	48 hrs.	72 hrs.	130 hrs.
1. 0.1 cc., 10% beef extract		5	-5	20
2. 20 gamma B_1 + N*	Auxiliary material added.	-5	-5	- 5
3. 0.3 cc, 3% peptone		10	10	15
4. (control)	7.5	8	10	15
5. (control)	7.5	10	10	15

10 cc. – 24 hr. culture
in 2% peptone.

1. 0.1 cc., 10% beef extract		-5	-5	10
2. 20 gamma B_1 + N	Auxiliary substances added	5	-5	-5
3. 0.3 cc, 3% peptone		5	5	25
4. (control)	7.5	-10	10	15
5. (control)	7.5	10	10	20

* B_1 + N - thiamin plus nicotinic acid.

- before a figure used to designate a survival time less than
the figure indicated.

TABLE XII (a)

Resistance measurements on the effect of adding auxiliary
substances to 48 hour culture of Staph. aureus.

Substances added to 10 cc. — 48 hr. culture in 1% peptone	Survival time in minutes in 1-60 phenol at 20°C.		
	48 hrs.	72 hrs.	120 hrs.
1. 0.1 cc.,10% beef extract	Auxiliary substances added	5	-10
2. 20 gamma B_1+N*		10	10
3. 0.3 cc., 3% peptone		5	15
4. Control	10	10	15
5. Control		15	25
To. 10 cc. 48 hr. culture in 2% peptone			
1. 0.1 cc, 10% beef extract		5	-10
2. 20 gamma B_1+N*	Auxiliary substances added	8	-10
3. 0.3 cc, 3% peptone		20	25
4. Control	10	10	15
5. Control		20	25

* B_1 + N — thiamin plus nicotinic acid.

— less than   + more than, the figure indicated.

TABLE XII (b)

Resistance measurements on the effect of adding
auxiliary substances to 72 hour culture of <u>Staph.</u>
<u>aureus.</u>

Substances added To 10 cc. 72 hour culture in 1% peptone	Survival time in minutes in 1-60 phenol at 20°C.		
	48 hrs.	72 hrs.	120 hrs.
1. 0.1 cc., 10% beef extract	Auxiliary substances added.		-10
2. 20 gamma B_1 +N*			-10
3. 0.3 cc., 3% peptone			+30
4. Control		15	20
5. Control			+40
To 10 cc. 72 hr. culture in 2% peptone			
1. 0.1 cc., 10% beef extract	Auxiliary substances added.		-10
2. 20 gamma B1 + N*			-10
3. 0.3 cc., 3% peptone			+30
4. Control		20	25
5. Control			+40

* B_1 + N - thiamin plus nicotinic acid.
- less than + more than the figure indicated.

It will be noted from the table that the addition of beef extract and of the growth promoting enzymes not only interfere with the normal resistance obtained in old peptone cultures, but the resistance is actually reduced, in contrast to the addition of peptone. In the first example in which beef extract was added to a 24 hour old culture to give a 0.1% concentration, it will be noted that the culture attained a high resistance 96 hours after the beef extract was added. This is not un-expected, for it has been repeatedly found that beef extract in such a concentration interferes very little with the attainment of high resistance, and cultures containing this amount of beef extract are generally more consistent in attaining a moderately good degree of resistance than are those containing only 1% peptone alone. If lack of resistance is assumed to be a characteristic of young cells, then in the absence of other known influences, the above results strongly support the supposition that old cultures, containing growth stimulating substances nevertheless contain a large proportion of young cells. This can be explained by the lowered resistance in cultures containing known growth stimulating substances. It is entirely possible that growth-promoting substances in beef-extract are similarly responsible for the comparatively low resistance of beef extract cultures.

DISCUSSION

In all germicidal and death rate studies it is difficult to obtain repeatedly uniform results. Chick (56) noted that Staph. aureus changed frequently in its resistance to phenol. Miller (57) stated that while working on germicidal tests a great deal of his time was consumed by his inability to obtain cultures of comparable resistance even when these were grown under identical conditions. Knaysi and Gordon (58) found that with the same strain under similar conditions it was difficult to obtain comparable results in death rate tests. Cohen (59) calculated a probable error of two or three per cent under ideal conditions with a customary error of 10%.

A number of factors may be sources of variation. The cultural background of the test organism is of great importance, some factors influencing this have just been considered in much detail. Smyth (42) has found that small variations in the dilution of phenol produced large errors in spite of the fact that phenol is comparatively little affected by the size of the inoculum as was pointed out by Eisenberg and Okolska (60). Smyth (42) further states that a two-hour difference in age of the culture or a 1% difference in incubation temperature will cause an important difference in the resistance of Staph. aureus. Other factors which per se are inconsequential may become of great importance as influences on the killing action of germicides. In previous studies by the author it has been found that cresols in amounts too small to be detected by official methods of titrating carbolic acid will invalidate the phenol test, and further that weak dilutions of alcohol, inert when tested for antiseptic

action, retard the action of some germicidal agents and greatly enhance others. Also in association with Ruehle (61) the author found that because of the salt content of R. W. broth, cultures grown in this medium gave exaggeratedly high phenol coefficients with certain disinfectants. Although the salt was indirectly responsible, the rapid change from a high to a low osmotic pressure was shown to be the direct cause. Again Stuart and James (62) have demonstrated a definite influence of sodium chloride on the functioning of reduction-oxidation potentials in cultures of certain bacteria. O'Meara (63) has pointed out the inimicable affect of traces of copper in peptone used for culture media.

In the testing of germicides it is almost invariably adopted as the criterion of the killing of the cell that of the inability of bacteria to reproduce. Consequently in germicidal studies, the sub-culture medium into which the treated organisms are inoculated becomes of very real importance. A medium which normally supports a good growth of an organism in the usual methods of transfer may not be suitable for use asa sub-culture medium when an inoculum containing but a few surviving cells of this organism is implanted in it. Hewlett and Hall (64) have shown that ordinary nutrient broth is a far less favorable sub-culture medium for anthrax spores previously exposed to formaldehyde than is nutrient agar. Curran and Evans (65) state that "enrichment substances incorporated in culture media are essential for the accurate enumeration of bacteria previously exposed to highly lethal factors ---. Spores which survive destructive influences when seeded in inadequate media lose their vitality rather rapidly". Morrison and Rettger (66) eliminated the lag period for heat treated spores by replacing nutrient broth as a sub-culture medium with enrichment media.

Baumgartner and Wallace (67) reported a direct relationship between survival time of mercuric chloride treated organisms and the concentration of peptone used in the sub-culture medium. Some of the date presented in this investigation showed beef extract broth to be better than peptone broth as a recovery medium for Staph. aureus after contact with Phenol (Table IX). The author has found that beef extract is extremely important in subculture medium for Staph. aureus after it has been exposed to mercury or formaldehyde.

Since it is generally accepted that bacteria that fail to multiply by sub-culture are dead and since the type of sub-culture medium influences the ability of treated bacteria to proliferate, it is highly important that a sub-culture medium that can be made with comparatively little variation should be employed in germicidal and death rate tests. Fildes (68) found a variation among different tubes of the same batch of media in the reduction oxidation potential. O'Meara (63) found adifferent reduction time among tubes of chopped horse meat media which greatly influenced the germination of Clostridium tetani. In testing germicidal action the writer has noted that age affects the efficacy of media used for sub-culturing purposes in Table X-a as demonstrated.

Had our knowledge been more complete concerning the mode of action exerted by germicides on bacterial cells, it is quite probable that considerably more attention would have been paid to the efficacy of sub-culture media.

Madsen and Lyman (69) and Chick (70) demonstrated that the death rate of bacteria being acted upon by a germicidal agent bears close analogy to the monomolecular chemical reaction, when the logarithms of the numbers

of surviving bacteria are plotted against time. Chick (56) later showed that the rate of coagulation of certain proteins (egg albumin and haemoglobin) by heat followed the same logarithmic path. This gave strong support to the popular theory that a number of germicidal agents act by coagulating the bacterial protoplasm. Although this theory does not conform with the evidence that treated bacteria will grow in one medium but not in another though both media support growth of untreated organisms, it is explained on the basis of "reversible coagulation" by the proponents of this theory of germicidal action. Burke (71) found that the spores of Cl. botulimum which survived the most severe exposure to heat exhibited the longest lag period. She therefore drew the conclusion that a thicker cell wall protected these organisms. Isaacs (72) and Virtanen (73) have stated such an explanation is untenable, since a cell wall many thousand times as thick as the wall of a spore could retard the passage of heat but a fraction of a second.

Isaacs (72) reported that, as early as 1899, Ducleaux advanced the theory that death in bacteria was caused by a disruption of their enzymic systems. Isaacs (72) himself has elaborated upon this theory and accounted for the longer survival of some cells by their possession of a more abundant enzyme supply. Although the theory of en zyme destruction as the initial process of death in bacteria appears to be the most plausable yet presented, it would seem to the writer even more logical if the theory of resistance had been placed not upon the amount of enzymes present but upon the inactivity and protected state of the enzymes in resistant cells.

Henrici (74) to whom we no doubt owe our greatest debt for the knowledge of the physiology and morphology of the various growth phases of bacteria points out that, "Apparently the aging of a culture, i. e., the partial exhaustion of the nutrients with resultant cessation of growth

affects the cells in two sorts of ways. Some of the cells are injured and die, while others go into a dormant or resting state, in which they are more resistant than the growing cells. In the spore-forming bacteria this dormant resistant form is a special body, the spore. But spores may be looked upon as merely a high development of a general phenomenon, for even with bacteria which do not form spores we know that the mature cells are more resistant than the growing cells. These cells also, then, may be looked upon as resting forms. The lag phase is probably best explained by assuming that these resting cells must be first transformed into growing types before growth may proceed; actually we may see such a morphological transformation occur. If an actively growing culture is transplanted, there is no lag in the new culture". In this investigation it was shown that cultures grown in peptone broth were found to decrease in population on aging but as there was an increase in resistance of the remaining cells for a period of at least 12 days it has been the experience of this writer to find that, in testing certain germicides the difference in resistance between a 24 hour culture of E. typhosa and an old dried culture of Staph. aureus is much greater than the difference in resistance between the Staph. aureus culture and some bacterial spores.

It would appear to the author that the enzymes remain close to the surface in a protected and inactive condition in spores rather than buried deeply beneath an impermeable coating since when placed in a favorable medium there is a rapid transformation of resistant spores into a more susceptible state - the vegetative form. The writer found it impractical to suspend test spores in nutrient broth rather than water or salt solution on account of the rapidity with which the spores lost their resistance in this medium.

Isaac's (72) has concluded from his data that the initial stage in the death of <u>Mycobacterium tuberculosis</u> exposed to the action of a lethal agent is the loss of an enzymic system which was capable of inducing growth on an acid medium. Quastel (75) has shown that death in bacteria cannot be considered as a rapidly culminating process but a step by step destruction of enzymic and vital processes. He found that bacteria no longer capable of proliferation (the ordinary criterion of bacterial death) were just about as active as normal resting cells in certain of their enzymic activities, but these too were lost one by one as the devitalizing process was continued. Hershey (76) has recently shown that on exposure to heat there is a decrease in respiration of the cells of <u>E. coli</u> a prolonged latent period with a final loss of vitality. Surviving cells show a regeneration of the respiratory function at a constant rate characteristic of normal growth except for degree. It would seem much more tenable to base Burke's observations concerning the longer lag phase of more resistant cells, on the more resistant and less active state of the enzymes rather than on the thickness and permeability of the cell coat or on the total amount of enzyme present as Isaac's has proposed.

Attention should perhaps be called to another important source of error in germicidal and death rate studies; that of the influence of the methods used in transferring the treated organisms to the subculture medium. The most common method is to carry over a portion of the culture under test to the subculture medium, usually with a loop. In the F. D. A. method a 4 mm. (inside diameter) 23 gauge platinum wire loop is used. Cade (77) has shown that representative numbers are rarely transferred by this method, especially as thr number of viable organisms diminishes toward the end of the test.

Dickson et al (78) have stated in connection with their studies on the

resistance of spores of Cl. botulinum that not more than 5 percent of the

spores in any batch are responsible for survival under drastic conditions,

95 percent being destroyed comparatively quickly and among the remaining

5 percent only a few are highly resistant. These few highly resistant

organisms are especially important in germicidal tests since they are

responsible for the unrepresentative results obtained with random sampling.

The only means of avoiding this error appears to be that used by Brewer (79)

which consists of separating the cells from the germicidal menstrum by

centrifugation and after washing, replanting the entire test batch in a

subculture medium. Such a procedure however is not feasible for ordinary

disinfectant testing or death rate sutdies and can be used only when one

or several widely separated time intervals are arbitrarily selected for

observing the lethal effect of germicides

The analogy between death-rates in bacteria and the monomolecular

reaction has already been mentioned. This analogy has been verified recently

by Watkins and Winslow (80). When the germicidal agent is in excess in

such a reaction and the only variable is the number of bacteria, the death

rate of the cells follows the Law of Mass Action — The greater the number

of cells the longer the survival time. When an effort was made in these

studies to increase the survival time of Staph. aureus, tested against

phenol, by increasing the number of cells through an additional food supply

conflicting results were obtained. When peptone broth was utilized for

growing the cultures, an increase in the number of cells per c.c. and a

corresponding increase in survival time was brought about by increasing

the concentrations of peptone. However, when an increase in the number

of cells per c.c. was produced by the addition of certain enrichment sub-

stances, particularly beef extract, there was a decrease inthe survival time of Staph. aureus in phenol. The reduction of survival time resulting from the addition of beef extract became more marked with increased concentration and this was particularly noticeable in older cultures. Some strongly influencing factor disrupted or completely covered the effect to be expected from the increase in numbers of organisms. When beef extract was added to peptone the type of growth was changed from a smooth even distribution to a type of growth showing pellicle formation along with a heavy flocculant deposit, so that it is highly probable that the actual number of cells was under-estimated in comparison with those in peptone. The clumps could not be entirely broken up with shaking so that colonies counted in estimating the number of cells by the plate method coubtless originated from groups of cells, rather than from individual cells, or at least from clumps far larger than was the case with peptone. An accurate estimation by direct counting in the Petroff-Hauser counting chamber was rendered extremely difficult for the same reason. Henrici (73) has stated that Staph. aureus is unfit for accurate death-rate determinations due to its propensity for forming clumps.

When the physical conditions under which the cultures in both beef extract and peptone broth were altered so that they were permitted access to an abundance of atmospheric oxygen, an increase in the number of cells together with an increase in resistance resulted in the cultures in both media. The increase in resistance in the beef extract cultures reached its limit in from 24 hrs. to 48 hrs. whereas the resistance of the peptone cultures continued to increase for a number of days. Both beef and peptone cultures grown under normal conditions react similarly but at a lower level of resistance. It would appear that an ample supply of oxygen allows

ready utilization of the nitritive substances contained in the media and permits a more rapid attainment of maturity. This supposition is supported by the following investigations. Working with E. coli Clifton (45) and Hershey and Bronfenbrenner (46) showed that the oxygen supply was the limiting growth factor of cultures in a media containing an ample supply of nutritive substances. Brunstetter and Magoon (31) found B. mycoides produced a larger and more resistant spore crop when aerated. Winslow, Walker and Ultmeister (49) showed a more rapid increase during the logarithmic growth phase of Es. coli when the cultures were aerated.

In these studies the cultures in beef extract broth failed to attain the resistance acquired by those in peptone broth at any age after 24 hrs. Furthermore after 24 hours of age in contrast to expectation they failed to gain materially in resistance yet Steinhaus and Birkelan (81) have shown recently that a two year old culture of Serratia marciens was three times as resistant as an eight hour culture of the same organism. It would appear that the beef-extract permits an earlier attainment of maturity of the culture as a whole than does peptone. It was shown that beef-extract broth is the better growth media and forther that beef-extract cultures have greater numbers of cells starting with the early stages of growth than do peptone cultures. Consequently, the beef extraΘt must contain something which interferes with the attainment of the resistance expected in old cultures. When the oxygen consumption of the beef-extract cultures was compared with that of peptone cultures, not only was it greater in the first 24 hour period, as would be expected, but continued at a higher level for several days. It appeared then, that metabolism of the cells continued at a higher rate in beef-extract than in peptone, even after a decrease in the number of viable

cells had begun. This would indicate that proliferation and autolysis were occurring in the beef extract cultures more rapidly than in peptone. The growth-promoting substances known to be contained in beef extract would be the most likely cause of this rapid metabolism and division in beef extract cultures.

When the pure growth promoting substances, thiamin and nicotinic acid, were added to peptone cultures they changed subcultures in appearance and resistance that they conformed to the appearance and resistance of beef extract cultures. Since the addition of certain growth-promoting substances to peptone cultures does reduce their resistance, it seems a fair assumption that the growth promoting substances in beef extract likewise are responsible for the reduction in the resistance of beef extract cultures.

In these studies it was shown that when a culture of Staph. aureus was grown in a peptone medium containing as little as .002 mg. per cc. of a growth promoting substance the resistance of the organisms in the culture was considerably reduced. Through the work of Sherman and Albus (55) and more recently that of Hershey (82) and Watkins and Winslow (81) and others it has become well established that old cultures are more resistant than young.

In view of these studies and in the absence of evidence to the contrary, it must be concluded that the cells in cultures containing the growth-promoting enzymes are younger and therefore less resistant than in cultures not containing growth-promoting enzymes. It would seem that in the cultures containing growth stimulating substances that a rapid procession of division and death is taking place so that at any age of the culture there are only young cells present.

Although thiamin and nicotinic acid when added together reduce the resistance of peptone cultures when either is added along this does not occur.

Indeed as has been shown the addition of thiamin increases the resistance of cultures, apparently by bringing about a more rapid metabolism in the presence of atmospheric oxygen with a consequent production of a culture of more uniformly mature cells. Tauber (83) has stated that co-carboxylase (pyroe phosphoric ester of thiamin) protects carboxylase from oxidation. The addition of nicotinic acid apparently had no obvious effect on the characteristics of peptone cultures. In speculating on the possible action of these two compounds it would seem plausible to assume that the thiamin, which indirectly activates metabolism, apparently is not readily reduced again to its active state so that rapid metabolism ceases. The early development of chromogenesis in cultures containing thiamin alone in contrast to normal cultures, strongly indicates not only that an early state of maturity is reached in these cultures but that the population remains in a statical condition. In the presence of nicotinic acid (the pyridine nitrogen of which is reversibly oxidized and reduced according to Stephenson (84) and auto-reversible system functions so that the metabolic activation by thiamin continues. Straub et al (85) have recently presented a scheme for the reversible oxidation and reduction of cozymase (which enzyme contains nicotinamide).

While the conclusion which has been advanced concerning the rapid "turn-over" in populations of cultures of Staph. aureus containing artificially concentrated sources of growth promoting substances, is quite attractive, there appears to be no method of proof that such a process actually occurs. There is no way of determining definitely the age of the individual cells in a culture, nor of determining rate of successions of populations among bacteria. Although it is a known fact that autolysis is a common phenomeum among bacterial populations there is no known method of determining its rate

in actively metabolizing and proliferating colonies. Certain observations on autolysis have been made but these were under special conditions. Autolysis of Staph. aureus has been observed by Jaumain (86) to take place more rapidly in an alkaline than an acid medium in sealed tubes. Northrop (87) was unable to demonstrate an increase in soluble protein after autolysis of Staph. aureus. Evangalinos and Wohlfeil (88) have demonstrated that autolysis may take place very rapidly in cell suspensions in the presence of certain salts and in alkaline medium a very appreciable lysis occurring in twenty-four hours. Loofbourow and Morgan (89) have found that certain fractions obtained from yeast will stimulate growth in one species of organisms and increase the rate of respiration in another. In view of the difficulty of measuring growth when rapid autolysis is occurring it would seem difficult to measure increased respiration alone as distinct from respiration and growth.

By careful staining, Staph. aureus cultures grown in extract broth present a picture quite different from those grown in peptone. Extract cultures beginning with a 24 hour growth show some large easily stained cells and some unorganized debris, by 48 and 72 hours the cells vary from large deeply stained units to unorganized unstained debris. Thus much of the deposit in the cultures which might easily be taken for cells is actually unorganized material. In contrast, cultures grown in peptone broth present a picture of uniform cells both in size and degree of staining with only small amounts of debris present even in 48 and 72 hour cultures. This microscopic picture indicates a regeneration and autolysis occurs in beef extract cultures in old cultures much more rapidly than in peptone cultures. Under seal, both cultures autolyse, the beef extract the more rapidly, until both show about the same degree of turbidity. Finally the increases uptake of oxygen in

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extract cultures as measured by manometric methods over that utilized by cells in peptone media, indicates a more rapid metabolic rate with a more rapid rate of proliferation and autolysis.

As a result of these studies certain conclusions have been derived and although the data presented strongly support these conclusions; never-theless, direct proof of them has not been presented. But to refrain from presenting a conclusion for want of unavailable, absolute proof, may be to neglect an obligation. Fifteen years of investigation and testing of disin-fectants and antiseptics has not only given a familiarity with the reactions exhibited by Staph. aureus under detrimental conditions, but has brought forward a group of facts based on the data obtained. A series of individual observa-tions regardless of their particular import, may be of greater value when considered together. It should be remembered that in spite of the tremendous volume of published material concerning the effect of outside influences upon bacteria, we have barely scratched the surface of knowledge regarding the detailed reactions of any of these.

A vigorous culture of bacteria in a laboratory test-tube is seldom thought of as an exquisitely sensitive manifestation of balance. But it is impossible to emphasize too strongly the extreme delicacy of adjustment which permits the cells comprising these cultures to rapidly perform a series of profound chemical transformations and physical reactions, simultaneously and jointly.

An appreciation of the complexity of reactions attending even the simplest germicidal action may render an interpretation based upon numerous observations of such an action more valuable than a mere statement of facts. When the combination of results gathered from a long series of observations and the findings of other investigators tend to confirm such an interpretation,

it would seem permissible to go a step beyond the limits of experimental proof and give consideration to the inferences and indications suggested by the observations, and the following statements attempt to give expression to these interpretations.

1. Two cultures of the same organism and of the same physical age may not be of the same physiological age, even though both have reached the so-called stationary phase of maximum numbers and both contain a comparable number of viable cells. The majority of individual cells comprising one of the cultures may be older than cells of the other; or the cells in one of the cultures may be of a uniform age while those of the other form a gradient from very young to old and resistant forms.

2. Cultures containing substances which stimulate the growth of its cells continue to do so even after the maximum number has been reabhed and a decline of viable organisms has become established; so that a rapid replacement of population constitutes a culture of continuously young cells, regardless of its physical age.

3. A more rapid death and autolysis accompanies the more rapid growth of the cells in cultures containing growth-stimulating substances thus allowing a more rapid renewal of populations.

SUMMARY AND CONCLUSIONS

It has been shown that an investigation of the substances in nutrient media affecting the resistance of Staph. aureus was fully warranted.

Evidence has been presented to show that the number of cells per cc. of the test-culture may be increased by the use of enrichment media but due, presumably, to more powerful factors, the number of cells in the test culture inoculum had no direct bearing on its resistance. Resistance to detrimental influences was not increased by substances used for enrichment purposes.

The use of increased amounts of beef-extract as an enrichment ingredient in media for culturing of Staph. aureus, it was found that a perceptible rise in bacterial count per cc. of the test culture resulted with increased concentrations; but that a decline in resistance of cultures of the test organism resulted.

A comparison of resistance between cultures grown in broth containing customary amounts of beef-extract and in plain peptone media showed the cultures in the peptone media to be the more resistant.

Increased concentrations of peptone improved the resistance of Staph. aureus cultures in contrast to the reduction in resistance which occurred when the concentration of beef extract was increased.

Cultures of Staph. aureus increased in resistance in proportion to the amount of available oxygen as measured by the area-depth ratio of media. Aereation of the cultures was found to increase their resistance.

Staph. aureus cultures grown in beef-extract media were found to utilize more atmospheric oxygen than those grown in peptone.

The increase in resistance resulting from prolonged incubation of peptone cultures did not occur in cultures containing beef-extract.

It was found that a combination of thiamin and nicotinic acid when added to peptone media inoculated with Staph. aureus or to 24 hour cultures of Staph. aureus in peptone media change the normal character and resistance of peptone culture to cultures resembling beef extract cultures in appearance and resistance.

It has been concluded, without direct proof, that the growth-stimulating substances contained in beef extract are the factors responsible for the lowered resistance of the Staph. aureus grown in media containing this material.

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